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A Mini Review on Components of Flax Seed and Their Effects on Breast Cancer

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ABSTRACT
Breast cancer is recognized as one of the most common cancers worldwide that can lead to death. Alternative treatment options are needed due to drug resistance caused by current treatment methods such as chemotherapy, inclusion of healthy cells in the target, and possible side effects. In this context, there is great interest in natural compounds and their active metabolites. One of these is flaxseed (FS), which is one of the most studied foods to be associated with breast cancer. FS is a functional food with high nutritional value. FS components (fatty acids, fiber, and lignans) and especially the basic lignan structure in FS content, secoisolariciresinol diglucoside (SDG) and its metabolites enterolactone (ENL) and enterodiol (END) have beneficial effects on breast cancer progression. This review aimed to develop a perspective for further research on this type of cancer in the future by giving some general information about FS and its components and evaluating some studies showing potential effects on breast cancer.

Keywords: Chemotherapy, Flaxseed, Lignan, Breast cancer, SDG

1. Introduction

Breast cancer is one of the most frequently diagnosed invasive cancer types, especially in women (Sharma et al. 2010). According to data in 2020, it has been reported that breast cancer with approximately 2.3 million cases is responsible for 11.7% of all cancer cases and 6.9% of cancer-related deaths worldwide (Global Cancer Observatory). According to the 2020 WHO-Turkey cancer profile, it has been reported that breast cancer has a rate of 46.6% in total cancer cases and 12.9% in cancer deaths (Cancer Today. Global Cancer Observatory).

It is estimated that a certain percentage of the occurrence of breast cancers (approximately 5-10%) is due to genetic factors based on mutations of some genes, and the presence of individuals with this disease in the family may cause the person to inherit genetic mutations that create the relevant risk factors (Kabel & Baali 2015; Smolarz et al. 2022). Related hormones (such as estradiol, and progesterone) are also the main determinants of cancer risk in women who do not have a genetic indicator, including gender, poor eating habits, bad lifestyles, alcohol or tobacco consumption, late first pregnancy, long-term hormone treatments, overweight and obesity are factors associated with the incidence of breast cancer (Yue et al. 2013; Britt et al. 2020; Smolarz et al. 2022).

The course of the disease and survival rates in breast cancer can vary greatly depending on the type of cancer, its stage, the types of treatment (such as surgical intervention, radiation, chemotherapy, and hormonal therapy), and even the geographical location of the patient (Sharma et al. 2010; Marghescu et al. 2012; Feng et al. 2018).

Related nuclear receptors for the steroid hormones estrogen (ER) and progesterone (PR) and human epidermal growth factor receptor 2 (HER2) are important hormone receptor markers used in breast cancer (da Luz et al. 2022). Breast tumors are divided into various subtypes based on hormone receptor expression and the amount of the cellular proliferation marker Ki67 (Januškevičienė & Petrikaitė 2019; Moar et al. 2023). These are hormone receptor-positive (HR+), HER2-enriched (HER2+), and triple-negative breast cancers (TNBC) (He et al. 2023). HR+'s are ER+ and PR+'s. They are divided into two subclasses: luminal A (ER+ and/or PR+ and HER2−) and luminal B tumors (ER+ and/or PR+ and HER2+) (or HER2− with high Ki-67) (Tang et al. 2016; da Luz et al. 2022; He et al. 2023; Moar et al. 2023).

Heterogeneity in breast cancer cases can cause difficulties in determining the course of this disease and in pursuing a successful treatment path (Wiggins et al. 2015; Feng et al. 2018). Although tumor cell molecular subtypes are different, the chemotherapy method applied remains the mainstay (Wang et al. 2010; Twelves et al. 2016; Xing et al. 2019). In order to get a stronger effect, chemotherapeutic drug combinations with different mechanisms of action are used, but increased toxicity is observed with this treatment method (Di et al. 2018).

Drug discovery from medicinal plants already plays an important role in cancer treatment. Natural compounds and their active metabolites are used as adjuvant therapy in breast cancer patients to eliminate chemotherapy and radiotherapy-induced side effects and to improve the quality of life of patients (Zhang et al. 2018). Flaxseed (*Linum usitatissimum*) (FS) is a rich functional food known for its high concentration of fiber, lignans, and omega-3 fatty acids (Rubilar et al. 2010; Bernacchia et al. 2014; Goyal et al. 2014). Lignans, from the phytoestrogen group, are diphenolic active natural compounds that exhibit various biological properties of plant origin and are present in many foods in small amounts, while they are found at high levels in FS (Kajla et al. 2015; Nikolić et al. 2017; Rodríguez-García et al. 2019). Studies involving lignans focus on their phytoestrogenic properties and their potential to affect estrogen-sensitive cancers, including breast cancer (Saarinen et al. 2007; Seibold et al. 2014; Chang et al. 2019).

In the review, the main information was presented about lignans and the basic properties of the components of FS, which is an important lignan type, and its connection with breast cancer. It aims to shed light on further studies on breast cancer by mentioning the studies based on its effects on breast cancer, which is a type of estrogen-sensitive cancer.

2. Estrogens and estrogen receptors

The steroid hormones estrogen and progesterone and their respective receptors are regulators of breast functions and are effective in the development of breast cancer (Daniel et al. 2011). In addition to many basic functions (bone homeostasis, modulation of brain functions, cardiovascular systems, and musculoskeletal system, etc.), there are three main forms of estrogens (17β-estradiol (E2), estrone(E1) and 16α-estriol (E3)), which are the primary sex hormones that perform the functions of the female reproductive systems. They are all derived from cholesterol and are known as C18 steroids. The most dominant and active estrogenic hormone among endogenous estrogens is known as E2 (Nazari & Suja 2016). E1 is produced in the ovaries, adrenal cortex, and testicles from androstenedione under the influence of aromatase. It is the main hormone during menopause in women and E1 is converted to E2 by the enzyme 17β-hydroxysteroid dehydrogenase. E2 is mainly secreted by the granulosa cells of the ovarian follicles and the corpus luteum. E3 is an almost completely inactive metabolite of E2 and E1 produced mainly in the liver and is active during pregnancy (Mansur et al. 2012; Samavat & Kurzer 2015; Fuentes & Silveyra 2019; Ceccarelli et al. 2022). While E2 synthesized in the ovaries in premenopausal women is the most important estrogen, endogenous estrogen production decreases in postmenopausal women, and mostly E1 plays a dominant role (Samavat & Kurzer 2015; Das et al. 2022). It is the type of estrogen with the strongest binding affinity for E2 receptors. E2 is known to increase cell proliferation, which is associated with breast cancer development through its receptor-mediated actions (Yue et al. 2013).

Steroid hormones act through receptors on target cells. The hormone binds to its corresponding receptor in the cytosol and the resulting complex acts as a transcription factor by binding to specific response elements or transcription initiation complexes on DNA. It activates or represses transcription and thus regulates cellular activity (Schwartz et al. 2016). Estrogens, a type of steroid hormone, perform their specific actions by binding to estrogen receptors (ERs) and initiate various estrogen-dependent physiological processes by activating transcriptional processes and/or signaling events (Fuentes & Silveyra 2019; Khan et al. 2022). Estradiol in steroid structure plays an active role in the development of breast cancer, binds to its receptor and translocates to the nucleus, and related gene transcriptions are stimulated. Most types of human breast cancer start with estrogen-related receptors expressed, and the expression of the receptors is recognized as important prognostic markers (Roy & Vadlamudi 2012; Hilton et al. 2018).

It has been reported that the most common type of breast cancer is estrogen receptor-positive (ER+) breast cancer, which accounts for a large percentage of all cases (Turner et al. 2017; Almeida et al. 2020). The physiological functions of estrogenic compounds are modulated by the alpha (ERα) and beta (ERβ) subtypes of estrogen receptors encoded by different genes (Mal et al. 2020; Božović et al. 2021). These receptors belong to the family of steroid receptors and bind certain ligands of different tissue distribution with different affinities (Almeida et al. 2020; Mal et al. 2020).

While ERα is found in the mammary gland, fatty tissues, prostate, uterus, male reproductive organs (testes and epididymis), liver, and ovaries, ERβ is mainly found in the bladder, prostate, ovary (granulosa cells), adipose tissue, and colon (Paterni et al. 2014; Khan et al. 2022). ERα appears to be the main arm of estrogen action in the breast. It has been reported that ERα+ has a very high rate (80%) among all breast cancer cases diagnosed in post-menopausal women (Poschner et al. 2019). Although not valid for all studies, it has been reported in general studies that ERα causes a proliferative effect in the breast, and ERβ mostly causes a pro-apoptotic and anti-proliferative effect and inhibits cell proliferation supported by ERα in tissues such as the uterus and breast (Paterni et al. 2014; Hilton et al. 2018).

3. Phytoestrogens and their estrogenic activities

Phytoestrogens are polyphenolic plant-derived compounds structurally similar to the mammalian estrogen 17-β-estradiol, which can exhibit both weak estrogenic and antiestrogenic properties and bind to their receptors (Power et al. 2008; Velentzis et al. 2008). Due to their nature, they can interfere with hormonal signaling by acting as weak estrogenic factors (Torrens-Mas & Roca 2020). Although phytoestrogens have many beneficial effects on health, the main mechanism of their action is their binding to ERs (Gorzkiewicz et al. 2021). Phytoestrogens are characterized by hydroxyl groups in their phenolic rings and these structures match the hydroxyl groups of the aromatic rings of E2 (Basu & Maier 2018; Ceccarelli et al. 2022). The estrogenic activity of phytoestrogens depends on their affinity for specific ERs in the body (Moreira et al. 2014). These compounds, which can bind weakly to both ERα and ERβ, induce estrogenic or antiestrogenic effects, competing with E2 for ligand binding sites of the receptors (Ceccarelli et al. 2022).

Phytoestrogens; classified as isoflavones, coumestans, stilbenes, and lignans, and the isoflavone and lignan family of phytoestrogens are the most frequently studied groups (Basu & Maier 2018; Tanwar et al. 2021). Soybean and soy-derived foods are the most important sources of isoflavone species in acetyl and malonyl glucoside forms (daidzin, glycitin, and genistin) (Helferich et al. 2008). Genistein, daidzein, glycitine, biochanin A, and farmononentin are compounds of the isoflavone group. While genistein and daidzein are the major isoflavones, biochanin A and farmonentin are the precursors that are metabolized to these two isoflavones, respectively (Tanwar et al. 2021).

The most important sources of lignans, a group included in the phytoestrogen family, can be listed as fiber-rich cereal bran, as well as fruits, vegetables, cereals, and seed pods (Velentzis et al. 2008; Touré et al. 2010). Lignans are chemically composed of two phenylpropane units linked by a β-β' bond between the central atoms of their respective side chains; are natural, biologically active compounds (Torrens-Mas & Roca 2020; Chhillar et al. 2021). They are found in small amounts in many foods (whole grains, sesame seeds, vegetables, and fruits) but at significant levels in FS (Di et al. 2018). After ingestion, plant lignans are metabolized by intestinal bacteria to enterolignans, enterodiol (END), and enterolactone (ENL) (Duffy et al. 2007). These metabolites, which are formed following the metabolism of plant lignans in the colon, pass into the circulation and target tissues (Patel et al. 2012).

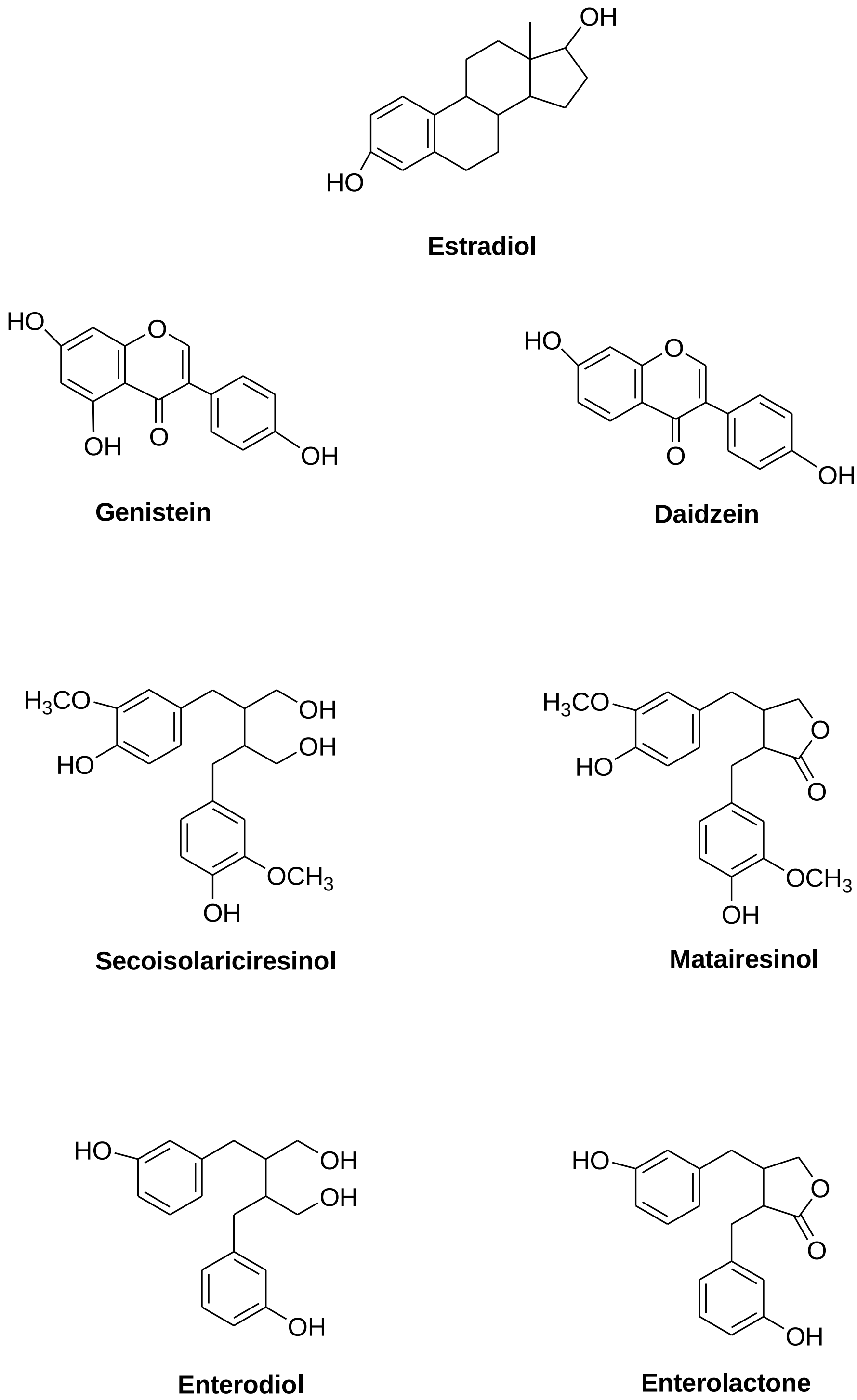

Figure 1- Chemical structures of 17β-estradiol and some isoflavones (Genistein and Daidzein) and lignans (Secoisolariciresinol, Matairesinol, Enterodiol, and Enterolactone)

4. Flaxseed content

Flax (*Linum usitatissimum*) in the form of seed or seed oil is a functional food known for its exceptional nutritional values (Buckner et al. 2019; Hu et al. 2019; Bhimjiyani et al. 2021). FS consists of two types, brown and golden, depending on the climatic conditions in which it grows (Calado et al. 2018). FS contains high levels of dietary fiber, lignans, abundant micronutrients, and omega-3 fatty acids (Khan et al. 2007; Truan et al. 2010; Lee & Cho 2012; Bak et al. 2016). It is unique among oilseeds because of the important fatty acids it contains, such as α-linolenic acid (ALA). It was determined that the concentration of ALA in FS was higher than the concentration of linoleic acid and oleic acid (Kavousi & Chavoshi 2020). It is very important for people to include foods high in ALA in their diet. This is because the human body lacks the desaturation enzymes necessary to produce these fatty acids (Simopoulos 2002). Omega-3 fatty acids are provided in the diet largely through the consumption of seafood. Therefore, both vegetarian and non-fish populations seem to be at a disadvantage in terms of omega-3 intake (Goyal et al. 2015). FS is a rich vegetarian source thought to meet this need. In addition, FS is rich in dietary fibers, which are known to have many beneficial effects such as modulation of the gut microbiota (Taibi et al. 2019).

5. Flaxseed lignan sources

FS is the richest source of the phytoestrogen secoisolariciresinol diglycoside (SDG), an important plant lignan that can be metabolized by bacteria in the animal or human colon to the mammalian lignans ENL and END (Chen et al. 2002; Chen et al. 2006; Power&Thompson 2007). SDG is a type of polyphenolic lignan found in FS and seeds rich in different oils, whole grains and legumes, and some fruits and vegetables (Dobrowolska&Regulska-Ilow 2021). The predominant lignan in FS is SDG, which makes up 95% of the lignan content of the seed. The remaining 5% consists of lariciresinol, pinoresinol, and matairesinol lignans (Westcott & Muír 2003; Tannous et al. 2020).

After oral ingestion of SDG, it is hydrolyzed to secoisolariciresinol (SECO) and *Peptostreptooccus* and *Eubacterium* bacteria in the colon convert this lignan to the mammalian lignans ENL and END by dehydroxylation and demethylation reactions (Khan et al. 2007; Patel et al. 2012; Bowers et al. 2019) (Figure 2). It is known that SDG and its metabolites enterolignans have preventive and protective effects on various types of cancer due to their antiproliferative, antiestrogenic, antioxidant, or inhibiting some enzymes (Alphonse & Aluko 2015). It has been reported that END and ENL are structurally similar to estrogen and bind to the estrogen receptor (ER), reducing the risk of cancer and exhibiting antiestrogenic effects (Saggar et al. 2010; Calado et al. 2018). It has also been shown that END and ENL can respond to benign prostatic hyperplasia, prostate tissue, and breast cancer by inhibiting 5α-reductase and aromatase-like enzymes (Brooks & Thompson 2005; Zhang et al. 2008).

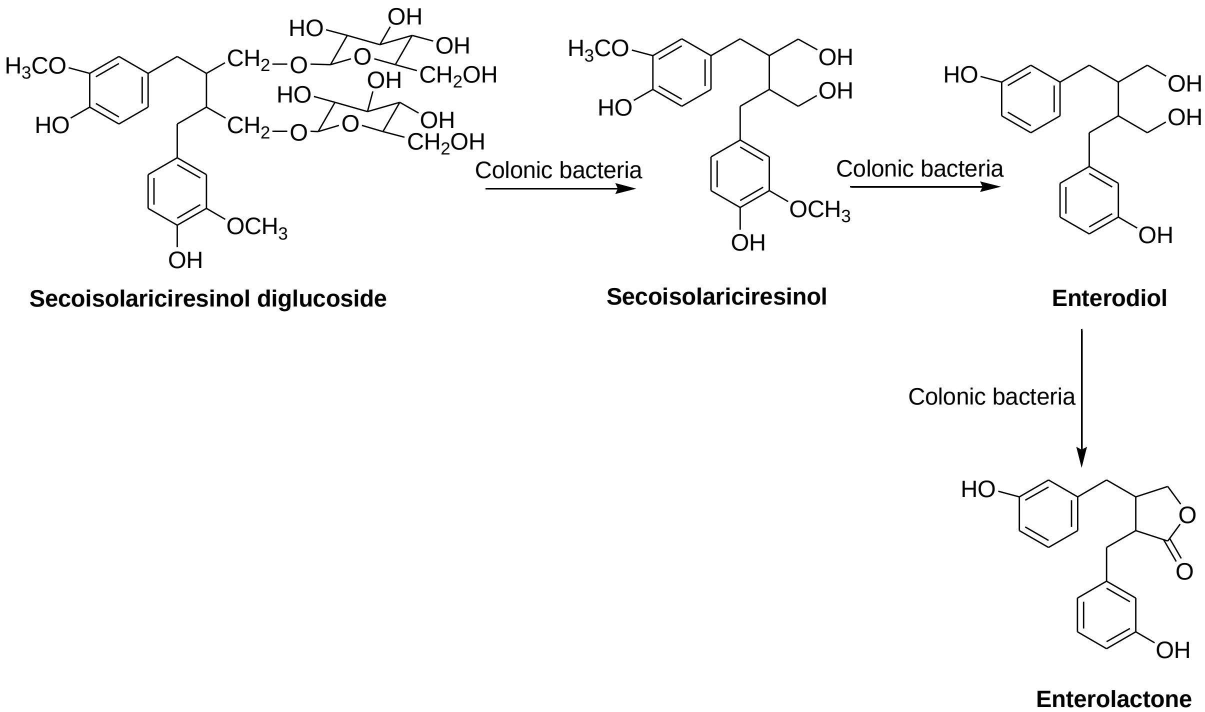

Figure 2- Transformation of SDG to END and ENL

6. Some studies on breast cancer with flaxseed components

Various studies have shown that dietary FS, flaxseed oil (FSO), and FS lignan components have inhibitory effects on the growth and development of breast cancer cells, and FS components combined with chemotherapeutic agents applied to cancer cells have a supportive effect on the cytotoxicity of drugs (Table 1). Chen et al. (2004) investigated the effects of dietary FS and tamoxifen (TAM) alone and in combination on the growth of estrogen-dependent human breast cancer (MCF-7) in athymic mice. It has been reported that FS highly regresses the tumor size before treatment at low E2 level. FS potentiated the tumor inhibitory effect of TAM at both low and high E2 levels. Di et al. (2018) proved in their study that FS lignans significantly increased the cytotoxic effect of classical chemotherapeutic agents (docetaxel, doxorubicin, and carboplatin) in metastatic breast cancer cell lines SKBR3 and MDA-MB-231. It has been observed that the combined treatment of trastuzumab and flaxseed oil, which is the primary treatment method for tumors, resulted in decreased cell proliferation of Akt and MAPK pathways and HER2 signaling, and activation of apoptosis. It has also been indicated that FSO alters the tumor fatty acid profile, which likely contributes to its effect on signaling pathways (Mason et al. 2015).

According to experimental studies, flaxseed has no interaction with drugs used in the treatment of breast cancer and may provide an additional protective effect when consumed with treatment. In animal studies, intake of flaxseed, flaxseed oil, or lignan SDG in combination with TAM has been confirmed to reduce tumor size more than TAM treatment alone (Calado et al. 2018). FS and FSO, a rich source of lignans and ALA, have been found to inhibit growth and metastasis, reduce tumor size, and increase apoptosis of human breast cancer implanted in athymic mouse models (Chen et al. 2002; Chen et al. 2006; Truan et al. 2010). The phenolic extract obtained from FS oil exhibits a strong synergistic effect to reduce proliferation in breast cancer cell lines, especially with chemotherapeutic treatment. It was seen in the study by Guerriero and coworkers that the application of the relevant extract in MCF-7 cells, when applied alone and in combination with the drug, induces apoptosis by increasing the mRNA expression of genes such as p53, Bax, p38, and caspase-3. They found that when combined treatment with DOX, especially in MCF-7 cells, it triggered both extrinsic and intrinsic apoptotic mechanisms. In MDA-MB-231 cells, on the other hand, it was shown that combined treatment induced only the genes related to the extrinsic apoptotic pathway, but both apoptotic pathways were activated when the extract was applied alone (Guerriero et al. 2017). Hu et al. (2019) showed that there was a dose- and time-dependent decrease in cell viability of MCF-7 cells treated with flaxseed extract, increased lipid peroxidation, and triggered apoptosis. In addition, it was determined that there was an increase in the expression of p53, cleaved caspase-3, cleaved-PARP, and cleaved caspase-7 in FS extract-induced apoptosis, and the mitochondrial membrane potential ($\triangle\Psi m$) decreased due to the activation of mitochondrial dysfunction.

Treatment with FSO in different cancer cell lines (cervical cancer, murine melanoma, leukemia, and breast cancer cell lines) has been shown to dose-dependently reduce the growth of malignant cells. In addition, it was determined that MCF-7 breast cancer and B16-BL6 murine melanoma cell lines regulate caspase activation by promoting caspase and PARP cleavage, increasing DNA fragmentation, and inducing apoptosis. The results revealed that cancer cell growth can be specifically inhibited by FSO (Buckner et al. 2019). Tumor size was reduced in mice receiving SDG supplementation. It was determined that phospho-p65 (p-p65), which is the activated form of a subunit of NFκB, was significantly reduced. In addition, *in vitro* ENL administration was found to inhibit viability, survival, and expression of NF-κB target genes in E0771, MDA-MB-231, and MCF-7 breast cancer cell lines (Bowers et al. 2019). Researchers have shown that SDG metabolites reduce proliferation, adhesion, migration, and invasion in estrogen receptor-negative (ER-) human breast cancer MDA-MB-231 cell line and increase the response of these cells to chemotherapy treatment (Mali et al. 2012; Di et al. 2018; Xiong et al. 2015; Bowers et al. 2019).

In a study examining the effect and potential mechanism(s) of TAM alone or in combination with SDG, and FO on the growth of established human estrogen receptor-positive (ER+) breast tumors, it was shown that FS components contribute to the efficacy of TAM by triggering decreased cell proliferation and increased apoptosis by modulation of ER and growth factor-mediated signaling pathways (Saggar et al. 2010). Chen et al. (2009) found that both FS and SDG can significantly reduce the growth of human mammary tumors located in athymic mice and mRNA expressions of some estrogen-sensitive genes, cyclin D1, pS2, ERα and Erβ, and biomarkers in signaling pathways such as epidermal growth factor receptor. SDG and its different derivatives have proven to be promising agents with antiestrogen and pro-apoptotic effects in hormone-dependent breast cancer cells (MCF-7) (Scherbakov et al. 2021).

ENL-induced cellular changes in MDA-MB-231 cells have been investigated and it has been reported that lignan has an antiproliferative effect in these cells and decreases the mRNA levels of Ki67, PCNA, and FoxM1 genes, which are related to cell proliferation (Xiong et al. 2015). It was determined that ENL decreased the viability of MDA-MB-231 and T47D breast cancer cell lines in a concentration- and time-dependent manner, significantly increased the radiosensitivity of the cells by abolishing G2/M blockade, impairing DNA repair, and increasing radiation-induced apoptosis (Bigdeli et al. 2016). It has been reported that the treatment of ER+ breast cancer cells with lignan extracts obtained from flaxseed changes possible estrogen signaling, increases estradiol production depending on the increase in concentration, and down/up-regulates ERα and ERβ expression depending on the concentrations, causing changes in cell development (Richter et al. 2010).

Table 1- Various studies on the effects of FS and its components on breast cancer

Model	***Dosage***	***Outcome***	***Reference***
Ovariectomized mice	10% dietary FS	Reduction in tumor area at both low and high E2 levels	Chen et al. 2004
SKBR3/ MDA-MB-231 cell lines	SECO with 1000µM with serial dilutions ENL (3.12-1000 µM)	Enhancing of FS lignans, especially ENL to the cytotoxic activity of chemotherapeutic drugs	Di et al. 2018
Ovariectomized athymic mice	4% FSO diet (1 or 2.5 mg/kg)	Modulation of combined therapy with reduction of HER2 signaling by AKT and MAPK pathways and decreased cell proliferation and increased apoptosis	Mason et al. 2015
Female athymic nude mice	10% FS diet	Inhibition of breast cancer growth and metastasis	Chen et al. 2002
Female athymic nude mice	SDG (0.2 g/kg) and FO (36.53 g/kg) in the 10% FS diet	Inhibition of tumor metastasis	Chen et al. 2006
Ovariectomized athymic mice	FSO (40 g/kg)	Reduction in tumor size and tumor proliferation, increased apoptosis, decreased expression of EGFR, EGFR2 and Akt	Truan et al. 2010
MCF-7 MDA-MB-231 cell lines	A phenolic extract from FS (3.9-258 µg/mL)	Decreased cell proliferation in both cell lines, induction of apoptosis by increasing mRNA expression of genes such as p53, Bax, p38, and caspase-3 of either cell type-dependent treatment alone or in combination	Guerriero et al. 2017
MCF-7 cell line	FS extract (0-320 µg/mL)	Dose and time-dependent cell growth inhibition, increase in apoptosis and lipid peroxidation; Increased expression of p53, cleaved-caspase 3, cleaved-PARP, and cleaved-caspase-7, decreased mitochondrial transmembrane potential	Hu et al. 2019
MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cell lines, and other cancer cell lines	0.3%-0.9% (v/v) of flaxseed	Inhibition of cancer cell growth, caspase activation, and induction of apoptosis in some cancer lines (MCF-7 and B16-BL6)	Buckner et al. 2019
C57BL/6 mice, MDA-MB-231, and MCF-7 human cell lines, EO771 mouse mammary tumor cell line	SDG, 100 mg/kg diet ENL (1 µM or 10 µM)	Reducing the expression of p-p65 in mice with SDG administration, reducing cell viability and expression of NF-kB target genes in breast cell lines with ENL application	Bowers et al. 2019
MCF-7, and MDA-MB-231 breast cancer cell lines	ENL (25 or 50 µM)	Inhibition of proliferation and migration of breast cancer cells; downregulation of MMP2, MMP9, and MMP14 gene expressions	Mali et al. 2012
MDA-MB-231 cell line	ENL (0-400 µM)	Accumulation of cells in the S phase and decreased cell proliferation, decrease of mRNA expression levels of Ki67, PCNA, and FoxM1 genes, which are genes associated with cell proliferation and expression of genes associated with the S and G2/M phases of the cell cycle, inhibition of migration and invasion	Xiong et al. 2015
BALB/c athymic mice	F0 (38.5 g/kg diet), SDG (1 g/kg diet) or their combination (10%FS diet)	Decrease of expression of related genes and proteins involved in ER- and growth factor-mediated signaling pathways and cell proliferation	Saggar et al. 2010
Ovariectomized athymic mice	FS (100 g/kg diet) SDG (1 g/kg diet)	Reduction of palpable tumor size, cell proliferation, induction of	Chen et al. 2009

	FH (lignan-rich fraction)(18 g/kg diet)	apoptosis; decrease of expressions of Bcl2, cyclin D1, pS2, ERα, ERβ, EGFR, HER2, and IGF-IR mRNA	
MDA-MB-231 T47D cell lines	ENL (1,10,50,100,200,500 µM)	Decrease of cell viability, elimination of G2/M arrest, DNA repair disruption, and increased radiation-induced apoptosis and radiosensitivity	Bigdeli et al. 2016
MCF-7 cell line	Flax root lignans (10-50-100 µg/mL)	Concentration-dependent increase in estradiol production, ERα and ERβ downregulation at high concentrations	Richter et al. 2010

7. Conclusions

FS is a nutritious functional food containing a high concentration of fiber-based lignans and omega fatty acids. Lignans, which are natural biologically active compounds, are a class of chemicals suitable for drug development. The predominant lignan in FS, SDG, is converted to its metabolites by bacteria in the colon after oral ingestion. Since SDG and its metabolites exhibit phytoestrogenic properties due to their special structures, their potential to affect estrogen-sensitive breast cancer-like cancer types has been focused on. Studies have proven that the nutraceutical application of FS components alone or in combination with drugs can show potent therapeutic efficacy in stopping or reducing breast cancer progression. Understanding how FS interacts with malignant cells offers hope for developing an anticancer drug or drug combination that can cause minimal side effects against cancer cells.

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Few-shot Learning in Intelligent Agriculture: A Review of Methods and Applications

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ABSTRACT
Due to the high cost of data acquisition in many specific fields, such as intelligent agriculture, the available data is insufficient for the typical deep learning paradigm to show its superior performance. As an important complement to deep learning, few-shot learning focuses on pattern recognition tasks under the constraint of limited data, which can be used to solve practical problems in many application fields with data scarcity. This survey summarizes the research status, main models and representative achievements of few-shot learning from four aspects: model fine-tuning, meta-learning, metric learning and data enhancement, and especially introduces the few-shot learning-driven typical applications in intelligent agriculture. Finally, the current challenges of few-shot learning and its development trends in intelligent agriculture are prospected.

Keywords: Few-shot learning; Intelligent agriculture; Meta-learning; Metric learning; Fine-tune; Data augmentation

1. Introduction

With the advancement and evolution of technologies, the development and application of machine learning have become more and more sophisticated (Yang et al. 2022). Represented by convolutional neural networks, machine learning has achieved results comparable to or surpassing humans in many tasks (Nie et al. 2023). Machine learning can achieve such proud results due to the massive data sets and prior knowledge behind it. Unlike humans, which can draw a tiger with a cat as a model, machine learning requires many datasets to train to ensure the accuracy of the results (Li et al. 2021; Li & Ercisli 2023). Currently, machine learning models usually rely on large datasets to ensure the accuracy of training results, which is difficult to achieve in some industries (Volkan et al. 2023). In particular, as represented by the agricultural field, the cost and difficulty of dataset collection and annotation are relatively high, and the construction of large datasets is expensive. In the traditional development of intelligent agriculture, a large amount of data is often required to support the control of the cultivation process of crops. By utilizing few-shot learning, the amount of data required can be significantly reduced, and the implementation cost is much cheaper. Therefore, in order to reduce the dependence on large datasets, the research on few-shot learning has begun to go into depth gradually, and how to reduce the massive demand of data for machine learning models through scientific methods has become a hot issue in current research (Parnami & Lee 2022; Yang et al. 2022).

Few-shot learning can be defined as machine learning with limited data supervision. A machine learning model learns feature information of relevant classes with only a few data samples (Wang et al. 2020). Inspired by the fact that human children can recognize an animal species through a few pictures in an encyclopedia, researchers want machine learning models to have such capabilities. Research on few-shot learning is gradually emerging. Especially in the field of intelligent agriculture, research on few-shot learning has been very popular, and many excellent modeling algorithms based on few-shot learning have been applied in the fields of crop image recognition and classification (Yang et al. 2022).

In the case of few-shot learning, researchers mostly conduct research from two perspectives. One is to start from the data, expand and supplement the data, and strengthen the diversity of data features. And the other is from the perspective of the network model. The network model plays a vital role in machine learning performance. A good network model framework structure and reasonable network model parameters can significantly improve the network model's accuracy, generalization ability, fast convergence ability, and other performance. Using different machine learning frameworks, researchers optimize and adjust the network model's parameters and structure to make it perform well in the case of few-shot data. Therefore, in the development of few-shot learning, it is possible to broadly classify few-shot learning into the following categories according to the specific methods used: few-shot learning based on model fine-tuning, few-shot learning based on meta-learning, few-shot learning based on metric learning, and few-shot learning based on data augmentation. The Basic ideas of each few-shot learning are shown in Table 1. At present, with the continuous development and optimization in few-shot learning, a large quantity of application cases of few-shot learning have emerged in many industries, such as remote sensing computer vision (Sun et al. 2021; Guo, Wang et al. 2022), intelligent agriculture (Li & Yang 2021; Nie, Wang et al. 2022a;), biomedicine (Yin et al. 2020), plant protection (Li et al. 2020; Li & Chao, 2021b), magnetic field physical and chemical parameter prediction (Nie et al. 2021; Nie et al. 2022), geological exploration (Liu et al. 2022), radar ranging (Yue Yang et al. 2021), point cloud segmentation (Guo et al. 2020), etc.

Table 1- The basic ideas and representative works of few-shot learning

Few-shot learning	*Basic ideas*	*Representative works*
Few-shot learning based on model fine-tuning	Fine-tuning the network model parameters to make the common network model still performs well with few-shot data	ULMFit (Howard & Ruder 2018) co-FCN (Rakelly et al. 2018) LM-BFF (Gao et al. 2020)
Few-shot learning based on meta-learning	Using the meta-knowledge learned by the model in various tasks to adjust the parameters of the model and improve the model's fast convergence in few-shot tasks	MAML (Finn et al. 2017) ANIL (Raghu et al. 2019) MTL (Sun et al. 2019) MnnFAST (Jang et al. 2019)
Few-shot learning based on metric learning	Using embedding space, determining similarity among samples to reduce the overfitting problem of the model with few-shot data.	FSLM (L. Yang et al. 2020) AMN (Mai et al. 2019) IPN (Zhong Ji et al. 2020) SARN (Hui et al. 2019)
Few-shot learning based on data augmentation	Improve few-shot learning by increasing the diversity of the data and enhancing the original characteristics of the data to make it easier to be classified	TPN (Y. Liu et al. 2018) DAGAN (Antoniou et al. 2017) FATTEN (B. Liu et al. 2018)

This article will outline and respond to the research questions below:

1) How is the present state of studies about few-shot learning?
2) What is the practical application of few-shot learning in intelligent agriculture?
3) What are the challenges that few-shot learning will face in the future?
4) What are the prospects of few-shot learning in intelligent agriculture?

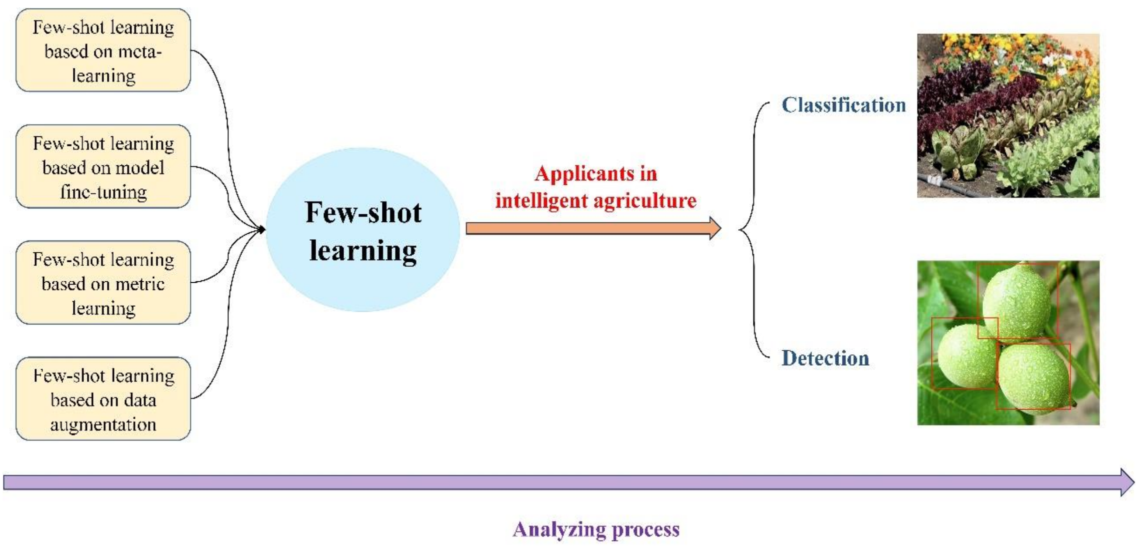

Figure 1- Narrative structure of article

The analyzing process of this paper is shown in Figure 1. We will first summarize the research status of few-shot learning based on model fine-tuning, few-shot learning based on meta-learning, few-shot learning based on metric learning, and few-shot learning based on data augmentation, then summarize the typical applications of few-shot learning in intelligent agriculture, and finally discuss and outlook the future challenges of few-shot learning and the prospect of its development in intelligent agriculture.

2. Few-shot learning based on model fine-tuning

The network model performance depends in part on the initialization parameters of the network model. Due to the small amount of data for few-shot learning, it is hard to train deep models. Many general models have poor convergence in few-shot learning, so since the advent of few-shot learning, the parameter optimization of network models has never stopped. Compared with the research of a new network model, it is easier to upgrade and optimize the key parameters of the old network model. Few-shot learning based on model fine-tuning will firstly train the network model on a dataset with a huge number of samples to generate a pre-trained model, and subsequently adjust the parameters of the top layer of the pre-trained model on the few-shot dataset so that the performance results of the parameter-adjusted network model on the few-shot dataset and the source dataset are similar to achieve the desired results. By fine-tuning the network model with parameters, the network's rapid convergence ability and the network generalization ability are improved. Few-shot learning based on model fine-tuning is shown in Figure 2.

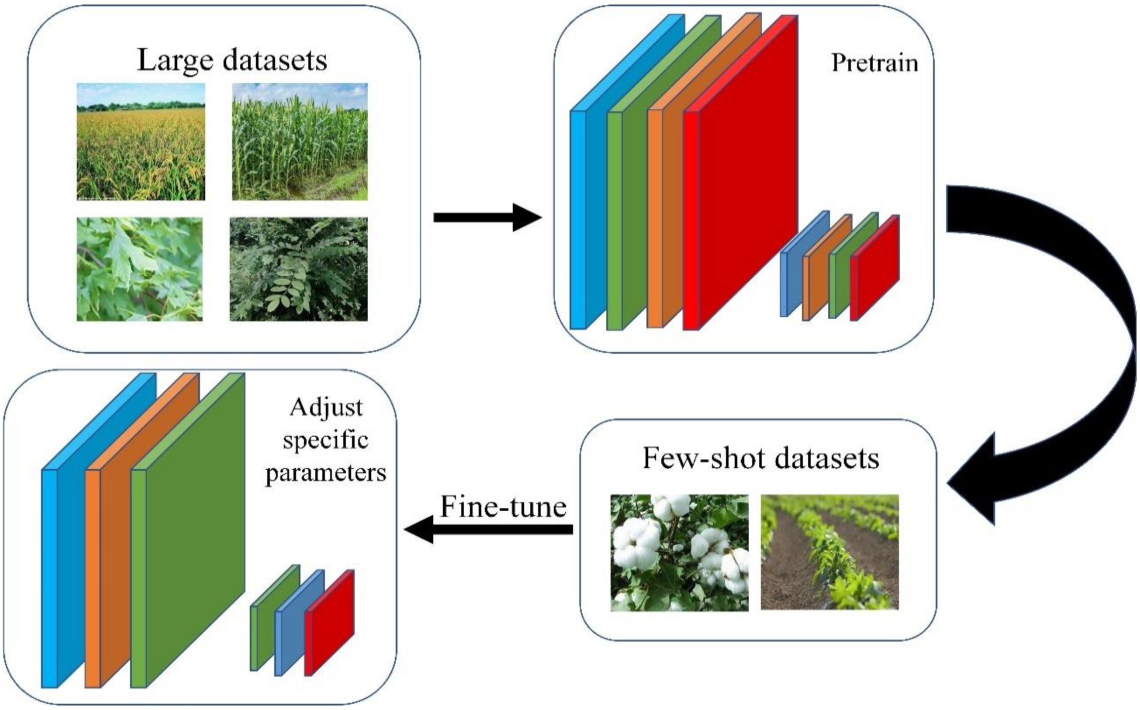

Figure 2- Few-shot learning based on model fine-tuning

Howard et al. (2018) proposed the Universal language model fine-tuning (ULMFit), a method mainly applied to text classification tasks, and obtained favorable results in multiple text classification missions. This method first pre-trains the language model, then fine-tunes the language model to achieve the expected effect by setting the learning rate and slanted triangular learning rates, and finally fine-tunes the classifier to further improve the text classification effect by adding a linear layer to the language model. ULMFit improves the convergence of the model on few-shot datasets and enhances the target classification effect by setting various learning rates for various layers of the language model and proposing slanted triangular learning rates. Rakelly et al. (2018) proposed co-FCN, by introducing conditional information, the network is able to perform semantic segmentation task with only a small amount of labeled data. The model fine-tuning method proposed by Nakamura & Harada (2019) improves the recognition accuracy and reduces the degree of model overfitting by reducing the learning rate when training on few-shot datasets, using an adaptive gradient optimizer, and adjusting the entire network. Chen et al. (2019) summarize and compare the existing methods. The authors study the influence of data, network structure and other factors on different methods, which provide directions and guidelines for subsequent research. Dhillon et al. (2019) verified the good effect of transductive fine-tuning for improving the performance of few-shot learning, analyzed the limitations of the current benchmarks, and proposed a new index to measure the performance of few-shot learning. Inspired by the GPT-3 model, Gao et al. (2020) proposed a model fine-tuning method LM-BFF based on a language model, which can fine-tune language models with few-shot samples. This method mainly includes automatically constructing templates and tag word maps, automatically combining templates and tag word maps into prompts, fine-tuning templates according to prompts, and dynamically selecting sample instances as input contexts. The authors' team conducted experiments on multiple few-shot datasets, and the results showed that LM-BFF method significantly outperformed other standard fine-tuning methods, and its average performance improvement could reach about 11%. Tian et al. (2020) concluded that the performance of embedding model plays an important role in few-shot classification. The authors used self-distillation to further enhance the feature extraction effect and realize the performance improvement. Boudiaf et al. (2021) found that the way in which inference is performed has a large impact on the performance of few-shot learning, which has often been overlooked by previous studies. The authors utilized a new loss containing complementary phases to improve the original performance. In order to solve the forgetting problem in few-shot learning, (Fan et al. 2021) designed Retentive R-CNN and tested its good performance through experiments. Kaul et al. (2022) presented a pseudo-labeling method for the target detection task. The method can generate high-precision pseudo-labels, eliminate the class imbalance problem, and improve the accuracy and effect of detection.

As a relatively simple and general method, few-shot learning based on model fine-tuning can significantly improve the problems of poor convergence, poor generalization ability, and poor recognition accuracy of ordinary networks in the face of few-shot datasets. Researchers have achieved fine-tuning of the network model by improving the pre-trained model or changing the learning rate of the network. However, it is notable that few-shot learning based on model fine-tuning is prone to overfitting problems, which will also become the focus of researchers' future research. For the intelligent agricultural field, using few-shot learning based on model fine-tuning to reduce the cost of data collection is a relatively easy way to realize. By changing the parameters and structure of traditional models, accurate analysis of agricultural data can be realized.

3. Few-shot learning based on meta-learning

Meta-learning focuses on allowing machine learning to acquire the ability to learn, allowing machine learning to learn, and its purpose is to make machine learning have the ability to carry out analogical associations like people (Wang et al.2022). Through this learning ability, machine learning can learn meta-knowledge in different tasks and apply this meta-knowledge to future tasks. Meta-knowledge includes loss, noise, optimizer, and so on. Few-shot learning based on meta-learning improves the effectiveness of few-shot learning by using meta-knowledge learned from other tasks to parameterize the target task model based on the target task training. At present, few-shot learning based on meta-learning includes model-agnostic meta-learning methods, meta-transfer learning methods, and memory-augmented neural network meta-learning methods.

3.1. Model- agnostic meta-learning method

The applicability of the model-agnostic meta-learning method is very broad, and most models can be used with this method. The model-agnostic meta-learning (MAML) framework proposed by Finn et al. (2017) is a typical representative, which optimizes the model by using gradient descent methods. The focus of MAML is to find a weight for the model with strong generality and universality, which enables the model to be well applied to the new task after several gradient descents and improves the model's fast convergence in the face of few-shot data tasks. But at the same time, MAML also has certain shortcomings. It is more sensitive to the network structure and its stability is not high during training. The model is also computationally intensive and takes a long time to train. In order to solve these problems, Antoniou et al. (2018) proposed various modifications to MAML by using Multi-Step Loss Optimization (MSL), Derivative-Order Annealing (DA), Learning Per-Layer Per-Step Learning Rates and Gradient Directions (LSLR), Cosine Annealing of Meta-Optimizer Learning Rate (CA) and other methods. Greatly improved its stability, generalization performance, convergence speed, and reduced its training time. Among them, MSL can mainly enhance the generalization performance and stability of MAML. DA can solve the gradient explosion problem in the training process. LSLR can reduce the computing memory and help alleviate the problems of overfitting. CA can adjust the meta-optimizer to improve the fitting effect and generalization performance of the model. Raghu et al. (2019) also optimized MAML, simplified and improved the MAML framework by removing the inner loop updates of all neural networks except the head, and proposed the Almost No Inner Loop (ANIL) algorithm.

3.2. Meta-transfer learning method

Meta-transfer learning method incorporates the advantages of meta-learning and transfer learning. A Meta-Transfer Learning (MTL) model was proposed by Sun et al. (2019). In general, deep neural networks are easy to overfit few-shot data, so meta-learning usually uses shallow neural networks, but shallow neural networks will harm the model's performance. MTL models can make good use of deep neural networks to improve model performance under the condition of normal fitfully. Since then, to continue to enhance the MTL model's performance, Sun et al. (2020) introduced the hard task (HT) meta-batch scheme after research, which improved the learning efficiency, fast convergence ability, and accuracy of MLT. Soh et al. (2020) extended meta-transfer learning to zero-sample learning, and found general initialization parameters suitable for internal learning by using the external and internal information of the image. The image recognition effect was good, and the recognition speed was fast.

3.3. Memory-augmented neural network meta-learning method

The memory-augmented neural network meta-learning method is an early proposed method for few-shot learning. Compared with traditional neural networks, memory-augmented neural networks add memory modules and reading and writing mechanisms, which can efficiently learn the display strategy of the corresponding task. Santoro et al. were inspired by the Neural Turing Machine (NTM) (Santoro et al. 2016) and used it to few-shot learning. The author uses an external memory module to store sample features and optimize the read and write mechanism using meta-learning to achieve effective few-shot classification and prediction. To further enhance memory-augmented neural networks performance, Jang et al. (2019) proposed the MnnFAST architecture, suitable for large-scale memory networks. MnnFAST can effectively reduce memory bandwidth consumption and eliminate cache contention. At present, the research on memory-augmented neural network meta-learning is mainly divided into two aspects: hardware (Rae et al. 2016) and network structure (Gulcehre et al. 2017). Relevant researchers hope to further improve memory-augmented neural network meta-learning by enhancing the capacity, speed, architecture or neural network structure of hardware memory modules.

As a popular few-shot learning method, the diversity of methods based on meta-learning is self-evident through the above combing. Allowing machine learning to gain the ability to learn has been a long-standing desire of relevant researchers and an important means of solving few-shot data tasks. Few-shot learning based on meta-learning has broad development prospects in the future. Relevant researchers will continue to optimize meta-learners, improve the interpretability of meta-learning theoretically, and enhance meta-learning performance when performing few-shot data tasks experimentally. In intelligent agriculture, there have been many cases and attempts to utilize few-shot learning based on meta-learning. By combining the features of meta-learning with other methods, related researchers have made good progress in crop detection, farmland segmentation, and so on.

4. Few-shot learning based on metric learning

Metric learning, also called similarity learning. It relies on a set distance function to measure the distance between different samples, and this distance represents the difference between two samples. A larger distance means that the features of the two samples are more different, and a smaller distance means that the features of the two samples are more similar. The distance function is a key part of metric learning as a standard for measuring the distance between samples and is often used as distance functions for deep learning, such as Mahalanobis distance, Euclidean distance, Manhattan distance, or cosine similarity. The metric learning framework generally includes an embedding module and a metric module. The embedding module is responsible for embedding samples into the vector space, and the metric module classifies samples by judging the similarity between samples according to the distance function. The few-shot learning methods based on metric learning mainly include the few-shot learning method based on Siamese neural network, the few-shot learning method based on Matching network, few-shot learning method based on Prototype network, and the few-shot learning method based on Relation network. A generic model for metric learning is shown in Figure 3.

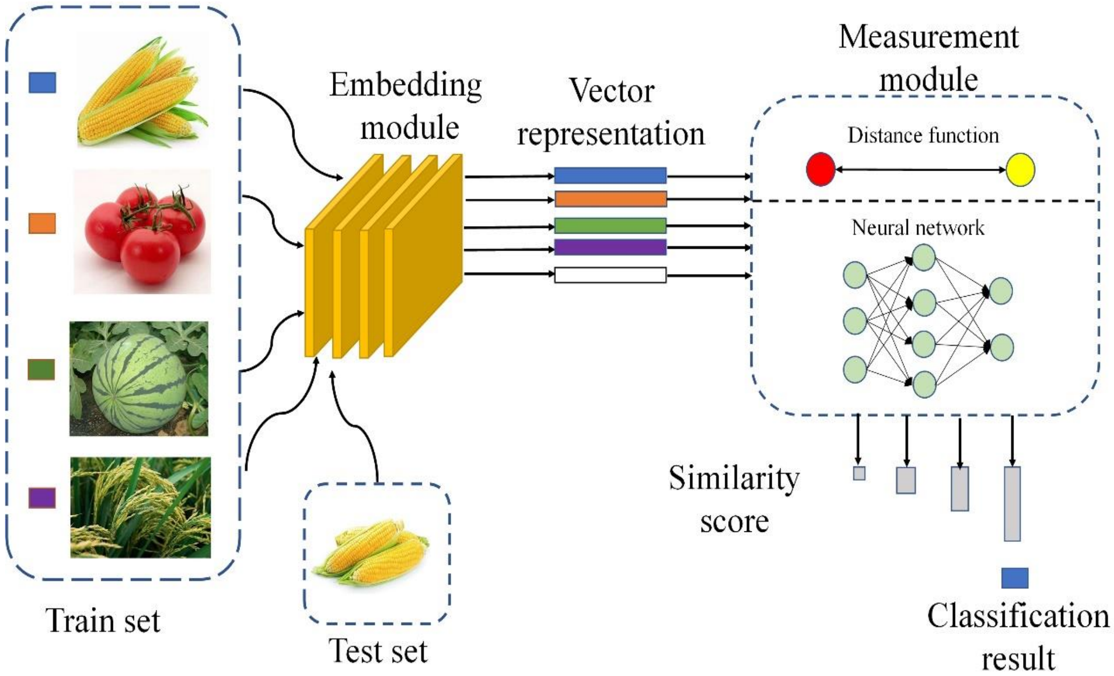

Figure 3- A generic model for metric learning

4.1. Few-shot learning based on Siamese neural network

A few-shot learning method based on Siamese neural network was first proposed by Koch et al. (2015) and the authors used Siamese neural networks to classify and identify single-sample data. The Siamese neural network can be viewed as two neural networks connected by the shared weight method. It contains two inputs, each of which maps one sample input to a high-dimensional feature space and outputs a representation. Then the distance function calculates the distance between the two samples to judge the similarity between the two samples. Based on Siamese neural network, Zhou et al. (2020) proposed a few-shot learning model FSL-SCNN, which is used for intelligent anomaly detection in industrial cyber-physical systems. The authors constructed a CNN-based Siamese neural network, which uses optimized features to calculate the distance between samples, alleviates the overfitting problem and improves anomaly detection accuracy. Yang et al. (2020) propose another model FSLM, in which the two subnetworks of the model consist of a self-attention model with the equal parameters. Compared with other few-shot learning methods, the FSLM model has superior accuracy and high robustness in text sentiment analyzing tasks. At present, the few-shot learning method based on Siamese neural network has developed relatively maturely, but its future still has a large development space due to the limitation of hardware conditions.

4.2. Few-shot learning based on Matching network

The most prominent feature of the Matching network is the introduction of an attention mechanism based on cosine distance. The cosine distance calculates the similarity between the training sample and the test sample, and then the similarity is normalized to obtain the attention distribution of the test sample on the training sample. Vinyals et al. (2016) proposed Matching network and applied it to one-shot learning problems in 2016. By introducing attention and memory mechanisms into learning tasks, the authors improved task accuracy on the ImageNet dataset by about 5%. Inspired by Matching network, Bartunov et al. (2018) came up with a Generative Matching Network. The authors improved the model's dynamic prediction performance by adjusting additional input datasets so that the model could quickly learn new concepts that were not in the training data. Mai et al. (2019) proposed an Attentive Matching Network (AMN) to solve the few-shot problem, which uses a feature-level attention mechanism to make the embedding network have better feature extraction capabilities. The authors also introduce a new complementary cosine into the loss function to boost the fit of the network on few-shot tasks.

4.3. Few-shot learning based on Prototype network

To continue to enhance the metric learning performance when facing few-shot tasks, a Prototype network model was proposed by Snell et al. (2017). The basic concept of the Prototype network is first to project the sample into the metric space, then calculate the center of each sample class, and determine the category to an input sample is judged according to the distance from input samples to the centers of different categories. Fort et al. (2017) used the Prototype network extension to generate Gaussian prototypical networks. Unlike prototype networks, a portion of the encoder output in Gaussian prototypical networks is represented as a Gaussian covariance matrix. The authors conducted experiments and tests on the Omniglot dataset, and the results were significantly better than the Prototype network. Ji et al. (2020) improved the Prototype network and proposed Improved Prototypical Networks (IPN). The author's work mainly includes two aspects. The first is proposing an attention analogy strategy, assigning different weights to different representative samples to get more representational weighting prototypes. The second is proposing a distance scaling strategy, which decreases the intra-class differences and enlarges the inter-class differences. These two works enhance the Prototype network's capability to use intra-class distribution information and enhance network's classification performance. Pahde et al. (2021) proposed a multimodal prototype network. The authors designed a cross-modal feature generation framework that can enrich the low-fill embedding space for few-shot data and better use auxiliary information. When processing computer vision tasks, multimodal prototype networks can simultaneously use data from other modalities represented by text to improve classification effects.

4.4. Few-shot learning based on Relation network

Siamese neural networks, Matching networks, and Prototype networks all require distance functions to calculate similarity, but the commonly used distance functions are not applicable to special tasks. To address those special issues, the Relation network came into being. In 2018, Sung et al. (2018) proposed a Relation network model. It calculates the distance between samples and analyzes the similarity between samples by constructing a neural network. The relationship network generally includes two sections. One is the embedding module, which is for withdrawing feature information of the sample. The other is the relation module, which calculates and compares similarities and outputs the similarity scores among samples. Hui et al. (2019) proposed a self-attention relation network (SARN) based on the Relation network model. The authors add an attention module to the Relation network model and use it to enhance the learned features. Wu et al. (2019) proposed a Position-aware relation network (PARN). The traditional Relation network uses CNN for distance calculation. The authors improved by introducing a deformable feature extractor (DFE) and designing a dual-correlation attention mechanism (DCA) to improve the detection accuracy of the network while making the network more lightweight.

Few-shot learning based on metric learning is now relatively mature. The emergence and development of the Relation network have made few-shot learning based on metric learning get rid of the shortcomings of only relying on a distance function. Compared with the traditional relying on distance function for similarity measurement, relying on deep neural networks for similarity measurement method has a wider range of application and higher classification accuracy. Relation network will also become the main direction of the few-shot learning based on metric learning in the future. As a comparatively well-established method, few-shot learning based on metric learning is playing an increasingly important role in intelligent agriculture. Especially in crop species, pest and disease identification, metric learning provides ideas and guidelines for solving agricultural few-shot problems.

5. Few-shot learning based on data augmentation

Data has a very important impact on the results of machine learning, and high-quality datasets are indispensable for satisfactory learning results, especially for few-shot learning (Li et al. 2022). To make few-shot learning have a high accuracy rate, relevant researchers began to study few-shot learning methods based on data augmentation. The researchers mainly start from two perspectives, one is to expand the original data, and the other is to enhance the characteristics of the data. Data enrichment refers to adding new data to the original data, such as unlabeled or synthetic labeled data. Feature enhancement refers to adding new features that are more obvious and easier to distinguish from the original data. There are three main methods of few-shot learning based on data augmentation: methods based on unlabeled data, methods based on data synthesis, and methods based on feature enhancement.

5.1. Methods based on unlabeled data

This method enriches few-shot dataset by adding unlabeled data to it. Common methods mainly include unsupervised learning, semi-supervised learning, and transductive learning. In this case, unsupervised learning refers to learning training samples without labels, which does not require the dataset author to label the dataset. The class of the training samples is unknown and needs to be classified according to the similarity between samples. Ji et al. (2019) studied a method for unsupervised few-shot learning by self-supervised training, which generates pseudo-labeled training examples by progressive clustering and optimizes data feature representation by episodic training to improve few-shot learning accuracy. Khodadadeh et al. (2019) investigated an UMTRA algorithm for few-shot data classification tasks. The algorithm combines the characteristics of meta-learning and unsupervised learning, and authors conducted experiments on Omniglot and Mini-Imagenet datasets with very good results. Lee & Chung (2021) proposed an Early-Stage Feature Reconstruction (ESFR) method for few-shot data classification. After a series of studies, the authors found that early generalization features during unsupervised training greatly help identify new few-shot sample classification.

Applying semi-supervised learning to few-shot learning is always a popular field for machine learning. Nassar et al. (2021) investigated SemCo, a semi-supervised learning approach combining label combination and joint training. It fully considers the visual similarity between categories and improves the accuracy of image classification by training two classifiers separately and training them jointly. Li et al. (2021a) came up with a semi-supervised learning approach using confidence interval adaptive selection of unlabeled samples for pseudo-labeling. The authors fully considered the problems of domain splitting and few-shot parameters and conducted experiments on the PlantVillage dataset. The average improvement rate obtained was satisfactory. Chao & Li (2022) put forward a semi-supervised few-shot classification approach on the basis of KNN distance entropy. It uses KNN distance entropy to automatically assign pseudo-labels and improve the data quality and the effect of few-shot learning.

Transductive learning is a sub-problem of semi-supervised learning, which will utilize unlabeled data as test data to enhance the generalization ability of the model. Through transductive learning, the model is exposed to not only training samples but also test samples while learning, fully utilizing various feature information from the data. Liu et al. (2018) presented the TPN that fully uses the idea of transductive learning. The network uses a graph construction module to learn how to pass labels to unlabeled samples. According to transductive learning, a cross-attention network was also proposed by Hou et al. (2019), which improved the recognition rate of features by introducing cross-attention modules to generate cross-attention maps for category features and query sample features. The authors also proposed a transformation reasoning algorithm to increase the representativeness of features through iterative methods. Ma et al. (2020) combined the idea of transductive learning with a graph neural network to propose a Transductive Relation-Propagation Network (TRPN). The authors modelled support-query pairs for the first time in the industry to consider support-query pairs relationships in few-shot learning problems.

5.2. Methods based on data synthesis

The method based on data synthesis mainly adds the synthesized new labeled data to the few-shot data to complete the expansion of the few-shot data. The common methods include Generative Adversarial Network (GAN), improving the optimization encoder, etc. The Generative Adversarial Network model is shown in Figure 4. Mehrotra & Dukkipati (2017) propose a generative adversarial residual pairwise network based on the GAN and uses the residual pair to measure the similarities among samples to solve single-sample problem. Antoniou et al. (2017) propose a data augmentation generative adversarial network (DAGAN) based on a Generative Adversarial Network, which can automatically learn augmented data and improve the performance of classifiers. In order to continue to enhance the generalization performance and feature interpretability of the generated data, (Xian et al. 2019) combined Variational autoEncoder (VAE) and GAN to develop a feature generation framework F-VAEGAN-D2. It generates features with good interpretability and can better complete the classification task of few-shot images. Zhou et al. (2021) put forward a data augmentation approach FlipDA that automatically performs label inversion. The authors found that label-flipping data had a greater impact on performance than other data, so using generative models and classifiers to generate label-flipping data greatly improved the effectiveness and robustness of data augmentation.

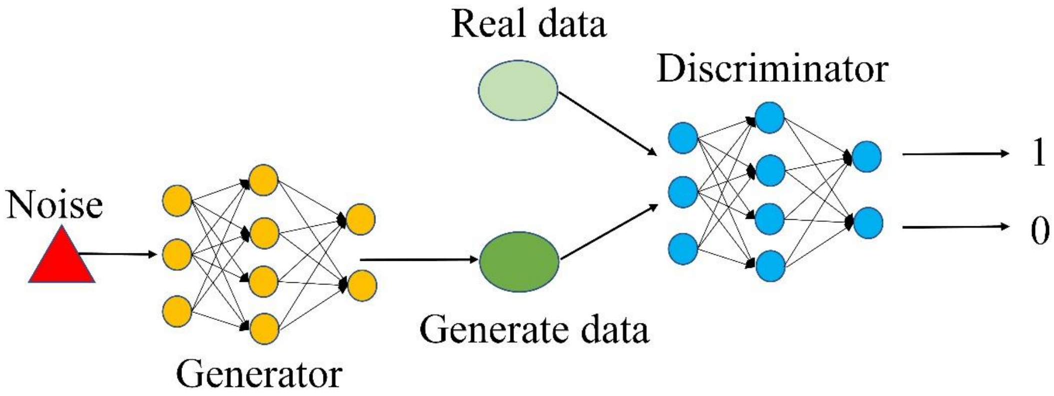

Figure 4- Generative Adversarial Network model

5.3. Methods based on feature enhancement

The main difficulties few-shot learning faces are the small amount of data and low sample diversity. As a common data augmentation method, the methods based on feature enhancement can make up for the lack of sample diversity. Its main principle is to improve the diversity of samples by enhancing the feature space of few-shot data.

In order to address the issue of data expansion in feature space, Liu et al. (2018) put forward a FeATure TransfEr Network (FATTEN). It is a feature transfer network that enables effective end-to-end training and can well record the feature trajectory of object attitude transformation. The authors conducted experiments on one-shot data and few-shot data to verify the good performance of FATTEN. Chen et al. (2018) achieved the enhancement of the feature space of few-shot data using semantic information. The authors propose a two-way network model TriNet. It first uses ResNet-18 network to withdraw the characteristics of input image, then maps the image characteristics to the semantic space for feature enhancement using TriNet's encoder, and finally maps the characteristics back to the image using the TriNet's decoder. Shen et al. (2019) used an uncertain attention mechanism on the model to improve model's generalization ability when facing few-shot data, and achieved optimization of network performance by using high-level features to guide the bottom-level features.

In summary, few-shot learning based on data augmentation has greatly progressed. Data is a crucial point in machine learning, and it is important to solve few-shot problems in terms of data viewpoint. Through data expansion and character enhancement, few-shot learning based on data augmentation can solve most few-shot issues with broad development prospects. From the perspective of data augmentation, further mining and expansion of agricultural data can reduce the cost of intelligent agriculture deployment and enhance the effectiveness and utilization of intelligent agriculture, leading to its wider application in some developing countries.

6. Applications of few-shot learning in intelligent agriculture

In the field of intelligent agriculture, researchers usually use devices represented by drones for data acquisition. The cost of data acquisition is high, the amount of data is few, and it is suitable to use few-shot learning for the research of related problems. Compared with traditional methods of intelligent agriculture, utilizing few-shot learning can reduce the difficulty and cost of data collection and further expand the scope of intelligent agriculture applications. At present, the application of few-shot learning in the field of intelligent agriculture mainly includes plant disease identification, pest identification, crop detection, and so on (Subburaj et al. 2023; Varol et al. 2022). We summarize few-shot learning’s common applications in intelligent agriculture and count the number of publications in the Web of Science (WOS) Core Collection database and Engineering Village (Ei Village) database over the last five years. The details are given in Figure 5 and Table 2. Also, the detailed specific applications of few-shot learning in intelligent agriculture over the past few years are further described.

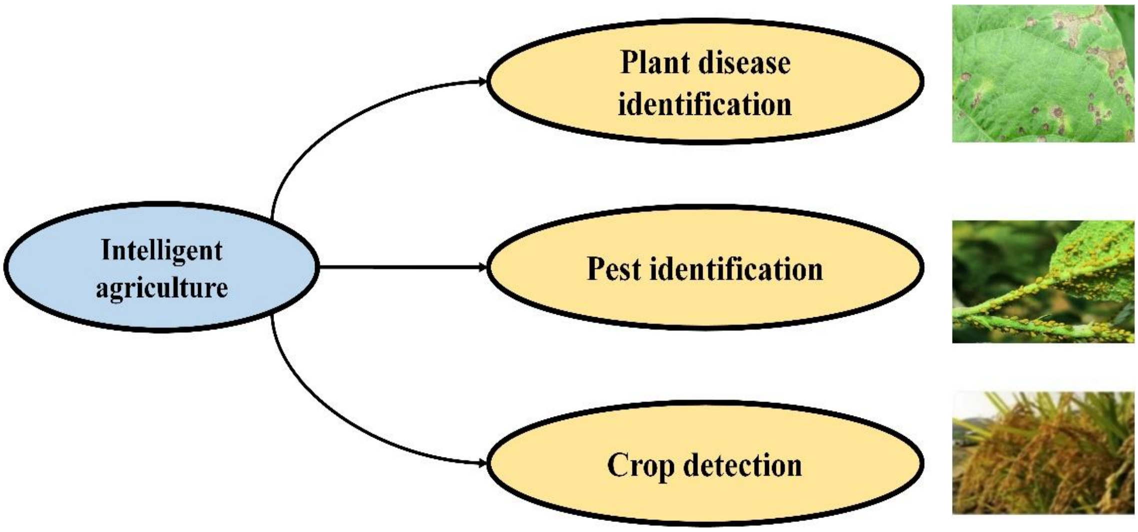

Figure 5- Common applications of few-shot learning in intelligent agriculture

Table 2- Literature publications on common applications of few-shot learning in intelligent agriculture

Common application	*Publish time*	*Search condition*	*Number of literatures*	
			WOS	*Ei Village*
Plant disease identification		Topic = few-shot learning And Topic = plant disease	37	31
Pest identification	2019-2023	Topic = few-shot learning And Topic = pest	18	14
Crop detection		Topic = few-shot learning And Topic = crop detection	25	25

6.1. Plant disease identification

Recognition and classification of plant diseases has always been an important problem facing intelligent agriculture, and how to extract disease features efficiently and realize high-precision classification is the direction of related researchers' continuous efforts. Argüeso et al. (2020) solved the problem of plant disease classification using few-shot learning. The authors used plant leaf images as the basis for the classification of different plant diseases and used a deep learning network model with triple loss, which greatly reduced the data sample requirements during training while improving the classification accuracy. Nuthalapati et al. (2021) realized the classification of plant diseases by using metric learning. The authors also produced a few-shot plant disease dataset, which provides some help for the subsequent related research. Chen et al. (2021) proposed an LFM-CNAPS model based on meta-learning. The model can predict unknown plant diseases with a small number of annotated samples, and at the same time, it can generate classification heatmaps for visualization. The authors produced a few-shot dataset of plant diseases and validated the LFM-CNAPS model on this dataset, obtaining good classification results. Wang et al. (2021) also improved meta-learning and proposed an IMAL model. The model has good fitting ability and generalization performance, and can achieve accurate classification of plant diseases. Aiming at the problem of limited feature extraction ability of few-shot learning, (Lin et al. 2022) enhanced the effect of plant disease feature extraction and improved the recognition of plant diseases by using the attention mechanism.

In the case of plant diseases, which are more diverse and have fewer samples, these problems can be adequately addressed using few-shot learning. Existing methods have demonstrated the bright future of few-shot learning in the field of plant disease identification.

6.2. Pest identification

Pests is a key factor affecting crop harvesting, using few-shot learning to accurately discover and identify pests is an important method to improve crop yield. Li et al. (2020) applied few-shot learning to identify cotton pests and diseases and greatly reduced the difficulty of collecting cotton pest and disease datasets by using few-shot learning. The authors extract the characteristics of disease and pest pictures and designed corresponding control programs and embedded terminals to realize the identification of pest species on cotton. Pandy et al. (2022) implemented rice pest recognition using meta-learning approach. The authors concluded that there are many species of rice pests and it is not realistic to collect a large amount of sample data for each pest, and this problem can be well solved by using few-shot learning based on meta-learning. Gomes et al. (2022) utilized a prototype network to achieve accurate recognition of pests. At present, the use of few-shot learning for pest identification and the construction of corresponding hardware terminals for control has become a popular direction for the development of few-shot learning in the field of intelligent agriculture.

Pests tend to be small in number and are often confused with the environmental context. Therefore, few-shot learning needs to further improve the ability to extract features in complex backgrounds to improve the recognition accuracy.

6.3. Crop detection

Crop detection first requires precise classification of the desired crop. Mixed planting is a popular way of planting nowadays. When working in large fields, farmers often mix several different crops to promote each other's growth and improve the yield and economic benefits. Thus, how to classify the crops in the field and how to accurately detect the desired crops have become a hot topic of concern. Bargiel et al. (2017) combined the climatic changes with remote sensing images, and proposed a high-precision crop classification method which is able to accurately classify crops such as maize and oilseed rape. Zhong et al. (2019) designed a one-dimensional convolutional layer deep learning model, which realizes an efficient classification. Once the crop has been classified, the location of the crop needs to be accurately located and the crop contours need to be precisely segmented. Hamuda et al. (2017) designed a crop detection network based on color features and morphological features with 99.04% detection accuracy. Zheng et al. (2019) constructed the CropDeep dataset, which contributes to few-shot crop classification and detection in intelligent agriculture. Zhao et al. (2020) designed a crop detection method based on transfer learning, which provides a reference for intelligent agriculture.

Few-shot learning has been relatively well developed for crop detection. In the future, researchers need to focus on data acquisition methods and conduct continuous research from the perspective of reducing data acquisition costs.

In summary, the application and development of few-shot learning in the field of intelligent agriculture has made great progress and has become an indispensable part of the development of intelligent agriculture. We have reason to believe that few-shot learning will make outstanding contributions to promoting the further development of intelligent agriculture.

7. Challenges and prospects

Based on the above summary of few-shot learning development and applications, we have the following views on the main challenges that few-shot learning will face going forward:

1) Smaller sample size means higher risk of overfitting. Further improving the over-fitting ability of few-shot learning is a major challenge for the future, and researchers need to continue to carry out in-depth research.

2) As technology continues to develop, data collection is becoming less difficult. At this time, the original few-shot data is likely to be expanded, just based on the amount of data defining few-shot may not be applicable. How to define few-shot and many-shot still need relevant scholars to study?

3) It is still worth exploring how to transfer few-shot learning methods from some specific fields to other emerging fields and increase their interpretability.

Based on the above summary of few-shot learning development and applications, we have the following views on the development prospects of few-shot learning in intelligent agriculture:

1) The overfitting problem is a severe test for few-shot learning based on model fine-tuning. The focus of future development will be to address the overfitting problem; researchers will continue to pay attention to the learning rate of different layers of the network to find ways to address the issue.

2) Concerning few-shot learning based on meta-learning, researchers will keep optimizing meta-learner, drive the continuous development of few-shot learning through meta-learning, apply few-shot learning based on meta-learning more diversified in intelligent agriculture.

3) For few-shot learning based on metric learning, the key research approach in the future will be the optimization and improvement of metric methods. Using neural networks for similarity calculation will be the mainstream trend of few-shot learning in intelligent agriculture.

4) For few-shot learning based on data augmentation, researchers need to make good use of unlabeled data further, make good use of a large amount of useful feature information hidden behind unlabeled data, and use various methods to enhance the ability to extract foreground objects in the complex background.

5) Overall, few-shot learning has a bright future in intelligent agriculture.

To summarize, the role of few-shot learning in intelligent agriculture will be more and more important. An essential feature of intelligent agriculture is to reduce the workload of related personnel and the cost of related work. By utilizing few-shot learning, the dependence of intelligent agriculture on a large amount of data will be reduced, and the degree of smartness will be further enhanced. For few-shot learning, the future focus is to further enhance the feature extraction ability and data augmentation ability, improve the accuracy of detection and segmentation, and increase the ability to accurately recognize the target in a complex background. Meanwhile, the existing literature mostly illustrates and improves the method of few-shot learning, and lacks research in hardware implementation. Therefore, it is necessary to further promote the hardware deployment effect of few-shot learning and reduce the cost of hardware deployment, so that it can be more widely used in intelligent agriculture.

8. Conclusions

In this paper, we summarize the main methods and development of few-shot learning from four aspects: few-shot learning based on model fine-tuning, few-shot learning based on meta-learning, few-shot learning based on metric learning, and few-shot learning based on data augmentation. In addition, we also describe and analyze the different applications of few-shot learning in intelligent agriculture, predict the future challenges and development prospects of few-shot learning. After exploring and summarizing, we can find that the development of few-shot learning is inseparable from people's desire to reduce the data collection workload and cost. Because some data are difficult to collect in large quantities, few-shot learning can achieve such rapid and high-quality development. As a typical representative of the high cost of data collection and the difficulty of data collection, the development of few-shot learning used in intelligent agriculture is even more diverse and novel. With the continuous advancement and development of machine learning technology, few-shot learning will eventually show its potentials in various fields represented by intelligent agriculture.

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Multidimensional Investigation of the Effect of Pre-treatment Solutions on Drying Characteristics and Raisin Quality

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ABSTRACT

This study aims to evaluate the relationships between pre-treatment solution, variety, drying characteristics, and raisin quality in raisin production, using multivariate analysis methods. The study was conducted on raisins obtained by dipping Bineteti and Zeyti local seed grape varieties in 13 different pre-treatment solutions which were obtained by mixing potassium carbonate and sodium bicarbonate with olive oil, hazelnut oil, and sesame oil at different concentrations. The dipped grapes were dried in the sun on a concrete drying platform. In the study, data of 15 numerical variables related to drying characteristics and raisin quality were reduced to four principal components (PC1, PC2, PC3 and PC4) using the principal component analysis (PCA), and their score values were numerically obtained. Then, two grape varieties, 13 pre-treatment solutions, and the four principal components were analyzed by non-linear principal component analysis (NLPCA). In addition, a cluster analysis was performed to determine the prominent pre-treatment solutions in terms of drying characteristics and raisin quality. It was determined that the pre-treatment solutions were effective on L^*, a^*, b^*, chroma (C^*), hue (h°), a/b values, antioxidant activity, total phenolic content, and drying time constituting PC1. It was remarkable that the colour parameters in prominent clusters in the cluster analysis also form PC1 in PCA analysis. The best pre-treatment solutions were found to be the "5% K_2CO_3 + 1% olive oil" solution for the Bineteti variety and the "5% K_2CO_3 + 2% hazelnut oil" solution for the Zeyti variety. It was determined that the pre-treatment solutions recommended for the varieties increased raisin quality and shortened the drying time, and had positive effects on the total phenolic content and antioxidant activity.

Keywords: Dimension reduction, Principal component analysis (PCA), Cluster analysis

1. Introduction

Increasing consumer awareness in food consumption has led to a rising interest in functional foods that positively affect health. Raisins, which contain many bioactive compounds, are considered functional foods and have continuously increasing consumption potential (Papadaki et al. 2021). Nutrients and bioactive compounds in functional foods lead to a healthier and longer life by protecting and controlling non-communicable diseases, especially in populations with genetic predispositions (Abuajah et al. 2015).

The wax layer on the grape berry is a protective barrier against fungal pathogens, controls gas exchange between the berry and the external environment, reduces water loss by transpiration, and provides protection against ultraviolet rays and physical injuries. The most notable disadvantage of the wax layer is that it prevents moisture removal during the drying process. Consequently, to remove the wax layer on grape berries and accelerate water diffusion, it is necessary to subject grapes to pre-treatment before drying (Esmaili et al. 2007). The pre-treatment performed before drying provides significant advantages in terms of shortening the drying time and improving the quality of raisins (Christensen & Peacock 2000). Physical and chemical pre-treatments are used to remove the wax layer on the grape berries (Wang et al. 2016). Sodium hydroxide (NaOH), potassium hydroxide (KOH), potassium mete bisulfate ($K_2S_2O_5$), potassium carbonate (K_2CO_3), sodium carbonate (Na_2CO_3), methyl and ethyl ester emulsions are the main chemical substances used in the preparation of pre-treatment solutions (Saravacos et al. 1988; Kassem et al. 2011; Doymaz & Altıner 2012; Patidar et al. 2021). Numerous studies have been conducted regarding the effects of pre-treatment solutions on raisin quality and drying characteristics (Khiari et al. 2021; Foshanji et al. 2022). The effects of various pre-treatments on hormones, enzymes, vitamins, minerals, and phenolic composition in raisins have been analyzed using PCA analysis (Keskin et al. 2022; Olivati et al. 2022).

Today, many of the chemical substances used in the preparation of pre-treatment solutions pose significant risks in terms of food safety and human health (Carranza-Concha et al. 2012; Farias et al. 2021). To minimize or entirely eliminate these risks is possible by using pre-treatment solutions prepared using chemicals and natural additives permissible in foods. In this study, pre-treatment solutions were used, which were obtained by mixing potassium carbonate and sodium bicarbonate with olive oil, hazelnut oil, and sesame oil at different concentrations. The use of potassium carbonate (K_2CO_3) (E501) and sodium bicarbonate ($NaHCO_3$) (E500) as food additives has been approved by both international organizations and the 'Turkish Food Codex Food Additives Regulation' (Anonymous 2013). As the pre-treatment solutions used in the study do not pose a risk in terms of human health, they will contribute to resolving residue problem in raisins. Many factors such as irrigation, nutrition, pruning, crop load, harvest time, disease and pest control, drying technique, pre-treatment solution, environmental conditions, variety, the sugar content of fresh grapes, and moisture content of raisins affect the drying characteristics and raisin quality (Jalili Marandi 1996; Çelik et al. 1998).

Physical, chemical, and sensory analyses are required to define drying characteristics and raisin quality. Although classical methods used to statistically evaluate the large number of data obtained from these analyses provide significant information according to each variable, they are insufficient to reveal the relationships between two or more variables (Doğan et al. 2021). By ignoring other variables, examining the relationships between variables as binary facilitates calculation and interpretation, but is insufficient to explain fully the original relationship structure. There are complex linear and non-linear relationship structures between variables. For this reason, there is a need for multivariate statistical analysis methods that can preserve the relationship structure between the original variables and facilitate interpretation, in other words, perform dimension reduction. One of these methods is the Principal Component Analysis (PCA). When the assumptions of PCA (linearity and numerical variables) cannot be met, non-linear a principal component analysis (NLPCA) is used as an alternative method (Linting et al. 2007; Kapucu 2016). NLPCA is a complementary and explanatory dimension reduction method that determines the direction and degree of relationships between variables in multivariate data sets with linear or non-linear relationships and shows the results numerically and visually (Kramer 1991; Kapucu 2016).

In the literature review conducted, it was observed that the use of multivariate analysis methods considering the categorical and continuous variables together and presenting the relationship structure between these variables in a simple and understandable way is limited. For this reason, NLPCA was used to determine the relationships between categorical, continuous, and ordinal variables in this study. In addition, a cluster analysis was performed to determine the prominent pre-treatment solutions in terms of drying characteristics and raisin quality.

This study aims to evaluate the relationships between pre-treatment solution, variety, drying characteristics, and raisin quality in raisin production using multivariate analysis methods (PCA, cluster analysis, and correlation analysis).

2. Material and Methods

2.1. Material

This study was carried out in 2020 on Bineteti and Zeyti local seed grape varieties considered as drying in Gercüş (Batman-Turkey) province. The cluster density is “dense” in the Bineteti variety and “medium” in the Zeyti variety, and the berries of both varieties are green-yellow in colour, short-oval shaped, juicy, and have a very thin skin and a weak wax layer. The average cluster and berry weights are 695.0±111.2 g and 3.63±0.52 g in the Bineteti variety and 659.5±128.02 g and 3.87±0.56 g in the Zeyti variety, respectively (Kırs 2019). The research material was obtained from a producer vineyard in Gercüş (Batman-Turkey) province. During the drying period, the average air temperature and humidity were measured as 28.18±2.07 °C and 21.01±4.74%, respectively.

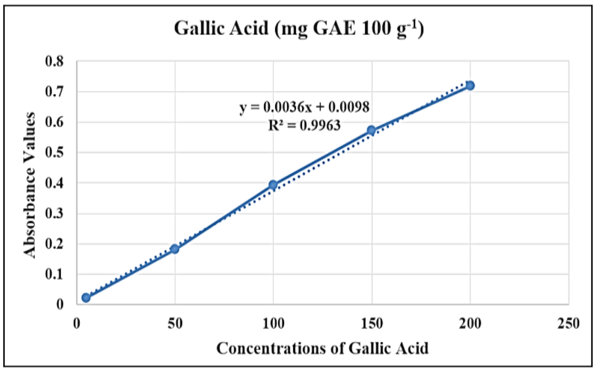
2.2. Method

The grapes were harvested when the soluble solid content (SSC) reached 22.26% in the Bineteti variety and 22.03% in the Zeyti variety. After the grapes were harvested, diseased and damaged berries were removed, and large bunches were separated into smaller bunches. The grapes were dipped in 13 different pre-treatment solutions 8-10 times and then dried in the sun on a concrete drying platform. In the preparation of pre-treatment solutions, high-purity (98-99% purity) potassium carbonate (K_2CO_3) and sodium bicarbonate ($NaHCO_3$) with high acidity (2-4%) olive oil, hazelnut oil, and sesame oil were used. The formulations of pre-treatment solutions used in the study are presented in Table 1. The drying process was stopped when the moisture content of the grapes reached 15-16% (Çelik et al. 1998; Anonymous 1979; 2002).

Table 1- The formulations of pre-treatment solutions used in the study

No	*The formulations of pre-treatment solutions*
1	Control
2	5% Potassium Carbonate (K_2CO_3) + 1% Olive Oil
3	5% Potassium Carbonate (K_2CO_3) + 2% Olive Oil
4	5% Potassium Carbonate (K_2CO_3) + 1% Hazelnut Oil
5	5% Potassium Carbonate (K_2CO_3) + 2% Hazelnut Oil
6	5% Potassium Carbonate (K_2CO_3) + 1% Sesame Oil
7	5% Potassium Carbonate (K_2CO_3) + 2% Sesame Oil
8	5% Sodium Bicarbonate ($NaHCO_3$) + 1% Olive Oil
9	5% Sodium Bicarbonate ($NaHCO_3$) + 2% Olive Oil
10	5% Sodium Bicarbonate ($NaHCO_3$) + 1% Hazelnut Oil
11	5% Sodium Bicarbonate ($NaHCO_3$) + 2% Hazelnut Oil
12	5% Sodium Bicarbonate ($NaHCO_3$) + 1% Sesame Oil
13	5% Sodium Bicarbonate ($NaHCO_3$) + 2% Sesame Oil

The drying time and drying yield were examined as drying characteristics, while moisture content, 100 raisin weight, surface colour values [L^*, a^*, b^*, *a/b*, chroma (C^*), hue ($h°$)], total acidity, SSC, pH value, total phenolic content, and antioxidant activity (total 15 numeric variables) were examined as raisin quality characteristics. The drying yield was calculated as the ratio of the total weight of fresh grapes to the total weight of raisins obtained after drying (Boztepe 2012). The moisture content of the raisins was determined by drying 50 g of the raisin sample in an oven at 65 °C until a constant weight was reached (Yıldırım 2018). The L^*, a^*, and b^* values of raisins were determined using Photoshop CS6 software from the JPEG format images taken at the same light intensity in the photo booth. Chroma (C^*) and hue ($h°$) values were obtained from the L^*, a^*, and b^* values by using the Ral Digital 5.0 software (Doğan & Uyak 2020). To determine the total acidity, 40 g of raisin sample was crushed in a mortar and soaked in 100 mL of distilled water for 4 hours. Then, these samples mixed with a mixer were filtered using filter paper. 10 mL of this mixture was taken and titrated until the pH reached a value of 8.1. Total acidity was calculated as tartaric acid (Köylü 1997). SSC measurements were conducted using a digital refractometer on the mixture prepared for the acid analysis. Then, the soluble solid content in the main sample was calculated by considering the dilution ratio (Cemeroğlu 1992). Raisin extract was prepared to determine the total phenolic content and antioxidant activity. For this purpose, 5 g of raisin samples were crushed in a mortar, and then 25 mL of methanol was added and homogenized for 2 minutes. Afterwards, the samples were kept in dark at room temperature for 30 minutes and centrifuged for 15 minutes, and the supernatant portions were transferred to eppendorf tubes and stored at -20°C. The total phenolic content was determined using the Folin-Ciocalteu calorimetric method (Swain & Hillis 1959). 150 µL of the raisin extract was taken and mixed with 2400 µL of distilled water, 150 µL of Folin solution, and 300 µL of 20% sodium carbonate (Na_2CO_3) solution, and this mixture was kept at room temperature for 1 hour. After this process, the absorbance values were read at a wavelength of 725 nm in the spectrophotometer, and the total phenolic content was calculated as gallic acid equivalent (GAE) mg 100 g^{-1} dry weight (Figure 1). The antioxidant activity was determined using the Ferric Reducing Antioxidant Power (FRAP) method (Benzie & Strain 1996). FRAP reagent was prepared by mixing acetate buffer, hydrochloric acid (HCl) solution, TPTZ (2,4,6-tripyridyl-s-triazine) solution, and ferric chloride ($FeCl_3.6H_2O$) solutions in a 10:1:1 ratio. The raisin extracts were first diluted 100 times with methanol, and then 150 µL of the sample was taken and mixed with 2850 µL of FRAP reagent, and this mixture was kept at room temperature for 30 minutes. Then, the absorbance values were read at a wavelength of 593 nm in the spectrophotometer, and the antioxidant activity values were calculated as µmol Trolox equivalent (TE) g^{-1} (Figure 1).



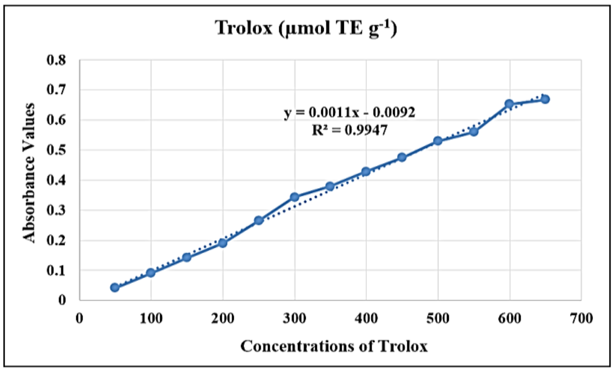


Figure 1- Calibration curves of gallic acid and trolox

2.3. Statistical analysis

The research was carried out according to a completely randomized design with 3 replications and 5 kg of fresh grapes in each replication. The PCA is a dimension reduction method used to obtain a fewer number of new variables (p), called principal components, from the linear combination of k number of original variables ($X1$, $X2$,…,Xk). In PCA, the first two or three principal components that explain most variation in the variables are considered. Thus, it is ensured that variables are summarized and interpreted significantly (Jollife 1986; Demir et al. 2016). To determine the number of appropriate principal components in the PCA, principal components with eigenvalues greater than one are considered (Alpar 2011). To apply PCA, it is required that the relationships between the variables are linear and the variables are on a numerical scale (Jolliffe 1986). However, in many studies, ordinal, categorical, or discrete variables are also used, as well as numerical variables. When the necessary conditions for PCA are not met, NLPCA is recommended as a more appropriate analysis method. NLPCA allows for variables to be scaled at different levels with an optimal scaling approach. As a result, categorical variables are appropriately scaled to the desired dimension. Thus, linear and non-linear relationships between variables can be modelled (Meulman & Heiser 2011; Mori et al. 2016).

In optimal scaling, analyses performed without considering the structure of the variables may not provide accurate results. Therefore, some transformations should be made by considering the variables' structures, and the variables should be transformed into a suitable form for optimal scaling. In this context, smaller dimensional solutions are obtained using non-linear methods by transforming a numerical variable into a categorical variable (Meulman & Heiser 2011; Güç 2015). The NLPCA method offers several notable advantages, including the absence of assumptions such as normality and linearity, and its ability to include nominal and ordinal scale variables in the analysis, unlike PCA. Thus, in data sets containing different types of variables, these variables can be considered together and the relationship structure between them can be presented numerically and visually in a two-dimensional space (Demir et al. 2021). In this study, NLPCA was used because the relationships between the variables in the nominal and ordinal scale were examined. In the study, binary Pearson correlation coefficients between the variables were first calculated in order to test the suitability of numeric variables for PCA. Because the variables for which correlations were calculated were numerical variables and the number of observations was over 30, the Pearson correlation coefficient, which is a parametric correlation, was calculated instead of the Spearman correlation coefficient, which is a non-parametric correlation coefficient. Then, Kaiser-Meyer-Olkin (KMO) and Bartlett Sphericity tests were used. The data of 15 numerical variables were analyzed using PCA. They were reduced to four principal components (PC1, PC2, PC3, and PC4) according to their eigenvalues, and the score values of the principal components were numerically obtained. Then, two varieties, 13 pre-treatment solutions, and the first four principal components obtained by PCA were analyzed by NLPCA. During the analysis, the scores of the first four principal components were converted into a two-group (high and low) categorical variable. The obtained results were interpreted numerically and graphically. PCA and NLPCA analyses were conducted on a total of 78 observations. In addition, a cluster analysis was performed using the JMP Pro software according to the Ward method. A correlation analysis was performed using the "corrplot" package (Wei & Simco 2017) in the R software, and PCA and NLPCA was performed with the SPSS (25.0 version) software. In this study, descriptive statistics for the continuous variables were presented as Mean ± Standard Deviation of Mean. A one-way ANOVA was performed for the comparison of group means. The Duncan multiple comparison test was also used to identify different groups. The statistical significance level was considered as 5%.

3. Results and Discussion

3.1. The effects of pre-treatment solutions on drying characteristics and raisin quality of varieties

It was determined that pre-treatment solutions were effective on drying characteristics (drying time and drying yield) and raisin quality (except total acidity in the Bineteti variety) in both varieties (Table 2). Pre-treatment solutions shortened the drying time in both varieties (Table 2). It has been reported that dipping solutions applied to grapes before drying accelerate the drying process (Esmaili et al. 2007; Matteo et al. 2000; Vázquez et al. 2000; Dev et al. 2008). The moisture content of raisins obtained from all treatments in both varieties varied between 15-16% (Table 2). Previous studies note that the moisture content of raisins should be between 13-18% (Anonymous 1979; 2002). Kapuci et al. (2022) has reported that the moisture contents of raisins varied between 14-15% depending on different drying sites and pre-treatment solutions in the Bineteti and Zeyti varieties. Drying yield varied between 23-25% in the Bineteti variety and 19-21% in the Zeyti variety (Table 2). Kapuci et al. (2022) determined that the drying yields varied between 24-26% for the Bineteti variety and 18-20% for the Zeyti variety depending on different drying sites and pre-treatment solutions. The 100 raisin weight varied between 124.18-133.03 g in the Bineteti variety and 77.32-86.77 g in the Zeyti variety (Table 2). Kapuci et al. (2022) calculated that the 100 raisin weights varied between 127.98-142.80 g in the Bineteti variety and 77.27-88.06 g in the Zeyti variety depending on different drying sites and pre-treatment solutions. It was determined that the L^*, a^*, b^*, chroma (C^*) and hue (h°) values of dipped raisins in both varieties were higher than natural (control) raisins, and their a/b values were lower than natural raisins (Table 2). Dipped raisins showed higher values than natural (control) raisins in terms of total phenolic content and antioxidant activity (Table 2). This may have stemmed from the natural (control) raisins not being subjected to any pre-treatment before drying and being exposed to intense enzymatic browning due to the longer drying times. Many researchers have reported that enzymatic activity reduces the total phenolic content and antioxidant activity in raisins (Yeung et al. 2003; Breksa et al. 2010; Foshanji et al. 2018). It has been stated that the effects of the drying process on phenolic compounds could vary depending on many factors such as drying time, drying environment, and grape variety (Mazlum & Nizamlıoğlu 2021).

Table 2- The effects of pre-treatment solutions on drying characteristics and raisin quality of varieties

Variables	*Drying Time (Day)*		*Moisture Content (%)*		*Drying Yield (%)*		*100 Raisin Weight (g)*	
Varieties	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*
Control	22.33±2.30 a	20.33±1.52 a	15.16±0.57 cd	15.22±0.23 bcd	23.49±0.63 c	20.25±0.97 abcde	126.09±1.96 bcd	80.36±3.05 bcd
%5K_2CO_3+1% Olive Oil	11.33±2.88 b	8.66±1.15 bc	15.32±0.38 bcd	15.71±0.77 abcd	25.83±0.84 a	21.90±0.99 a	133.03±3.29 a	80.89±4.31 bcd
%5K_2CO_3+2% Olive Oil	11.00±1.00 b	8.00±0.00 c	16.36±0.36 a	15.41±0.74 abcd	25.50±0.51 a	20.02±0.86 bcde	124.73±2.29 cd	79.75±3.93 bcd
%5K_2CO_3+1% Hazelnut Oil	12.00±1.73 b	10.00±0.00 b	15.50±0.63 abcd	15.10±0.40 cd	25.56±0.71 a	21.03±0.62 abcd	131.81±3.36 ab	77.39±2.66 cd
%5K_2CO_3+2% Hazelnut Oil	13.00±0.00 b	8.00±0.00 c	15.08±0.39 d	16.19±0.64 ab	24.66±0.63 ab	19.77±0.82 cde	125.03±3.13 cd	80.72±2.44 bcd
%5K_2CO_3+1% Sesame Oil	11.33±1.66 b	9.33±1.15 bc	16.03±0.34 abcd	15.28±0.15 bcd	24.84±0.73 a	20.92±0.28 abcd	124.18±1.83 d	77.56±2.66 cd
%5K_2CO_3+2% Sesame Oil	13.00±0.00 b	8.00±0.00 c	15.11±0.34 d	16.06±0.23 abc	24.73±0.33 a	21.59±0.52 ab	127.55±2.93 abcd	86.77±1.32 a
5%$NaHCO_3$+1% Olive Oil	13.00±0.00 b	9.33±1.15 bc	15.52±0.75 abcd	15.98±0.64 abcd	25.36±0.66 a	21.01±1.09 abcd	131.56±2.74 ab	80.03±2.03 bcd
5%$NaHCO_3$+2% Olive Oil	12.00±1.73 b	8.00±0.00 c	15.48±0.50 abcd	16.33±0.21 a	25.00±0.64 a	19.32±0.63 de	130.21±3.02 abc	77.32±3.54 d
5%$NaHCO_3$+1% Hazelnut Oil	11.33±2.88 b	10.00±0.00 b	16.16±0.65 ab	16.08±0.42 abc	24.63±0.20 ab	21.48±1.58 abc	126.21±2.46 bcd	85.45±1.16 ab
5%$NaHCO_3$+2% Hazelnut Oil	13.00±0.00 b	8.66±1.15 bc	15.10 ±0.17 d	15.54±0.63 abcd	23.58±0.92 bc	19.85±1.11 bcde	126.75±3.47 bcd	83.19±4.73 abc
5%$NaHCO_3$+1% Sesame Oil	11.33±2.88 b	9.33±1.15 bc	16.12±0.40 abc	15.80±0.50 abcd	24.95±0.11 a	20.79±0.68 abcde	126.05±3.27 bcd	80.96±2.40 bcd
5%$NaHCO_3$+2% Ssesame Oil	11.33±2.88 b	9.33±1.15 bc	15.58±0.74	15.03±0.53 d	23.13±0.56 c	19.10±1.12 e	125.52±4.93 cd	78.29±1.72 cd

Variables	**Total Acidity (g/l)**		**Soluble Solids Content (%)**		**pH**		**Total Phenolic Content**	
Varieties	**Bineteti**	**Zeyti**	**Bineteti**	**Zeyti**	**Bineteti**	**Zeyti**	**Bineteti**	**Zeyti**
Control	2.78±0.11 ns	3.39±0.69 ab	80.77±1.67 abc	76.33±2.08 bc	3.87±0.04 c	3.86±0.01 d	253.47±7.77 e	245.03±6.08 c
%5K_2CO_3+1% Olive Oil	2.67±0.32	3.09±0.24abcd	78.22±1.68 cd	78.33±1.85 abc	4.00±0.02 abc	3.95±0.04 abcd	273.96±8.43 bcd	284.69±6.36 ab
%5K_2CO_3+2% Olive Oil	2.54±0.03	3.59±0.03 a	80.55±1.38 abcd	76.44±2.03 bc	3.95±0.03 bc	3.87±0.07 d	280.70±8.64 abcd	290.55±7.96 ab
%5K_2CO_3+1% Hazelnut Oil	2.46±0.22	3.19±0.31 abc	78.99±1.20 bcd	75.88±2.54 bc	3.94±0.04 bc	3.99±0.07 abc	282.82±6.13 abcd	282.72±7.34 ab
%5K_2CO_3+2% Hazelnut Oil	2.73±0.29	3.58±0.16 a	81.33±1.76 ab	79.21±2.14 ab	4.00±0.04 abc	3.88±0.04 cd	278.75±9.03 bcd	284.30±4.47 ab
%5K_2CO_3+1% Sesame Oil	2.57±0.24	3.36±0.44 ab	79.44±1.07 bcd	80.10±1.38 a	3.97±0.08 bc	3.90±0.08 cd	272.10±9.87 cd	289.86±6.51 ab
%5K_2CO_3+2% Sesame Oil	2.99±0.20	2.85±0.34 bcd	80.44±1.39 abcd	76.21±1.26 bc	3.94±0.09 bc	3.89±0.02 cd	298.80±8.87 a	283.91±7.07 ab
5%$NaHCO_3$+1% Olive Oil	2.61±0.28	2.57±0.12 d	79.99±1.85 abcd	77.21±1.50 abc	3.91±0.08 bc	4.02±0.09 ab	269.27±8.36 de	289.16±4.20 ab
5%$NaHCO_3$+2% Olive Oil	2.58±0.07	3.03±0.13abcd	82.33±1.19 a	74.88±1.01 c	3.92±0.06 bc	3.90±0.04 cd	289.86±6.91 abc	282.92±4.79 ab
5%$NaHCO_3$+1% Hazelnut Oil	2.47±0.27	2.64±0.22 cd	80.88±1.01 abc	76.10±1.38 bc	4.09±0.08 a	4.05±0.03 a	271.80±6.75 cd	280.33±7.03 ab
5%$NaHCO_3$+2% Hazelnut Oil	2.67±0.20	2.73±0.35 cd	79.10±1.50 bcd	77.99±1.85 abc	4.00±0.06 abc	3.93±0.09 bcd	280.11±7.73 abcd	292.64±7.31 a
5%$NaHCO_3$+1% Sesame Oil	2.68±0.24	3.08±0.20abcd	78.10±1.67 cd	75.10±1.07 c	3.94±0.03 bc	3.96±0.04 abcd	292.89±5.69 ab	280.14±6.12 ab
5%$NaHCO_3$+2% Sesame Oil	2.55±0.31	2.70±0.19 cd	77.83±0.70 d	76.21±2.21 bc	4.04±0.08 ab	3.97±0.05 abcd	281.81±5.98 abcd	275.02±5.73 ab

a, b, c ↓ The differences between treatments with different letters in the same column are statistically significant ($P<0.05$), ns: No significant

Table 2- The effects of pre-treatment solutions on drying characteristics and raisin quality of varieties (Continued)

Variables	*Antioxidant Activity*		L^*		a^*		b^*	
Varieties	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*	*Bineteti*	*Zeyti*
Control	8.20±1.13 c	7.20±1.12 d	23.33±0.36 h	21.87±0.52 g	17.25±0.09 c	18.56±0.09 h	14.55±0.11 g	15.15±0.21 g
%5K_2CO_3+1% Olive Oil	10.55±0.91 bc	9.00±1.45 cd	35.51±0.90 a	33.60±0.60 ab	25.53±0.03 a	26.63±0.44 a	29.26±0.83 a	30.48±0.53 a
%5K_2CO_3+2% Olive Oil	12.09±0.94 ab	12.66±0.95 a	30.90±0.45 fg	31.90±0.81 cd	23.32±0.75 b	19.96±0.38 g	26.23±0.62 e	23.81±0.15 f
%5K_2CO_3+1% Hazelnut Oil	12.52±0.96 ab	9.55±1.11 abcd	31.78±0.93 def	30.86±0.85 def	25.09±0.27 a	24.39±0.28 cd	27.50±0.57 cd	27.04±0.81 cd
%5K_2CO_3+2% Hazelnut Oil	10.12±1.01 bc	12.50±1.32 ab	32.38±1.03 cde	32.93±0.42 bc	24.85±0.35 a	25.44±0.18 b	28.91±0.74 ab	26.99±0.51 cd
%5K_2CO_3+1% Sesame Oil	11.34±1.20 ab	9.84±1.35 abcd	34.55±0.80 ab	31.67±0.89 de	24.71±0.16 a	23.75±0.21 e	29.77±0.07 a	26.34±0.25 de
%5K_2CO_3+2% Sesame Oil	13.23±1.16 a	9.48±1.61 bcd	32.41±0.73 cde	34.35±0.43 a	23.84±0.38 b	25.38±0.46 b	26.70 0.33 de	29.04±0.56 b
5%$NaHCO_3$+1% Olive Oil	10.16±0.95 bc	9.20±1.51 cd	33.69±0.85 bc	32.08±0.56 cd	25.38±0.16 a	24.56±0.21 c	28.86±0.19 ab	26.45±0.24 de
5%$NaHCO_3$+2% Olive Oil	11.88±1.30 ab	10.81±1.06 abc	33.63±0.96 bc	30.31±0.70 f	24.95±0.25 a	23.94±0.61 de	28.03±0.29 bc	25.74±0.87 e
5%$NaHCO_3$+1% Hazelnut Oil	11.73±1.26 ab	9.30±1.26 cd	31.18±0.56 efg	30.51±0.42 ef	24.73±0.16 a	24.47±0.41 cd	27.19±0.68 cd	25.45±0.89 e
5%$NaHCO_3$+2% Hazelnut Oil	10.12±1.08 bc	12.48±1.30 ab	31.17±0.73 efg	33.55±0.85 ab	23.35±0.20 b	26.19±0.46 a	25.07±0.84 f	27.56±0.45 c
5%$NaHCO_3$+1% Sesame Oil	10.13±0.90 bc	10.80±1.03 abc	30.27±0.43 g	30.38±0.98 f	23.79±0.93 b	21.42±0.11 f	26.05±0.23 e	24.29±0.71 f
5%$NaHCO_3$+2% Ssesame Oil	10.31±1.06 bc	9.98±1.41 abcd	32.64±0.71 cd	34.32±0.22 a	25.16±0.54 a	24.71±0.13 c	27.61±0.28 cd	27.11±0.69 cd

Variables	*a/b*		**Croma (C^*)**		**Hue (h°)**	
Varieties	**Bineteti**	**Zeyti**	**Bineteti**	**Zeyti**	**Bineteti**	**Zeyti**
Control	1.18±0.01 a	1.22±0.01 a	22.57±0.06 g	23.96±0.16 i	40.14±0.33 g	39.23±0.38 g
%5K_2CO_3+1% Olive Oil	0.87±0.01 cd	0.87±0.02 ef	38.83±0.85 a	40.48±0.36 a	48.88±0.43 bc	48.86±0.57 b
%5K_2CO_3+2% Olive Oil	0.88±0.01 bcd	0.83±0.01 f	35.10±0.66 ef	31.07±0.21 h	48.33±0.47 cde	50.02±0.63 a
%5K_2CO_3+1% Hazelnut Oil	0.91±0.02 bc	0.90±0.01 de	37.24±0.68 cd	36.42±0.78 cd	47.60±0.57 e	47.93±0.56 bcd
%5K_2CO_3+2% Hazelnut Oil	0.86±0.03 de	0.94±0.01 bcd	38.14±0.46 abc	37.10±0.43 c	49.31±0.59 b	46.68±0.52 ef
%5K_2CO_3+1% Sesame Oil	0.83±0.03 e	0.90±0.01 de	38.69±0.15 a	35.47±0.06 ef	50.30±0.11 a	47.96±0.52 bcd
%5K_2CO_3+2% Sesame Oil	0.89±0.04 bcd	0.87±0.03 ef	35.80±0.50 e	38.58±0.20 b	48.23±0.15 cde	48.84±0.58 b
5%$NaHCO_3$+1% Olive Oil	0.87±0.05 cd	0.92±0.04 bcd	38.43±0.22 ab	36.10±0.31 de	48.67±0.17 bcd	47.11±0.12 def
5%$NaHCO_3$+2% Olive Oil	0.89±0.01 bcd	0.93±0.03 bcd	37.52±0.07 bcd	35.16±0.60 f	48.32±0.58 cde	47.06±0.54 def
5%$NaHCO_3$+1% Hazelnut Oil	0.91±0.02 bc	0.96±0.04 b	36.75±0.54 d	35.55±0.27 ef	47.70±0.68 de	46.50±0.73 f
5%$NaHCO_3$+2% Hazelnut Oil	0.93±0.03 b	0.95±0.03 bc	34.27±0.54 f	38.03±0.31 b	45.35±0.49 f	46.44±0.53 f
5%$NaHCO_3$+1% Sesame Oil	0.91±0.04 bc	0.88±0.03 ef	34.79±1.06 f	32.33±0.80 g	48.87±0.70 bc	48.65±0.53 bc
5%$NaHCO_3$+2% Sesame Oil	0.91±0.02 bc	0.91±0.01 cde	37.35±0.25 cd	36.68±0.59 cd	47.66±0.86 e	47.64±0.611 cde

a, b, c ↓ The differences between treatments with different letters in the same column are statistically significant ($P<0.05$).

3.2. Principal component analysis

In the study, the Kaiser-Meyer-Olkin (KMO) value was found to be 0.777, and accordingly, it was observed that 15 original continuous variables were dimensionally reducible with the principal component or factorable (suitable for PCA analysis). This value is considered sufficient when it is above 0.50 by Field (2009) and classified in the 'good' category among 0.70-0.80. The result of the Bartlett Sphericity test ($\chi^2(105)=1683.701$; $P<0.001$) indicated that the correlations among the variables were large enough for PCA analysis.

According to the PCA result, while PC1 explained 45.1% of the total variance, PC2, PC3, and PC4 explained 20.2%, 9.9%, and 6.8% of the total variance, respectively. The first four principal components explained 82.1% of the variation in the original variables. In other words, 15 variables were reduced to four principal components, explaining approximately 82% of the total variance (Table 3). Loading values equal to or greater than 0.40 are accepted as ideal in determining the contribution of variables to principal components (Field 2009). Accordingly, L^*, a^*, b^*, chroma (C^*), hue (h°), a/b, antioxidant activity, total phenolic content, and drying time were the variables that contributed the most to PC1. The variables of a/b value and drying time had a negative effect on the PC1, whereas the other variables had a positive effect. The total acidity, SSC, drying yield, 100 raisin weight, and drying time were the variables that contributed the most to PC2. The total acidity variable had a negative effect on the PC2, whereas the other variables had a positive effect. Antioxidant activity, total acidity, SSC, and pH were the variables that contributed the most to PC3. The pH variable had a negative effect on the PC3, whereas the other variables had a positive effect. Only the moisture content variable provided the highest positive contribution to PC4 (Table 3).

Table 3- Load values of the first four principal components and variance ratios explained by them

Variables	*Unit*	*PC1*	*PC2*	*PC3*	*PC4*
L^*	-	**0.937**	-0.030	0.039	-0.140
a^*	-	**0.859**	0.027	-0.204	-0.131
b^*	-	**0.972**	0.029	-0.056	-0.127
Croma (C^*)	-	**0.946**	0.027	-0.121	-0.130
hue (*h*°)	-	**0.911**	-0.053	0.131	0.005
a/b	-	**-0.923**	0.041	-0.123	0.048
AA	µmol TE g^{-1}	**0.527**	0.079	**0.501**	0.345
TPC	mg GAE 100 g^{-1}	**0.694**	-0.231	0.296	-0.022
TA	g l^{-1}	-0.250	**-0.648**	**0.519**	-0.061
SSC	%	0.092	**0.671**	**0.436**	0.079
pH	-	0.322	0.240	**-0.711**	-0.034
MC	%	0.235	-0.141	-0.264	**0.888**
DY	%	0.219	**0.910**	0.092	0.051
100 RW	g	0.127	**0.936**	0.118	0.063
DT	Day	**-0.758**	**0.567**	0.063	-0.162
Eigenvalue	-	6.765	3.042	1.492	1.025
Explained variance	(%)	45.1	20.2	9.9	6.8
Cumulative variance	(%)	45.1	65.3	75.3	82.1

AA: Antioxidant Activity; **TPC**: Total Phenolic Content; **TA**: Total Acidity; **SSC**: Soluble Solids Content; **MC**: Moisture Content; **DY**: Drying Yield; **RW**: Raisin Weight; **DT**: Drying Time; **TE**: Trolox Equivalent; **GAE**: Gallic Acid Equivalent

The relationship structure between two factors (variety and pre-treatment solution) and the four principal components obtained from the reduction of 15 variables was examined using NLPCA. The score values of the principal components obtained through PCA were obtained numerically. However, the scores of the first four principal components used for NLPCA were transformed into a two-group categorical variable for a more understandable interpretation. As discriminative power and contribution of the categories to the dimensions increase, the coefficient values of the dimensions also increase. In other words, as the values any category takes on the dimensions deviate from the origin, the effect of this category in determining the dimension increase also (Demir et al. 2021). The categories of the variables and their vector coordinates in two-dimensional space were presented in Table 4. When both dimensions were evaluated together, the pre-treatment solution strongly affected the variables that made up PC1, while it weakly affected the other variables. According to this analysis, it was observed that the pre-treatment solution had a significant effect on the colour values, total phenolic content, antioxidant activity, and drying time. Similar results have also been reported by other researchers (Doymaz & Altıner 2012; İşçi & Altındişli 2016; Zemni et al. 2017; Çelik 2019; Khiari et al. 2021; Kapuci et al. 2022). A positive correlation was observed between the 1^{st}, 3^{rd}, 10^{th}, 11^{th}, and 12^{th} pre-treatment solutions and the '-3-0' category of PC1, the '0-2' categories of PC3 and PC4 (Table 4). In the same way a positive relationship was found between the 2^{nd}, 5^{th}, 6^{th}, 7^{th}, 8^{th}, 9^{th}, and 13^{th} pre-treatment solutions and '0-0' category of PC1, '-2-0' categories of PC3 and PC4. A negative relationship was determined between these two groups of variables with a positive relationship. Furthermore, a strong relationship was found between the variety and PC2. It was observed that Bineteti variety had a positive effect on the total acidity, SSC, drying yield, 100 raisin weight, and drying time variables that form PC2. The NLPCA showed which variety is more suitable for drying. Variables that cause PC2 discrimination are among the factors affecting the drying yield and raisin quality. Many studies note that these factors are positively or negatively associated with the variety (Yalçınkaya 2016; Çelik 2019; Kapuci et al. 2022). However, Table 4 shows that the relationships between variety and

PC2 variables and other variables were weak. It was determined that the Bineteti variety was positively correlated with the '0-1' category of PC2, and the Zeyti variety was positively correlated with the '-1-0' category of the same principal component (Table 4). This analysis results revealed that the Bineteti variety had a higher performance than the Zeyti variety in terms of drying yield and raisin quality. In PCA, the PC1 always has the highest variance explanation ratio, while the last principal component has the lowest variance explanation ratio (Demir et al. 2021). Therefore, in NLPCA, the low variance explanation ratios of PC3 and PC4 compared to other principal components also affects the relationship of these variables with other variables. In this context, it was observed that the PC3 and PC4 variables were weakly correlated with the other variables (Table 4).

Table 4- Categories of variables and their vector coordinates in two-dimensional space

		Vector Coordinates	
Variables	***Category***	***1st Dimension***	***2nd Dimension***
Variety	Bineteti	0.986	-0.044
	Zeyti	-0.986	0.044
Pretreatment Solutions	1.Control	0.039	1.246
	2.5%K_2CO_3+1% Olive Oil	-0.029	-0.905
	3. 5%K_2CO_3+2% Olive Oil	0.003	0.085
	4. 5%K_2CO_3+1% Hazelnut Oil	-0.001	-0.027
	5. 5%K_2CO_3+2% Hazelnut Oil	-0.025	-0.793
	6. 5%K_2CO_3+1% Sesame Oil	-0.023	-0.736
	7. 5%K_2CO_3+2% Sesame Oil	-0.025	-0.793
	8. 5%$NaHCO_3$+1% Olive Oil	-0.013	-0.424
	9. 5%$NaHCO_3$+2% Olive Oil	-0.010	-0.312
	10.5%$NaHCO_3$+1%Hazelnut Oil	0.066	2.100
	11.5%$NaHCO_3$+2%Hazelnut Oil	0.012	0.370
	12.5%$NaHCO_3$+1%Sesame Oil	0.034	1.077
	13.5%$NaHCO_3$+2%Sesame Oil	-0.028	-0.889
PC1	-3.53061- -0.00036	0.112	1.328
	0.02334 - 0.93847	-0.053	-0.626
PC2	-1.67041- -0.02001	-0.958	0.029
	0.16779 - 1.78391	1.008	-0.030
PC3	-2.61137- -0.02024	-0.280	-0.144
	0.00396 - 2.12636	0.266	0.136
PC4	-2.03316- -0.03591	-0.028	-0.325
	0.00988 - 2.11238	0.032	0.379

After optimal scaling, the total variance explanation ratios of the first and second dimensions were 34.0 and 29.9%, respectively, and these two dimensions explained 64.0% of the total variance. The eigenvalues were found to be 2.044 for the first dimension and 1.796 for the second dimension. According to the first dimension, variety and PC1 constituted positive loading variables, while others constituted negative loading variables. According to the second dimension, PC1 and PC2 constituted positive loading variables, while others constituted negative loading variables. Variety (0.925) and PC2 (-0.926) were the most effective variables in determining the first dimension, while pre-treatment solution (-0.864) and PC1 (0.844) were the most effective variables in determining the second dimension (Table 5).

Table 5- After optimal scaling, component loadings of variables in two-dimensional space and variance ratios explained by dimensions

	Component Loadings	
Variables	***1st Dimension***	***2nd Dimension***
Variety	**0.925**	-0.345
Pre-treatment Solution	-0.310	**-0.864**
PC1	0.354	**0.844**
PC2	**-0.926**	0.330
PC3	-0.303	-0.049
PC4	-0.136	-0.325
Eigenvalue	2.044	1.796
Explained variance (%)	34.0	29.9
Cumulative variance (%)	**64.0**	

A strong negative relationship was found between variety and PC2 and pre-treatment solution and PC1. It was observed that the variety and PC2 were not associated with PC1, PC4, and pre-treatment solution. It was seen that variety was also partially associated with PC3, but this relationship was weaker than that between variety and PC2. Similarly, it was observed PC1 was associated with PC4, but this relationship was weaker than that between PC1 and the pre-treatment solution. In general, variables far from the origin have higher variance explanation ratios, while variables close to the origin have lower variance explanation ratios (Demir et al. 2021). In this context, it was observed that the variance explanation ratios of the PC3 and PC4 variables due to their proximity to the origin were low, and their relationships with the other variables were very weak (Figure 2).

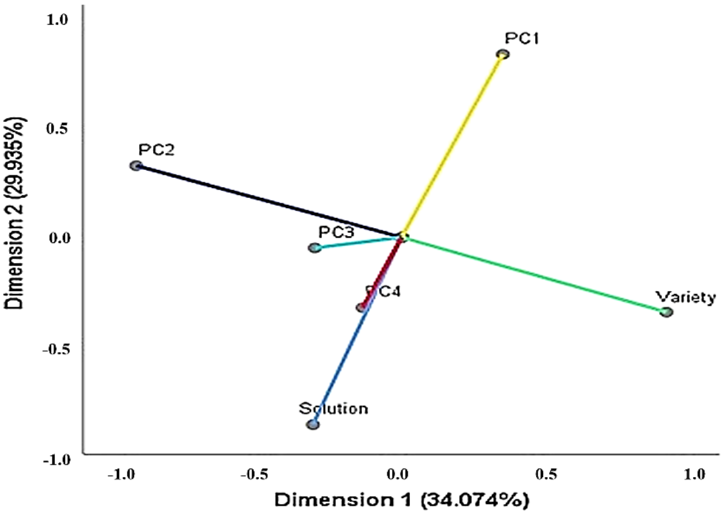


Figure 2- Configuration of variables in two-dimensional space after optimal scaling

3.3. Cluster analysis

In the study, the pre-treatment solutions in both cultivars yielded significant results compared to the control application. According to cluster analysis, control applications in both varieties were separated from other applications as a distinct group. This distinction clearly indicated that treating the grapes with the pre-treatment solution was necessary before drying. When the cluster separation values were examined in terms of the effectiveness of the pre-treatment solutions, the B cluster in the Bineteti variety and the C cluster in the Zeyti variety were the prominent groups (Table 6). For the Bineteti variety, the best pre-treatment solutions were found to be "5% K_2CO_3 + 1% Olive oil", "5% $NaHCO_3$ + 1% Olive oil", "5% K_2CO_3 + 1% Sesame oil", "5% K_2CO_3 + 2% Hazelnut oil" and "5% $NaHCO_3$ + 2% Sesame oil" solutions. For the Zeyti variety, the best pre-treatment solutions were found to be "5% K_2CO_3 + 2% Hazelnut oil" and "5% $NaHCO_3$ - 2% Hazelnut oil" solutions. It was observed that the "5% K_2CO_3 + 2% Hazelnut oil" pre-treatment solution took place in both prominent clusters for varieties (Figure 3).

Table 6- Cluster analysis according to some quality characteristics and drying times of the two varieties

Variety	***BİNETETİ***				***ZEYTİ***				
Cluster	***A***	***B***	***C***	***D***	***Cluster***	***A***	***B***	***C***	***D***
Count	*1*	*5*	*5*	*2*	***Count***	*1*	*8*	*2*	*2*
L*	23.33 c	33.75 a	31.98 b	30.72 b	***L****	21.87 c	32.21 b	33.24 a	31.14 b
a*	17.25 c	25.12 a	24.38b	23.57ab	***a****	18.56d	24.72 b	25.81 a	20.69 c
b*	14.55 d	28.88 a	27.13 b	25.56c	***b****	15.15 c	27.20 a	27.27 a	24.05 b
a/b	1.18 a	0.87 c	0.89 c	0.92b	***a/b***	1.22 a	0.94 b	0.91 b	0.86 c
Croma(C*)	22.57 d	38.28 a	36.48 b	34.53c	**Croma(C*)**	23.96 c	36.80 a	37.56 a	31.70 b
hue (*h*°)	40.14 c	48.96 a	48.03 a	47.11b	**hue (*h*°)**	39.23 c	46.56 b	47.73 b	49.33 a
TPC	253.47c	275.17b	285.39a	286.50a	**TPC**	245.0c	283.57ab	288.47a	285.34ab
DT	22.33 a	11.99ab	11.86ab	12.16b	**DT**	20.33 a	9.08 b	8.33 c	8.66 c
AA	8.20 c	10.49 b	12.29 a	10.12b	**AA**	7.20 d	9.64 c	12.49 a	11.73 b

a, b, c → The differences between cluster averages with different letters in the same row are statistically significant (P<0.05). **TPC:** Total Phenolic Content; **DT:** Drying Time; **AA:** Antioxidant Activity

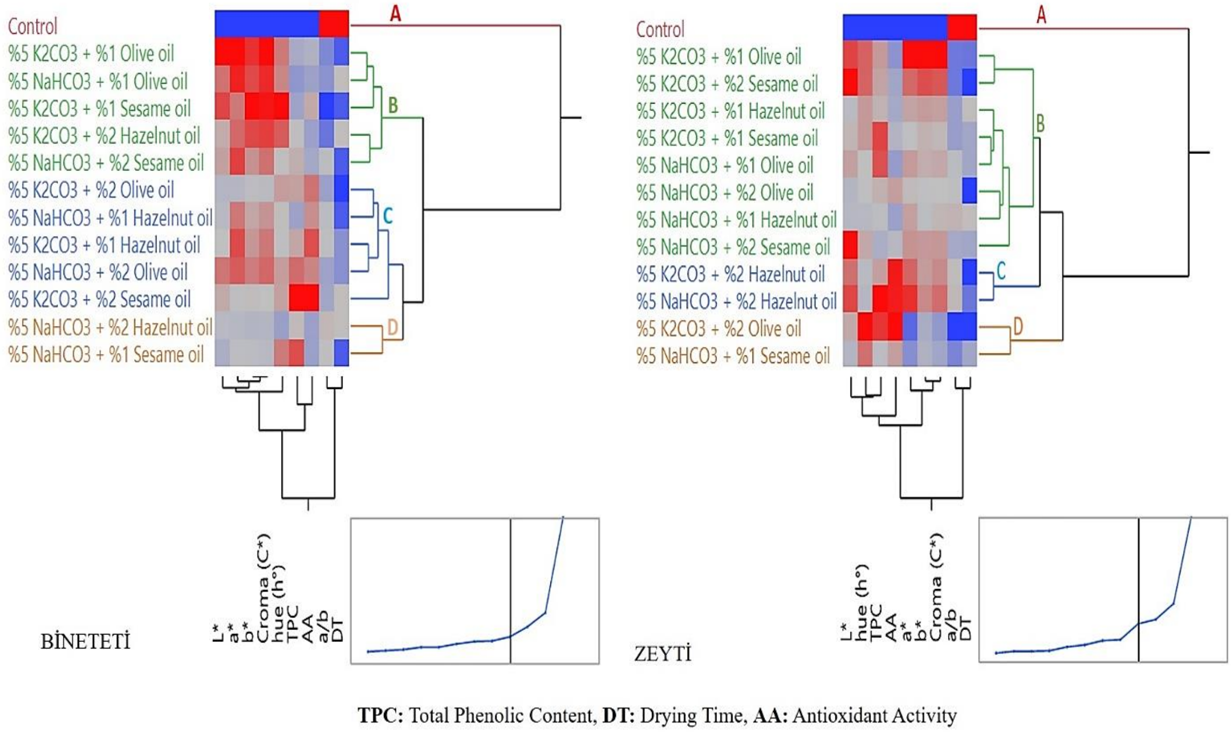


Figure 3- Dendogram of cluster analysis for the two varieties

It was observed that the mean values taken in terms of variables of the other pre-treatment solutions in the prominent clusters were close to each other. In this case, the pre-treatment solutions in the prominent clusters can be used as alternatives to each other. It has been reported that the olive oil used in pre-treatment solutions increase raisin's drying speed, elasticity and quality (Doymaz & Pala 2002; Akdeniz 2011). The disadvantage of olive oil used in pre-treatment solutions is that it causes an undesirable taste, aroma, and odour in dipped raisins. Sesame and hazelnut oils can be mixed with many medicinal and aromatic oils due to their indistinctive structure in terms of odour and colour and their soft structure. As a result of the sensory analysis, it was determined that sesame and hazelnut oils could be used as an alternative to olive oil in the preparation of pre-treatment solutions. It was observed that the pre-treatment solutions significantly affected the total phenolic content and antioxidant activity. This effect is thought to be due to the reduction of the drying time by pre-treatment solutions and the additional contribution of the oil components in the solution to the total phenolic content and antioxidant activity. It has been stated that a high L^* value and low a/b value in raisins obtained from varieties with a green-yellow skin colour caused a brighter and more yellow colour formation (İsmail 2005; Chayjan et al. 2011; Doymaz & Altıner 2012). The average L^* and a/b values in the prominent classes for the varieties were found to be compatible with the literature. The chroma (C^*) value, which indicates the saturation of the colour and directly appeals to colour perception, shows low values in dull colours and high values in vivid colours (Mc Guire 1992). It was observed that the average chroma (C^*) values in the prominent clusters in our study were higher than those in the other clusters. The evaluation of L^*, a/b, and chroma (C^*) values showed that the pre-treatment solutions used in our study caused more bright, vivid, and yellow-coloured raisins to be obtained. It should be considered that colour parameters may vary according to grape varieties. A colour quality criterion for grape varieties with a green-yellow berry skin colour can be formed based on high L^*, chroma (C^*), hue (h°) values, and low a/b values. It was observed that the effectiveness of the pre-treatment solutions used in our study might differ according to the berry characteristics and contents of the varieties. In this study, variables showing close values to each other in the groups forming in cluster separation were not included in the cluster analysis.

3.4. Correlation analysis

Statistically significant (P<0.01-0.001) positive and negative correlations were determined between the drying time and other variables (except for total acidity and pH value). Statistically significant (P<0.001) negative correlations (r=from -0.60 to 0.71) were found between drying time and L^*, a^*, b^*, chroma (C^*), hue (h°) values, and total phenolic content. A statistically significant (P<0.001) positive correlation (r=0.72) was observed between the drying time and a/b value (Figure 4). It has been reported that the colour values of raisins were adversely affected depending on the length of the drying time (Özel & İlhan 1980; Akdeniz 2011). In addition, previous studies found that enzymatic activity decreased the total phenolic content and antioxidant activity in raisins depending on the drying time (Yeung et al. 2003; Breksa et al. 2010; Foshanji et al. 2018). The strong negative correlations found between the drying time and colour values, and total phenolic content in the study supported the results of other researchers. Statistically significant (P<0.001) negative correlations (r=from -0.63 to 0.97) were determined between the a/b value and L^*, a^*, b^*, chroma (C^*), hue (h°) values, and total phenolic content. (Figure 4). It was observed that a/b and hue (h°) values were a lower correlation with a^* value and a higher correlation with b^* value. It was found that the b^* value was more effective than the a^* value on the colour formed after drying in the grape varieties examined. Statistically significant (P<0.001) negative correlations (r= from -0.50 to 0.55) were determined between total acidity and drying yield, 100 raisin weight, and pH value (Figure 4). Statistically significant (P<0.001) positive correlations (r= from 0.50 to 0.64) were also found between total phenolic content and a^*, b^*, chroma (C^*), hue (h°) values, and antioxidant activity (Figure 4). The effect of phenolic compounds on colour can explain the correlations between the total phenolic content and colour variables. It has been reported that phenolic compounds are effective in colour formation in raisins (Yueng et al. 2003). The correlations between the total phenolic content and antioxidant activity originated from the antioxidant properties of phenolic compounds. Statistically significant (P<0.001) positive correlations (r= from 0.62 to 0.87) were found between hue (h°) value and a^*, b^*, chroma (C^*), and L^* values (Figure 4). Statistically significant (P<0.001) positive correlations were determined between L^* value and a^* (r=0.81), b^* (r=0.93) and chroma (C^*) (r=0.91) values (Figure 4). Statistically significant (P<0.001) positive correlations were found between the chroma (C^*) value and a^* (r=0.96) and b^* (r=0.99) values (Figure 4). A statistically significant (P<0.001) positive correlation was determined between the b^* value and the a^* value (r=0.91) (Figure 4). Positive correlations between colour values (except for a/b) demonstrated that these variables affected each other. Considering that three main colours form the foundation of the colour space, it can be expressed that these variables are prone to interact with each other. Statistically significant (P<0.001) positive correlations were determined between 100 raisin weight and the SSC (r=0.60) and drying yield (r=0.92) (Figure 4). A statistically significant (P<0.001) positive correlation was determined between the drying yield and the SSC (r=0.59) (Figure 4). While the increase in the soluble solids content has a positive effect on the drying yield, the increase in the total acidity has a negative effect on the drying yield. It has been reported that there was a positive relationship between the SSC and the drying yield (Çelik et al. 1998).

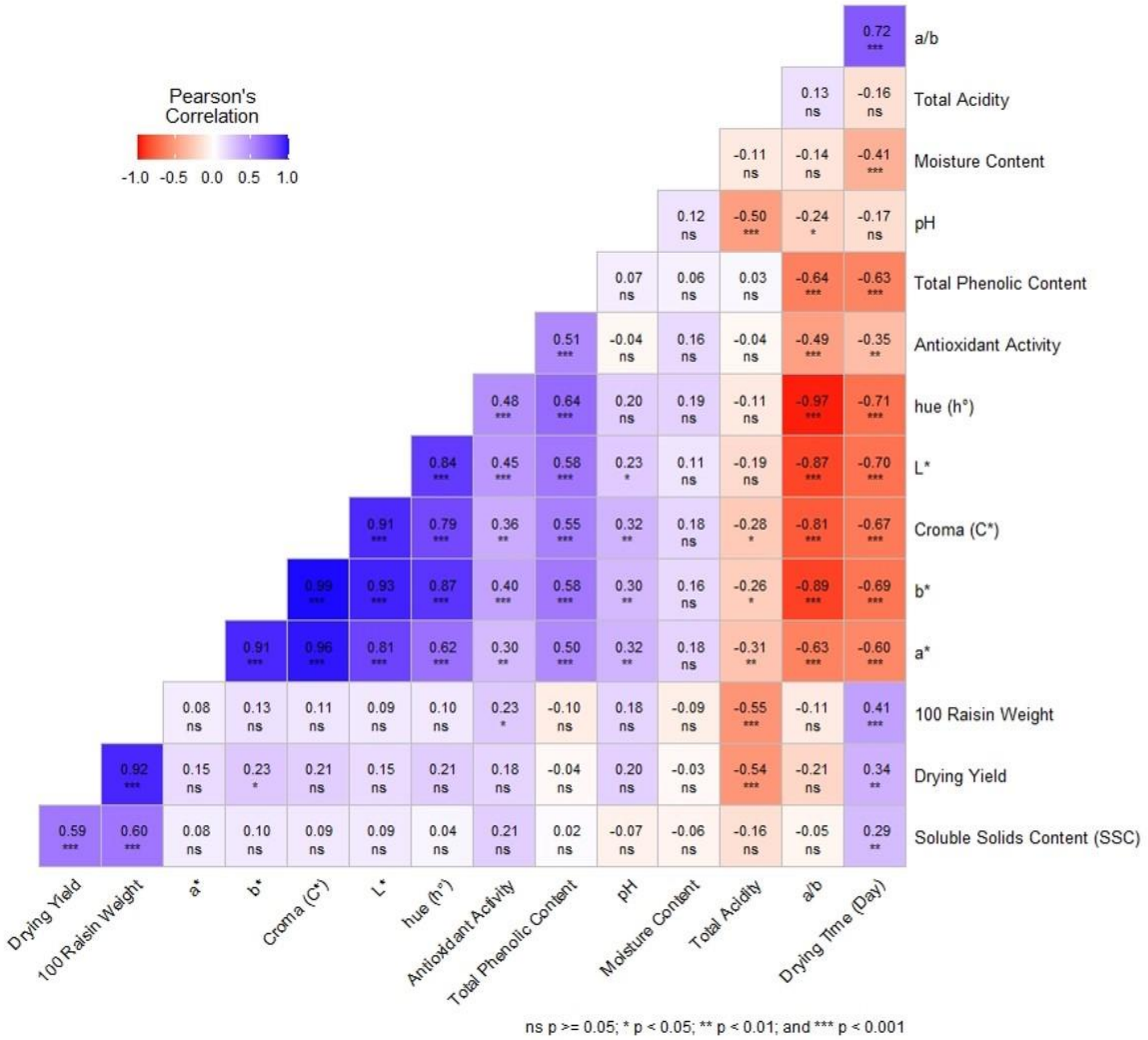


Figure 4- Correlations between variables

4. Conclusions

In this study, a dimension reduction was made by PCA on 15 variables being drying characteristics and raisin quality. According to this analysis, 15 variables were reduced to four principal components with an explainable variance ratio of 82.1%.

Subsequently, NLPCA was performed to determine the relationship of the four principal components with the pre-treatment solutions and varieties. As a result of the analysis, six variables consisting of variety, pre-treatment solution, and four produced variables (PC1, PC2, PC3, and PC4) were reduced to two dimensions with an explainable variance ratio of 64.0%. Thus, the linear and/or non-linear relationships between a total of 19 variables, consisting of two varieties, 13 pre-treatment solutions, and the first four principal components, were reduced to a two-dimensional space and presented visually in an easy to understandable and interpretable way. It was determined that the pre-treatment solutions were effective on L^*, a^*, b^*, chroma (C^*), hue (h°), a/b values, antioxidant activity, total phenolic content, and drying time constituting PC1. It was remarkable that the colour parameters in prominent clusters in cluster analysis were also the parameters forming PC1 in PCA analysis. This shows that the results obtained according to both methods (PCA and NLPCA) supported each other. Similarly, it was determined that the total acidity, SSC, drying yield, 100 raisin weight, drying time, antioxidant activity, and pH value, constituting PC2 and PC3, varied depending on the variety. The best pre-treatment solutions were found to be the "5% K_2CO_3 + 1% Olive oil" solution for the Bineteti variety and the "5% K_2CO_3 + 2% Hazelnut oil" solution for the Zeyti variety. Alternative solutions to these solutions were found to be "5% $NaHCO_3$ + 1% Olive oil", "5% $NaHCO_3$ + 2% Sesame oil", "5% K_2CO_3 + 1% Sesame oil", and "5% K_2CO_3 + 2% Hazelnut oil" solutions for the Bineteti variety and "5% $NaHCO_3$ + 2% Hazelnut oil" solution for the Zeyti variety. It was determined that the pre-treatment solutions recommended for the varieties caused brighter, more vibrant, and yellow-coloured raisins to be obtained, shortened the drying time, and positively affected the total phenolic content and antioxidant activity. According to the correlation analysis, it was determined that the drying time correlated with all variables except pH and total acidity, and the colour parameters correlated with each other, as well as the total phenolic content and antioxidant activity. It was determined that the moisture content did not correlate with any variable except drying time, and the SSC correlated with the drying time, drying yield, and 100 raisin weight, and the drying yield correlated with the drying time, total acidity, and 100 raisin weight.

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Drivers of Capia Pepper Farmers' Intentions and Behaviors on Pesticide Use in Turkey: A Structural Equation Model

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ABSTRACT
The main actors responsible for pesticide use behavior are farmers. Still, factors are influencing the pesticide use behavior of farmers in the background. The theory of planned behavior is a widely used concept that explains farmers' pesticide use behavioral intention. However, previous studies show that behavior is not included in the model. As a novelty, it was investigated whether behavioral intention transforms into behavior in this study. In this context, it is the first study conducted in Turkey. Turkey is in the top three for pepper production in the world. In addition, pepper is among the agricultural products that use the most pesticides. This study aims to determine and explain Capia pepper farmers' pesticide reduction intention and pesticide use behaviors in Turkey. For this purpose, the pesticide use behaviors of farmers in the province of Çanakkale, which ranks first in Capia pepper production in Turkey, were analyzed with the Structural Equation Model. Face-to-face surveys were conducted with 206 Capia pepper farmers selected by a proportional sampling method. The research results show that subjective norms and attitudes are important determinants of farmers' willingness to reduce pesticide use. Likewise, perceived behavioral control is a unique factor in farmers' use of pesticides. However, farmers' intentions to reduce pesticide use were not reflected in their actions. It is important to increase awareness among farmers about pesticide behavior. Agricultural policy implications should be planned that target the perceived behavior, subjective norms, and attitudes of farmers regarding pesticide use.

Keywords: Farmer, Theory of planned behavior, Survey, Smart PLS, Pesticide use

1. Introduction

The agricultural sector's role in feeding the global population is paramount, as emphasized by the United Nations (UN 2022). However, as the world population continues to expand, the corresponding demand for increased agricultural production presents a pressing challenge (WB 2022). To meet this demand, the utilization of pesticides in plant cultivation has become a widespread practice since the inception of the Green Revolution, aimed at enhancing yield per unit area (FAO 2017). Farmers have embraced extensive farming methodologies to optimize yields while mitigating the impact of pests and diseases (Govindharaj et al. 2021; Wang & Liu 2021).

1.1. Pesticide use and its significance

Pesticides, chemical agents employed in agricultural production to combat weeds, diseases, and various pests, are central to this perspective (Sharma et al. 2019). The term "pesticide" encompasses any substance or blend designed to prevent, eliminate, repel, or mitigate pests. Pesticides are also deployed as plant regulators, defoliants, or desiccants (USEPA 2022). Remarkably, the global revenue generated from pesticides, encompassing crop protection chemicals, herbicides, insecticides, and fungicides, surpassed $103 billion in 2020. Notably, the revenue from insecticides alone is projected to reach $188 billion by 2031. As of 2020, the top three global consumers of pesticides were China, the United States, and Brazil, jointly constituting 66.0% of the worldwide pesticide consumption (FAO 2022a).

1.2. Challenges and safety concerns

Within Europe, the years spanning 2015 to 2020 saw the reporting of 2 473 food safety incidents linked to pesticide residues within fruit and vegetable categories (RASFF 2020; Pan et al. 2021). The issue of pesticide residues in agricultural produce remains a paramount concern for food safety (Niyaz & Demirbaş 2018). Paradoxically, while pesticides were initially introduced to bolster agricultural productivity, their indiscriminate usage over time has posed threats to human health and the sustainability of environmental practices. The inappropriate application of pesticides in terms of quantity, timing, and frequency jeopardizes not only environmental sustainability but also human well-being. Moreover, the excessive utilization of pesticides hampers the optimal allocation of natural and economic resources (FAO 2022b; WHO 2022).

1.3. Turkey's agricultural landscape

Turkey has recently emerged as a formidable player in global agricultural production, securing a spot among the top ten nations (FAO 2022b). In 2020, Turkey was ranked 12th worldwide in terms of pesticide consumption, utilizing a substantial 53 672 tons. The distribution of plant protection products in the same year encompassed fungicides (38.4%, 20 600 tons), herbicides (24.7%, 13 250 tons), insecticides (23.1%, 12 437 tons), plant activators (9.3%), and other groups such as plant growth regulators, insect attractants, fumigants, acaricides (4.0%, 4 995 tons), and rodenticides and molluscicides (0.5%, 280 tons) (TRAFM 2022).

1.4. Pesticide usage in fruit and vegetable production

The application of pesticide residues finds its most intensive use in the production of fruits and vegetables on a global scale (Pan et al. 2021). This practice is particularly widespread in the cultivation of annual vegetables like tomatoes and peppers in Turkey (EURASIANET 2022).

1.5. The focus of the study

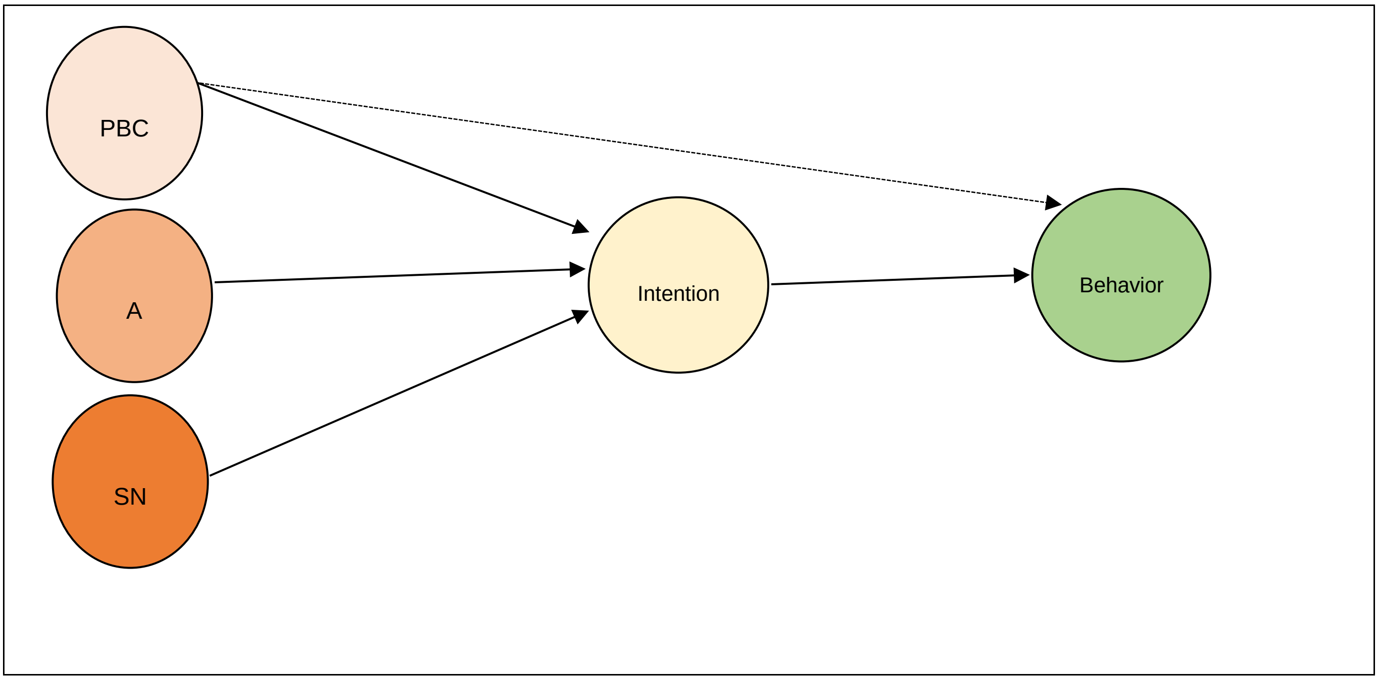
Turkey is a significant contributor to global pepper production, ranking third alongside China and Mexico. In 2019, the country's pepper production reached an impressive 2.59 million kg, contributing to 6.9% of the world's total pepper output. Diverse varieties of peppers, including table, dried, and canned types, thrive in Turkey (FAO 2022a). The present study aims to understand the determinants that shape pesticide use intentions and behaviors among Capia pepper farmers in Turkey. Positioned within the framework of the Theory of Planned Behavior (TPB), this study holds significance in the realm of world pepper production due to its unique focus on pesticide utilization. Central to this investigation is the fundamental inquiry: "What factors influence the pesticide use intentions and behaviors of Capia pepper farmers in Turkey?"

2. Previous Studies and Theoretical Background

The use of pesticides is applied by farmers producing agricultural products to combat organisms that harm the development of agricultural plants (Rezaei et al. 2020; Ataei et al. 2021; Damalas 2021; Lou et al. 2021). It is important to use pesticides for agricultural purposes at the optimum dose. Accordingly, the final actors that are effective in deciding the type and amount of pesticide use are farmers (Bakker et al. 2021; Govindharaj et al. 2021; Wang and Liu 2021). Agricultural pesticide residues and related problems mainly stem from farmers' pesticide use behavior. Therefore, it is necessary to explain the behavior of farmers when using pesticides and the factors that influence this behavior. Within the framework of behavioral economics, there are many behavioral theories designed to understand human behavior in the literature (Kwon & Silva 2019). TPB is one of the most frequently studied models on the pesticide use behavior of farmers (Beedell & Rehman 2000; Colémont & Broucke 2008; Fan et al. 2015; Asadollahpour et al. 2016; Wang et al. 2017; Rezaei et al. 2018; Bagheri et al. 2019; Despotovic et al. 2019; Farani et al. 2019; Rezaei et al. 2019a; Rezaei et al. 2019b; Yazdanpanah et al. 2019; Kahramanoğlu et al. 2020; Savari & Gharechaee 2020; Ataei et al. 2021; Bagheri et al. 2021a; Bagheri et al. 2021b; Damalas 2021; Govindharaj et al. 2021; Imani et al. 2021; Lou et al. 2021; Semuroh & Sumin 2021; Tama et al. 2021). Somewhat akin similar to this study, only Çakırlı Akyüz & Theuvsen (2020) modeled the organic farming intentions of grape farmers in Turkey with TPB. Contrary to the international literature, no study has been found in which the direct pesticide use intention and behavior of farmers in Turkey's field has been theoretically examined within the framework of the Planned Behavior Theory.

TPB (Figure 1) was produced and developed by Ajzen in 1991 (Ajzen 1991; Ajzen 2002). It is a social-psychological theory that links intention and behavior. Accordingly, three main factors affect people's intentions and behaviors. These are Perceived Behavior Control (PBC), Subjective Norms (SN) and Attitudes (A). PBC refers to the perception of individuals' skills and possibilities, whether they are under their control or not (Ajzen 1991; Ajzen 2002; Damalas 2021). According to the TPB, PBC can be expected to affect both intention and behavior. Subjective norms are defined as the effect of the thoughts of people they care about on the attitudes and behaviors of individuals (Ajzen 1991; Ajzen 2002; Fan et al. 2015; Rezaei et al. 2018; Tama et al. 2021). Accordingly, the attitude of the people around can be a guide for any behavior of the individual. Subjective norms are expected to have a direct effect on an individual's intention and indirectly on his behavior. Attitudes, for if behavior is perceived as positive, it increases the probability of that behavior is implemented. Attitudes are expected to have a direct effect on the individual's intention and indirectly on his behavior (Ajzen 1991; Ajzen 2002; Asadollahpour et al. 2016; Pahang et al. 2021).

Figure 1- Theory of Planned Behavior Structure (Ajzen, 1991, 2002)



3. Material and Methods

This study utilized both primary and secondary data sources. The primary data consisted of a questionnaire specifically designed for the purpose of this study. Secondary data were collected from official statistical sources and macro reports. The primary data for the study were gathered through face-to-face surveys conducted with Capia pepper farmers.

3.1. Research design, questionnaire, variables, and scale

This research employed a quantitative approach to investigate the pesticide use behavior related to the TPB among Capia pepper farmers. A structured questionnaire was developed and administered between February & March 2020. The questionnaire was constructed based on previous research on pesticide use behavior and TPB applications (Ajzen 2002; Cheah & Phau 2011; Yadav & Pathak 2016; Kim et al. 2017; Farani et al. 2019; Savari & Gharechaee 2020; Ataei et. al. 2021).

The data collected from the structured questionnaires included information about the socio-demographic characteristics of Capia pepper farmers and contextual variables related to their pesticide use behaviors. To measure TPB-related items, a 5-Point Likert Scale (ranging from 5=strongly agree to 1=strongly disagree) was employed, consistent with previous studies in the field.

To assess the reliability and validity of the items on the 5-Point Likert Scale, a Reliability Analysis was conducted. Reliability Analysis is a method used to evaluate the consistency of responses to survey questions, providing insights into the reliability and validity of the collected data (Eisinga et al. 2012). In this study, the Cronbach's Alpha for the seventeen items used was calculated as 0.702, indicating high reliability for the entire scale used in the research.

3.2. Research area

Çanakkale holds a significant position in various economic sectors in Turkey, including agriculture and logistics, due to its strategic location. The province connects Asia and Europe, and its unique geographical and climatic characteristics contribute to a wide range of agricultural products. Çanakkale ranks among the top ten provinces in Turkey for the production of over forty agricultural products, notably leading in Capia peppers, peaches, and nectarine production (Çanakkale Commodity Exchange 2022).

Çanakkale province ranks 1st in Capia pepper production in Turkey, accounting for approximately 20.0% of the national total in 2018. Capia pepper represents 94.0% of the total pepper production in Çanakkale (TSI 2018; TSI 2020). According to 2018 data from the Turkish Statistical Institute (TSI), Çanakkale (218 591 Tons) is ranked first Turkey's Capia pepper production for tomato paste (1 128 060 Tons).

3.3. Sampling

According to data gathered by the Çanakkale Provincial Directorate of Agriculture and Forestry, the Yenice district accounts for 60.0% of Çanakkale province's entire Capia pepper production. For this reason, the research was carried out in the Yenice district of Çanakkale province (Çanakkale Provincial Directorate of Agriculture and Forestry 2019). The number of farmers who are members of the Chamber, producing Capia pepper in the Yenice district of Çanakkale, was determined to be 841. The sampling formula was taken into account over this number. According to the results of the Proportional Sampling formula, it was decided to conduct a face-to-face survey with 206 Capia pepper producers in the Yenice district. 90% confidence interval for 0.05 margin of error,

$1.645\,\sigma_p = 0.05\quad \sigma_p = 0.03039$

$$n = \frac{N.p\,(1-p)}{(N-1)\,\sigma^2{}_{p_x} + p\,(1-p)} \quad (Yamane, 2010). \tag{1}$$

$$n = \frac{841\,(0.5)(0.5)}{840\,(0.03039)^2 + (0.5)(0.5)} = 206$$

Due to the inadequacy of data from the Çanakkale Provincial Directorate of Agriculture and Forestry, data from the Yenice Chamber of Agriculture were used to determine the total number of Capia pepper producers in the Yenice district and other information from village to village. Accordingly, there are 7 548 farmers and 841 Capia pepper producers in the Yenice district. When determining the villages where the surveys were to be conducted, villages with 30 or more Capia pepper producers were selected. Accordingly, the distribution of the 12 villages where the survey was conducted and the proportional distribution of the surveys according to the data are given by Yenice Chamber of Agriculture 2019.

3.4. Structural equation modeling

This study aimed to elucidate the pesticide use behaviors of Capia pepper producers in Çanakkale, the leading region in Capia pepper production in Turkey. This was achieved through the application of Structural Equation Modeling (SEM) within the framework of the TPB.

SEM is a robust statistical method for testing models that involve concurrent causal and reciprocal relationships between observable and latent variables. It allows for a comprehensive evaluation and quantification of significant theories. SEM is particularly useful for modeling interactions between theoretical constructs, accounting for measurement errors and relationships between errors, making it distinct from simple regression analysis (Hwang et al. 2020). SEM comprises two fundamental components: the Structural Model and the Measurement Model. The measurement model estimates latent variables using observable variables, displaying the relationships between latent variables and observable ones. In contrast, the structural model assesses relationships between latent variables. In this study, the SEM was created to test hypotheses (H_1, H_2, H_3, H_4, H_5) regarding the factors affecting pesticide use behaviors and intentions among Capia pepper producers within the TPB framework. In the course of the research, hypotheses (H_1, H_2, H_3, H_4, H_5) were examined using the model.

H_1= Intention affects behavior (Ajzen 2002; Bagheri et al. 2021b).

H_2= PBC is effective on behaviors (Ajzen 2002; Farani et al. 2019; Bagheri et al. 2021b).

H_3= PBC affects intention (Ajzen, 2002; Yazdanpanah et al. 2019; Savari and Gharechaee 2020; Ataei et al. 2021; Damalas 2021; Semuroh & Sumin 2021).

H_4= Subjective Norms are effective on intention (Ajzen 2002; Yazdanpanah et al. 2019; Savari & Gharechaee 2020; Ataei et al. 2021; Damalas 2021; Semuroh & Sumin 2021).

H_5= Attitudes have an impact on intention (Ajzen 2002; Yazdanpanah et al. 2019; Savari & Gharechaee 2020; Ataei et al. 2021; Damalas 2021; Semuroh & Sumin 2021).

In the SEM, which will be based on the Planned Behavior Theory, the factors that are expected to have an indirect or direct effect on both pesticide use intention and pesticide use behavior will be included (Rezaei et al. 2018; Bagheri et al. 2019; Despotovic et al. 2019; Farani et al. 2019; Rezaei et al. 2019a; Rezaei et al. 2019b; Yazdanpanah et al. 2019). Firstly, within the framework of the Planned Behavior Theory, the Intent factor is calculated with the following formula;

$$I = w_A\,A + w_{SN}\,SN + w_{PBS}PBS \tag{2}$$

The calculation of the three basic factors in the formula is as follows:

$$A = \sum_{i=1}^{n} b_i\;e_i \tag{3}$$

$$SN = \sum_{i=1}^{n} n_i m_i \tag{4}$$

$$PBC = \sum_{i=1}^{n} c_i \; p_i \tag{5}$$

Behavior is defined by the formula below. The effect of intention and PBC variables on the behavior variable is tested in this way.

$$B = w_{\iota} I + w_{PBC} PBC \tag{6}$$

b, n, c= the strength of every item about an outcome or quality,
m, p= evaluation of the result and the characteristic
B= Behaviors, I= Intentions, A= Attitudes, SN= Subjective Norms,
PBC= Perceived Behavior Control
w= empirically derived coefficient (Ajzen 1991; Ajzen 2002; Damalas 2021; Govindharaj et al. 2021; Lou et al. 2021).

4. Results

4.1. Demographics and background variables

The demographic characteristics of Capia pepper farmers are given in Table 1. Accordingly, 99.5% of the surveyed farmers are male. As part of the research, the survey participants' ages were asked for and subsequently categorized into specific age groups. According to the data obtained, the average age of the farmers was 46.3 years, and the standard deviation was calculated as 10.7 years. In addition, the youngest farmer was 20 years old with the oldest farmer being 72 years old. Table 1 displays the distribution of Capia pepper producers in the research categorized by age groups. According to this, 7.8% of Capia pepper producers are between 20 and 30 years old, 23.3% are between 31 and 40 years old, 35.4% are between 41 and 50 years old, and 24.3% are between 51 and 60 years old and 9.2% are over 61 years old. Considering the distribution of Capia pepper producers within the scope of the research according to their educational status, it is seen that 61.6% of Capia pepper producers are primary school graduates, 19.4% are middle school graduates, and 13.6% are high school graduates with only 5.4% being university graduates. The number of people living in the households of Capia pepper producers in the research region was requested to be constantly variable and then grouped. While the average number of people in the households of the farmers was calculated as 3.4, the standard deviation was calculated as 1. The number of people living in the households of the farmers was determined as at least 1 and at most 6. So, 24.3% of the farmers live in households with 1-2 persons, 64.6% in households with 3-4 persons and 11.2% in households with 5-6 persons. More than half of the Capia pepper farmers, 54.9% have been farming for 25 years or less, and 45.1% have been farming for more than 25 years. More than three-quarters of the farmers (75.7%) have social security. Again, the majority of Capia pepper farmers (70.9%) are members of at least one agricultural cooperative. Finally, 42.7% of the farmers have non-agricultural income (rent, earned from other jobs, etc.).

Table 1- Demographics of Capia farmers

Variables	***Total (n)***	***(%)***
	206	100.0
Gender		
Male	205	99.5
Female	1	0.5
Age Groups		
20-30 years	16	7.8
31-40 years	48	23.3
41-50 years	73	35.4
51-60 years	50	24.3
61 and up	19	9.2
Descriptive statistics of age= Min.=20, Max=72, Mean= 46,3, Standart Deviation (S.D.)=10,7		
Education groups		
Primary school graduate (5 years)	127	61.6
Secondary school graduate (6-8 years)	40	19.4
High school graduate (9-11 years)	28	13.6
Graduated from a university (12 years and +)	11	5.4
Number of households groups		
1-2 person	50	24.3
3-4 person	133	64.6
5-6 person	23	11.2
Descriptive statistics of household number= Min=1, Max= 6, Mean= 3,4, S.D.= 1,0		
Farming experience status		
3-25 years	113	54.9
More than 25 years	93	45.1
Descriptive statistics of farmin experience= Min=3, Max= 55, Mean= 25,5, S.D.= 11,6		
Farmers' social security status		
Yes	156	75.7
No	50	24.3
Farmers' cooperative membership status		
Member	146	70.9
Not member	60	29.1
Non-farm income status		
Yes	88	42.7
No	118	57.3

Table 2 presents the particulars of farmers with non-agricultural income. Interestingly, 83.0% of the research participants opted not to disclose their total annual income, encompassing both agricultural and non-agricultural earnings. Therefore, questions about income are reduced to annual non-agricultural income and income from Capia pepper farming. The average annual non-agricultural income of Capia pepper producers with non-agricultural income was calculated as $ 4 710.

Table 2-Annual non-farm income of Capia pepper farmers

Annual non-farm income	***N***	***(%)***
4 710 $* and less	49	57.0
Over 4 710 $	37	43.0
Total	86**	100.0

Descriptive statistics of annual non-farm income= Min=316.9 $, Max= 14,084.5 $, Mean= 4,710.9 $, S.D.= 2,301.9 $;
*: 1 American $= 5.68 Turkish Liras in 2019 (CBRT, 2022).**: Two producers could not state their annual non-farm income.

Table 3 provides information on the annual income of farmers from Capia pepper production in 2019. Accordingly, the annual average income of Capia pepper farmers from Capia pepper is around $11.302. 62.8 % of the farmers earn around this figure or below.

Table 3- Income of annual Capia pepper production

Income of annual capia pepper	***N***	***(%)***
11 302 $ and less	103	62.8
Over 11 302 $	61	37.2
Total	164*	100.0

Descriptive statistics of annual Capia pepper production income= Min=1 056.3 $, Max=35,211.3$, Mean= 11,302.99 $, S.D.=7,715.8 $: ***;** Forty-two producers could not state their annual Capia pepper income

Table 4 provides descriptive statistics pertaining to Capia pepper production among the participants included in the research. Accordingly, the average size of the land is 20.9 decares, the average number of parcels is 3.1 parcels, and the average Capia pepper yield per decare is 3 199.3 kg.

Table 4- Descriptive statistics for Capia pepper production within the research's scope

Descriptives	*Minimum*	*Maximum*	*Mean*	*S.D.*	*N*
Capia Pepper Produced Area (Ownership+Rent)	1 da	100 da	20.9 da	15.9 da	206
Number of Parcels Produced Capia Pepper	1 parcel	15 parcels	3.1 parcels	2.4 parcels	206
Capia Pepper Average Yield/Decare (da)	2 000 kg	5 000 kg	3 199.3 kg	715.2 kg	206

Worldwide, 40.0% of pesticides are used as to herbicides, 17.0% to insecticides, and 10.0% to fungicides in 2020 (EUROSTAT 2022). According to 2018 data, the average pesticide use in Turkey is 15 005 Tons. 38.0% of this is fungicides, 25.0% herbicides, 23.0% insecticides and 14.0% other pesticide groups (TSI 2020). According to the effect assessment on diseases and pests given in Table 5, 42.2% of the farmers find herbicides (herbicides) effective, while 30.6% consider them very effective. 35.0% of the farmers find fungicides less effective, 30.1% ineffective, 20.4% effective and 14.1% very effective. 41.7% of the farmers stated that they found pesticides very effective, 31.1% effective and 20.9% less effective.

Table 5- Assessment of the impact of pesticides employed by Capia pepper producers in the research on diseases and pests (%)

N=206	*Very Efficient (% 75-100)*	*Efficient (% 50-75)*	*Less Efficient (Less than % 50)*	*Not Efficient*
Herbicide	30.6	42.2	21.4	5.8
Fungicide	14.1	20.4	35.0	30.5
Insecticide	41.7	31.1	20.9	6.3

4.2. Data analysis

In this research, Partial Least Squares (PLS) software (Smart PLS 3.2.3) has been used for SEM to determine the measurement and structural model.

4.2.1. Measurement model

First, the relationship between the measurement model and the latent variables and their measurements will be explored. Table 6 suggests the results of the measurement model. The measurement model provides the conditions for validity and reliability. Cronbach Alfa (α), Composite Reliability (CR), and average Variance Extracted (AVE) must offer certain values for convergent validity. According to the related literature, Cronbach Alfa and AVE is above 0.55 while CR is above 0.70 for convergent validity (Hair et al. 2017). The measurement model results provide these criteria (Table 6).

Table 6- Measurement model results

Variables of measurement model	*Factor Loadings*	*Cronbach's α*	*CR*	*AVE*
Pesticide Use Behaviors (B) [a,b]		0.874	0.924	0.803
B1. I grow Capia peppers using heavy amounts of pesticides.	0.963			
B2. I often grow Capia peppers using pesticides.	0.958			
B3. I grow Capia peppers using the most effective pesticides.	0.750			
Pesticide Reduction Intention (I) [a,c,d,e,f]		0.894	0.925	0.755
I1. I would like to grow Capia peppers using fewer pesticides.	0.855			
I2. I would like to grow Capia peppers using pesticides less often (with less scheduling).	0.905			
I3. I would like to grow peppers using pesticides that are less dangerous and do the least harm to nature.	0.898			
I4. I would like to grow peppers using pesticides that are less dangerous and do the least harm to humans.	0.817			
Attitudes (A) [a,e,f,g]		0.878	0.906	0.617
A1. I believe that excessive use of pesticides pollutes the soil, water and air.	0.786			

Table 6 (Continue)- Measurement model results

Variables of measurement model	*Factor Loadings*	*Cronbach's α*	*CR*	*AVE*
A2. I believe that excessive use of pesticides adversely affects consumer health.	0.781			
A3. Excessive use of pesticides can affect my health. I believe it has a negative effect.	0.786			
A4. I feel guilty when I use more pesticides than necessary.	0.743			
A5. Today, I feel a moral obligation to use extensive pesticides.	0.823			
A6. I feel a moral obligation to use extensive pesticides for future generations.	0.792			
Subjective Norms (SN) [e,f,g]		0.604	0.740	0.589
SN1. Farmers around me try to use pesticides less.	0.812			
SN2. We talk to the farmers around me about the use of pesticides.	0.720			
Perceived Behavior Control (PBC) [e,f]		0.618	0.743	0.614
PBC1. I believe I can lower my pesticide use in Capia pepper if I want.	0.984			
PBC2. I can manage to use exactly as much (no more or less) pesticides as the pepper needs.	0.510			

References*:* [a]Ajzen 2002; [b]Farani et al. 2019; [c]Kim et al. 2017; [d]Yadav & Pathak 2016, [e]Savari & Gharechaee 2020; [f]Ataei et. al. 2021; [g]Cheah & Phau 2011

4.2.2. Structural model

After the validity and reliability of the measurement model are achieved, the structural model results are presented. A bootstrap resampling method based on 5 000 repetitions and 300 cases was employed to assess their significance (Hair et al. 2017). Table 7 shows the result of the hypothesis tests and the SEM. H_2, H_4, and H_5 are supported, but H_1 and H_3 are not supported from the opposite side.

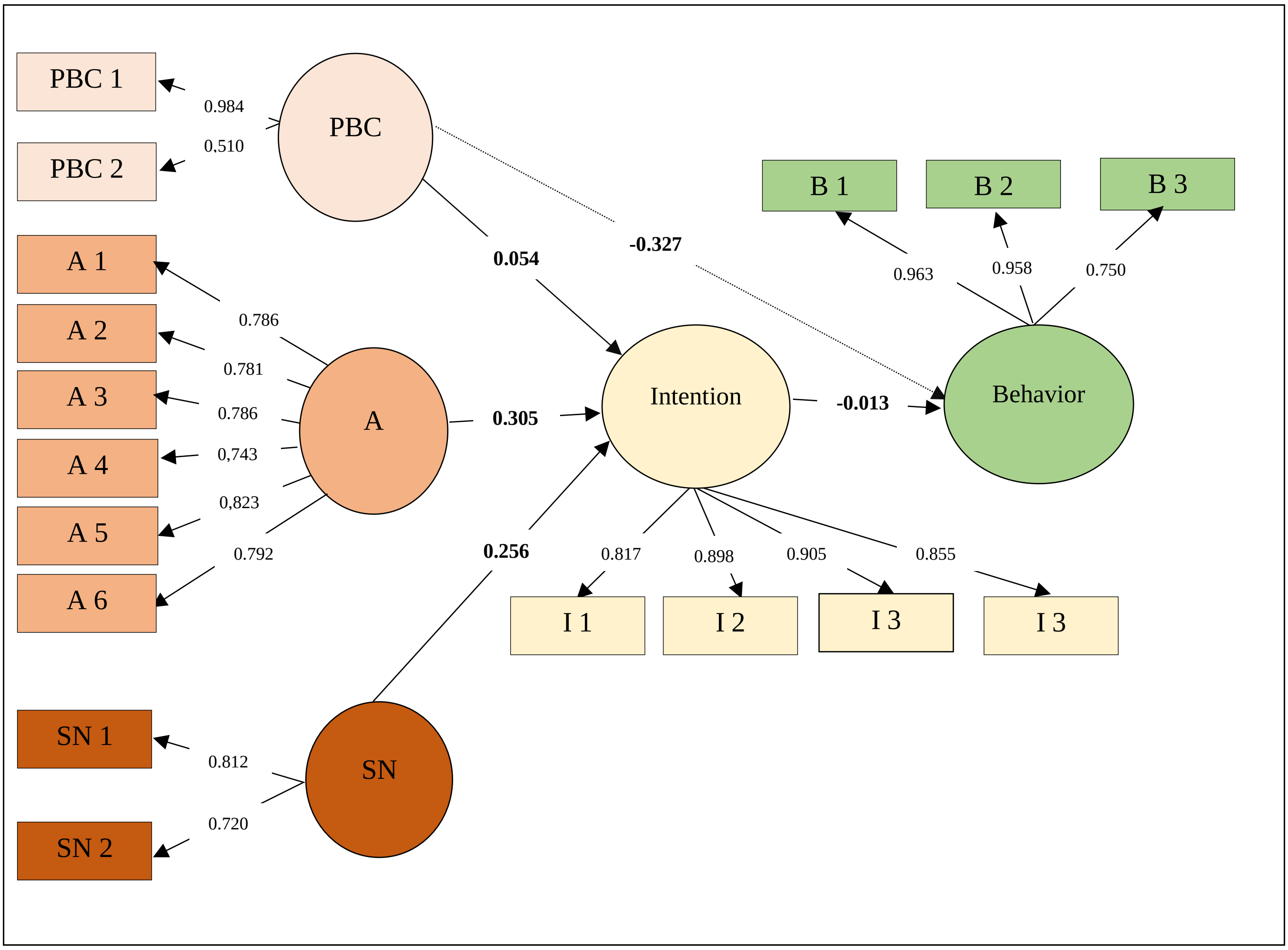
The Capia pepper pesticide use behavior is not influenced by the intention (H_1). This is because Capia pepper farmers generally refuse to believe that they use too much pesticide.
However, pesticide reduction intention is affected by attitudes and subjective norms (H_4, H_5). PCB has a negative effect on pesticide use behavior (H_2), while it is not effective on pesticide reduction intention (H_3).

Table 7- Structural model results

Hypothesis	*Regression Path*	*Path Coefficient*	*t-statistics*	*P values*	*Remarks*
H_1	I -> B	-0.013	0.182	0.856	Not supported
H_2	PBC -> B	-0.327	4.472	0.000*	Supported
H_3	PBC -> I	0.054	0.606	0.545	Not supported
H_4	SN -> I	0.256	3.496	0.000*	Supported
H_5	A-> I	0.305	3.086	0.002*	Supported

The visual diagram of the obtained SEM is given in Figure 2. Path coefficients between latent variables in Table 7 are shown schematically. Again, the schematic representation of the factor loadings of the latent variables, which are given in Table 6 and constitute the lantent variables, are provided.

Figure 2- Visual Results of SEM



5. Discussion

5.1. Reiterating the research problem

This study investigates the pesticide usage behavior and the intention to reduce pesticide use among Capia pepper farmers, employing the TPB framework. The research problem stems from the concern that pesticide residues in Turkey's fruit and vegetable exports have increased, as indicated by the Rapid Alert System for Food and Feed (RASSF) portal data, revealing a threefold rise compared to the previous year (RASSF 2022). The study addresses the critical issue of pesticide use in fruit and vegetable production and the discrepancies between farmers' intentions and actual behaviors in this regard.

5.2. Major findings

Intentions vs. Behaviors: Farmers generally expressed their intention to use fewer and less intensive pesticides. However, they did not acknowledge intensive pesticide usage in their behaviors. Notably, the intention to reduce pesticide use did not significantly translate into corresponding behavior. This discrepancy highlights a challenge in aligning farmers' intentions with their actual pesticide usage behaviors.

Perceived Behavioral Control (PBC): PBC, representing individuals' perceptions of their control over a behavior, was not statistically significant concerning the intention to reduce pesticide use. This suggests that farmers' perceptions of their abilities and possibilities in reducing pesticide use did not significantly influence their intentions in this study. However, PBC was found to be statistically significant regarding pesticide use behavior, indicating that farmers' perceived control over their actions affected their actual pesticide usage.

Subjective Norms and Attitudes: Subjective norms, reflecting the social pressure farmers felt regarding their intentions, played a significant role in influencing their pesticide reduction intentions. Farmers' attitudes towards pesticide use were also significant in shaping their intentions. Both subjective norms and attitudes appeared to be strong drivers of farmers' intentions to reduce pesticide use.

5.3. Explanatory power and contribution to literature

This study stands out in examining the interplay between intention and behavior in the context of pesticide use among Capia pepper farmers. While many previous studies have focused solely on modeling the intention to use pesticides, our research extends the TPB model to analyze the impact of intention on actual behavior. This distinction is critical as it provides insights into the challenges of translating intention into action in the context of pesticide use.

5.4. Meaning of the findings and their importance

The findings underscore the complexity of influencing farmers to reduce pesticide use in practice. Despite their expressed intention to use fewer and less intensive pesticides, farmers' actual behaviors do not align with these intentions. This suggests that interventions aimed at changing farmers' pesticide use behaviors may face barriers beyond their intentions. Understanding these barriers is vital for policymakers and agricultural extension services seeking to promote sustainable and responsible pesticide use.

5.6. Relating the findings to similar studies

Comparing our results to similar studies in the literature reveals both consistencies and discrepancies. For instance, subjective norms and attitudes consistently emerged as significant factors influencing intention across various studies (Savari & Gharechaee 2020; Bagheri et al. 2021b; Damalas 2021; Govindharaj et al. 2021). However, the significance of PBC varies, with some studies reporting it as significant (Savari & Gharechaee 2020; Bagheri et al. 2021b; Govindharaj et al. 2021) and others, including our study, not finding it significant concerning intention (Yazdanpanah et al. 2019; Ataei et al. 2021).

Furthermore, our research extends the analysis to behavior, highlighting the significance of PBC on pesticide use behavior, which aligns with findings from some previous studies (Farani et al., 2019; Bagheri et al., 2021b). However, the non-significant relationship between intention and behavior observed in our study contrasts with some prior research that found a significant association between these variables (Savari & Gharechaee 2020; Govindharaj et al. 2021).

5.7. Alternative explanations of the findings

The inconsistency in the significance of PBC and the non-significant relationship between intention and behavior in our study may suggest that factors beyond individual intention and perceived control play a role in shaping farmers' pesticide use behaviors. Possible alternative explanations may include external factors such as economic incentives, agricultural practices, and the availability of alternative pest management strategies. Further research is needed to explore these additional factors and their interactions with the TPB constructs.

In summary, this study provides valuable insights into the complexities of influencing pesticide use behaviors among Capia pepper farmers. It underscores the importance of considering not only farmers' intentions but also external factors that may impact their actual pesticide usage. Policymakers and agricultural extension services should take into account these findings when developing strategies to promote sustainable and responsible pesticide use in fruit and vegetable production.

6. Conclusions

This research aimed to examine the drivers of farmers' pesticide use intention and behaviors. It has shown that farmers' positive intentions to reduce pesticide use do not necessarily turn into behaviors. The research has also shown that SN and attitudes are important drivers of farmers' pesticide reduction intentions while PBC is effective on their behaviors.

No TPB modeling approach has been used before to measure Turkish farmers' intentions and behaviors toward pesticide use. For this reason, these results provide new information fully revealing the TPB model as a suitable method to explain the intention and behavior Cappia pepper farmers in the study area to use pesticides in an effective way.

The results provide novel information about Capia pepper farmers' intentions and behaviors to reduce pesticide use. The discovery of these new insights, derived from a compelling survey on the intentions and behaviors of Capia pepper farmers in the study area, gives rise to another significant finding: this study found TPB to be a sufficiently robust model to study pesticide use intentions and behavior in Capia pepper. The results will help future research investigate: "Why can't farmers' intent to reduce pesticides be a positive behavior?"

Greater efforts are needed to ensure agricultural policies that will increase the awareness of farmers on pesticide use behaviors. In this context, it is thought that agricultural extension implications involving leading farmers and campaigns and public service announcements targeting farmers' attitudes may be beneficial.

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Nematicidal Activity of Various Aqueous Extracts against Root-Knot Nematodes (*Meloidogyne chitwoodi*)

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ABSTRACT

In this study, the effects of twelve aqueous extracts on the hatching and mortality of second-stage juveniles (J2s) of *Meloidogyne chitwoodi* were evaluated under in vitro and growth chamber conditions in 2020-2021. The mortality of the J2s increased with the increasing exposure time and concentration for all extracts. Maximum mortality was observed in 50% of all aqueous extracts. Up to 100% maximum mortality was observed in 10% of extracts of *Anethum graveolens* (100%), *Eruca sativa* (100%), *Ficus carica* (100%), *Juglans regia* (100%), *Melia azedarach* (100%), *Mentha piperita* (100%), *Vitex agnus-castus* (98%), *Asphodelus aestivus* (96.4%), *Eucalyptus camaldulensis* (95.6%) after 24 hours and minimum mortality was found in 1% of extracts of *Laurus nobilis* (6.6%) after 6 hours. Moreover, maximum suppression of hatching was found at 64.2%, 61.0%, and 59.4% with extract of *A. aestivus*, *Nerium oleander*, and *V. agnus-castus* after 7 days, respectively.

The pot experiments showed that the gall index was the lowest in soils treated with *Ficus carica*, *Nerium oleander*, *Laurus nobilis*, *Eucalyptus camaldulensis* and *Zingiber officinale* extracts. The chemical composition of five aqueous extracts was analysed using gas chromatography-mass spectrometry (GC-MS), and the main components of five aqueous extracts were identified as eucalyptol, 2-methoxy-6,10-dimethyl-dodeca-2E,6Z,10Z-trienoic acid, 12-acetoxy-, dihydroedulan II and α-Zingiberene. The study confirms the potential of mainly *L. nobilis, E. camaldulensis, F. carica*, *Z. officinale* and *N. oleander* extracts for the formulation of new products for controlling *M. chitwoodi*

Keywords: *Eucalyptus camaldulensis*, *Nerium oleander*, *Zingiber officinale*, Mortality, Management, GC-MS

1. Introduction

Plant-parasitic nematodes cause 12.6% of global crop yield loss which is equivalent to an estimated 157 billion dollars per year (Nicol et al. 2011; Das et al. 2021; Marin-Bruzos et al. 2021). In particular, root-knot nematodes (*Meloidogyne* spp.) have a large host plant spectrum and cause low crop yields. They rapidly reproduce under favourable conditions, and the increment of the population causes low crop yields (Perry et al. 2009). Over the years, researchers have developed a variety of cultural, mechanical, and chemical applications to control root-knot nematodes (Sharma et al. 2020). Nematicides can be effectively used to prevent crop losses from plant-parasitic nematodes. Chemical nematicides are still the most effective means of plant-parasitic nematode control. However, intensive nematicide use destroys beneficial microorganisms in the soil; therefore, nematicide usage is neither a healthy nor an economical solution for growers. In order to overcome these problems, it is crucial to adopt effective and environmentally friendly methods to control nematodes (Nolling & Becker 1994). One such practice would be the use of plant extracts that have nematicidal properties (Aydınlı & Mennan 2014). The flowers, shoots, and leaves of *Nerium oleander* L. have been shown to have nematicidal effects on *Meloidogyne javanica* (Treub 1885; Chitwood 1949; Moosavi 2012). Plant crude extracts have a potential nematicidal effect against *M. incognita* (Sithole et al. 2021).

Columbia root-knot nematode, *Meloidogyne chitwoodi* Golden et al. 1980 is one of the most widespread root-knot nematodes that infect potatoes, vegetables, wheat, corn, tomatoes, alfalfa, and numerous weeds (Evlice & Bayram 2019). For this reason, a few nematicides should be applied at each cropping season. Currently, research on alternative methods to nematicide is primarily focused on plant extracts and their components. The effect of plant extracts and essential oils in controlling plant-parasitic nematodes is well documented (D'Addabbo et al. 2020). However, few studies have been published on the control of *M. chitwoodi* using plant extracts (Golec 2019). The study focused on the effects of aqueous extracts on the control of *Meloidogyne chitwoodi*, one of the most widespread root-knot nematodes that infects numerous crops worldwide. The study evaluated the efficacy of the extracts in vitro and pot experiments and analysed five of the extracts using gas chromatography-mass spectrometry (GC-MS). The results showed that certain plant extracts had significant nematicidal effects on *M. chitwoodi*, and these extracts could be potentially used as alternatives to synthetic nematicides.

2. Material and Methods

2.1. Nematode cultures

Meloidogyne chitwoodi was initially isolated from infested potato tubers collected from Niğde province (Devran et al. 2009). To obtain the pure culture, 20 egg masses were handpicked using forceps and then surface-sterilized. The egg masses were then inoculated onto four-leaf stage tomato seedlings (Cüsseli F1) and the plants were grown for 8 weeks in a growth chamber at 23±1 °C (Hussey & Barker 1973). After 8 weeks, egg masses were collected from the infested tomato roots.

2.2. Preparation of aqueous extracts of plant materials

The plant species were harvested from (March-June) 2019-2020 in the Adana and Hatay provinces. Rhizome (*Zingiber officinale*), whole plants (*Anethum graveolens*), and leaves (other plants) were cut, washed with tap water, and then dried in an oven at 60 °C for 72 h (Abbas et al. 2009). The dried plants were ground with a blender and the plants powder stored in a glass bottle at 4 °C. For the preparation of aqueous extracts, 100 g powder was added to 900 mL distilled water, and thoroughly mixed on a shaker for 4 h. The suspension was then filtered with muslin cloth and then centrifuged at 5,000 rpm for 15 min. The resulting supernatant was collected and stored at 4 °C in opaque bottles until used as stock solutions (Elbadri et al. 2008; Abbas et al. 2009; Oka et al. 2012). With these stock solutions, dilutions of 1%, 2.5%, 5%, and 10% were prepared by adding the necessary amount of sterile distilled water.

2.3. Effect of aqueous extracts on juveniles mortality

To test for juvenile mortality, J2s were exposed for 6, 12, and 24 h to the 12 extracts at 1%, 2.5%, 5%, and 10% concentrations. The effect of plant extracts was evaluated in 96-well plate using suspensions of 100 J2/mL in distilled water at 22 °C. Distilled water was used as a control. The experiments included five replicates of each treatment. The mortality of the J2s was evaluated based on the number of dead nematodes observed under a microscope. The viability of the nematodes was determined using a needle.

2.4. Effect of aqueous extracts on egg hatching

Hatching was assessed after 24 h, 72 h, and 7 d in 10% concentration of plant extracts using five egg masses of the same age (Moosavi 2012). This method was used because better results were obtained at a concentration of 10%. The experiments included four replicates of each treatment, including control with distilled water, and were incubated at 21-22 °C in a growth chamber.

2.5. Effect of aqueous extracts on Meloidogyne chitwoodi in pot experiments

Experiments were set up in the growth chamber. Tomato (Cüsseli F1) seedlings at the four-leaf stage were grown in a one-kg capacity with autoclaved soil and 2,000 newly hatched J2s of *M. chitwoodi* were inoculated (Elbadri et al. 2009). Then, 100 mL of each plant extract was added to the soil. The negative control was distilled water, the positive control included nematodes only. Each treatment was replicated four times, and pots were arranged in a completely randomized design. The inoculated plants were placed in a growth room at 23 °C±1, 60±10% RH, and 16:8 h L:D photoperiod and watered regularly for 8 weeks. The plant roots were evaluated according to the 0-5 gall index (Hartman & Sasser 1985). Additionally, the fresh and dry weights of roots and plant height were measured according to Aydınlı & Mennan (2014).

2.6. Data analysis

All experiments in the study were were repeated twice. The data were analysed using one-way ANOVA (SPSS version 25) and the means were separated using Duncan's multiple range test. The pooled data from the in vitro and nematode data from the in-pot experiments were Arcsin and Ln (x+1) transformed for the homogenize error variances. Additionally, repeated measures ANOVA was used to determine the relation with time, plant species and concentration. The relative suppression rate was calculated as illustrated in Equation 1 (Yang et al. 2016).

$$= \frac{Relative\ suppression\ rate\ (\%)\ number\ of\ J2\ in\ sterilized\ water\ -\ number\ of\ J2\ in\ root\ exudate}{number\ of\ J2\ in\ sterilized\ water} x100 \qquad (1)$$

2.7. Gas chromatography-mass spectrometry (*GC-MS*)

The composition of *Laurus nobilis* L. (Laurales: Lauraceae), *Eucalyptus camaldulensis* Dehnh (Rosids: Myrtaceae), *Ficus carica* L. (Rosales: Moraceae), *Nerium oleander* (Gentianales: Apocynaceae), *Zingiber officinale* (Zingiberales: Zingiberaceae) was analysed by GC-MS. The Agilent Brand 7890B, GC 7010B MS system was used for the GC-MS analysis. Using the solid phase micro extraction (SPME) method, 3 mL of plant extract was placed in a 20 mL vial, and kept at 50 °C for 10 min. Then, samples were adsorbed for 30 min using the SPME apparatus 50/30 µm fiber coated with divinylbenzene/carboxene/polydimethylsiloxane

(DVB/CAR/PDMS) Agilent. DB-Wax (60 m x 0.25 mm i.d. x 0.25 µm; J&W Scientific-Folsom, USA) was then injected into the capillary column by desorbing for 5 min. At first, the injection temperature was kept at 250 °C, column oven temperature was kept at 40 °C for 4 min, after which the temperature was increased at a rate of 3 °C/min to 90 °C. It was then increased at a rate of 4 °C/min up to 130 °C for 4 min.

3. Results and Discussion

Plants produce phytochemicals that provide significant defense against pathogenic organisms and pests. The present study investigated the effects of twelve plants on *Melodiogyne chitwoodi*. Few plant extract studies have been written on the subject of *M. chitwoodi* and, for this reason, this study seeks to provide new analyses and data. Plant extracts were tested for suppression of egg hatching, mortality of juveniles and growth of tomato plants inoculated with *M. chitwoodi*. The plants' chemical components which have the most suppressive effect were determined by GC-MS. The results indicated that aqueous extracts of *F. carica* resulted in 100% mortality of J2s at 1% concentration after 12 h under laboratory conditions. The best concentration was found in 10% of all plants.

3.1. Effect of aqueous extracts on the mortality of juveniles

Table 1 shows the1% and 2.5% mortality of the J2s for the various extracts while Table 2 presents the 5% and 10% mortality of the J2s for the various extracts. In all treatments, the mortality of the J2 increased with increasing exposure time. All plant extracts showed higher mortality with 24 h exposure compared to 12 h. After 24 h, more than 50% nematode mortality was induced by 2.5% concentration of all plant extracts. Complete mortality was observed after 12 h with 1% extracts of *F. carica*. Additionally, the most effective concentration and exposure time of plant extracts were those of 10% concentration and after 24 h.

Table 1- Effects of aqueous extracts on mortality of second-stage juveniles (J2) of *Meloidogyne chitwoodi (1% and 2.5%)**

Conc. %	***1%***			***2.50%***		
Exposure time	***6 h***	***12 h***	***24 h***	***6 h***	***12 h***	***24 h***
Control	1.6±0.5^{g}	19.0±0.7^{g}	28.6±0.9^{j}	1.6±0.5^{h}	19.0±0.7^{g}	28.6±0.9^{i}
Anethum graveolens L.	10.8±1.2^{de}	38.6±1.2^{c}	48.4±1.3^{f}	18.2±1.8^{f}	48.2±0.8^{d}	76.4±0.7^{d}
Asphodelus aestivus Brot.	7.4±1.5^{ef}	45.2±1.0^{b}	60.6±0.9^{d}	16.2±1.4^{f}	51.6±0.9^{c}	72.4±1.3^{e}
Eruca sativa Mill	13.2±1.0^{d}	35.2±1.5^{d}	53.4±1.4^{e}	22.2±1.3^{de}	51.6±0.7^{c}	75.8±0.8^{d}
Eucalyptus camaldulensis Dehnh	13.8±0.9^{d}	35.0±0.9^{d}	48.8±1.2^{f}	23.8±1.3^{cd}	41.2±1.6^{e}	64.0±0.8^{f}
Ficus carica L.	51.4±1.0^{a}	100±0.0^{a}	100±0.0^{a}	94.6±0.5^{a}	100±0.0^{a}	100±0.0^{a}
Juglans regia L.	22.2±1.3^{b}	40.6±1.5^{c}	57.8±1.2^{d}	25.4±1.2^{bcd}	75.4±1.0b	94.8±0.4^{b}
Laurus nobilis L.	6.6±1.2^{f}	31.8±1.2^{de}	38.2±0.9^{i}	9.6±0.9^{g}	34.8±0.9^{f}	54.0±0.9^{h}
Melia azedarach L.	11.2±1.6^{d}	41.4±1.4^{c}	69.6±1.2^{b}	17.8±1.1^{f}	49.2±0.7^{cd}	81.0±1.1^{c}
Mentha piperita L.	21.0±1.1^{bc}	44.8±1.4^{b}	63.8±1.0^{c}	28.2±1.5^{b}	51.0±1.1^{cd}	80.8±0.7^{c}
Nerium oleander L.	10.6±0.9^{de}	24.0±1.0^{f}	44.0±1.1^{gh}	18.8±1.1^{ef}	35.0±1.2^{f}	60.8±0.8^{g}
Vitex agnus-castus L.	18.6±1.6^{c}	30.0±1.0^{e}	42.6±1.4^{h}	26.4±0.9^{bc}	35.6±1.0^{f}	65.0±1.3^{f}
Zingiber officinale Roscoe	12.2±1.0^{d}	32.4±1.3^{de}	46.4±0.9^{fg}	18.4±1.1^{f}	39.6±1.1^{e}	60.4±0.9^{g}

*: Means followed by the same letters within columns are not significantly different according to Duncan test at P<0.05. The results are expressed as mean ± standard error

Table 2- Effects of aqueous extracts on mortality of second-stage juveniles (J2) of *Meloidogyne chitwoodi (5% and 10%)**

Conc. %	***5%***			***10%***		
Exposure time	***6 h***	***12 h***	***24 h***	***6 h***	***12 h***	***24 h***
Control	1.6±0.5^{i}	19.0±0.7^{h}	28.6±0.9^{i}	1.6±0.5^{h}	19.0±0.7^{h}	28.6±0.9^{f}
Anethum graveolens L.	26.4±1.3^{de}	56.0±1.0^{d}	89.2±0.9^{d}	33.2±0.9^{e}	64.2±1.3^{d}	100±0.0^{a}
Asphodelus aestivus Brot.	18.6±1.6^{g}	57.4±0.9^{d}	81.4±0.8^{f}	58.2±1.4^{b}	90.0±0.8^{c}	96.4±0.6^{c}
Eruca sativa Mill	25.0±0.9^{ef}	68.0±1.0^{c}	87.6±0.5^{d}	28.2±0.7^{f}	89.4±0.7^{c}	100±0.0^{a}
Eucalyptus camaldulensis Dehnh	29.2±0.9^{cd}	50.6±1.0^{e}	84.4±0.6^{e}	32.8±0.7^{e}	55.6±1.2^{f}	95.6±0.4^{c}
Ficus carica L.	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}
Juglans regia L.	37.2±0.9^{b}	88.2±1.2^{b}	100±0.0^{a}	45.0±0.8^{c}	95.0±0.7^{b}	100±0.0^{a}
Laurus nobilis L.	14.8±0.9^{h}	38.2±1.0^{g}	62.4±1.1^{h}	19.8±1.1^{g}	45.2±1.2^{g}	79.8±0.9^{e}
Melia azedarach L.	23.2±1.2^{f}	55.6±0.9^{d}	94.6±0.7^{c}	27.4±0.9^{f}	59.2±1.4^{ef}	100±0.0^{a}
Mentha piperita L.	35.0±1.2^{b}	55.6±0.7^{d}	98.2±0.6^{b}	38.0±0.7^{d}	62.8±1.2^{de}	100±0.0^{a}
Nerium oleander L.	25.4±0.9^{ef}	47.8±0.9^{ef}	75.6±0.7^{g}	29.8±1.0^{f}	62.6±1.5^{de}	86.8±0.9^{d}
Vitex agnus-castus L.	31.6±1.1^{c}	54.4±1.2^{d}	84.6±0.8^{e}	37.2±0.8^{d}	65.2±0.9^{d}	97.6±0.7^{b}
Zingiber officinale Roscoe	27.8±0.7^{de}	46.4±0.9^{f}	73.8±1.0^{g}	29.4±1.2^{f}	56.6±1.7^{f}	88.6±0.9^{d}

*: Means followed by the same letters within columns are not significantly different according to Duncan test at P<0.05. The results are expressed as mean ± standard error

3.2. Effect of aqueous extracts on the egg hatching

All tested samples were found to increase and decrease the egg hatching rate in egg masses of *M. chitwoodi* compared to the control group (distilled water). The extract from *Asphodelus aestivus* had the highest effect on the hatching at all times and reduced egg hatching by 64.2% at the end of the seventh day. This was followed by *Nerium oleander*, *Vitex agnus-castus*, and *Anethum graveolens* with 61%, 59.4%, and 53.6% respectively. The lowest effect was found in the *M. azedarach* extract with 15.2% at the end of the seventh day. The results are presented in and Table 3.

Table 3- Effect of aqueous extracts on egg hatching and relative suppression rate (%) to control of *Meloidogyne chitwoodi* in vitro condition*

Plant species	***24 h (Mean± SE)***	***Relative suppression rate (%)***	***72 h (Mean± SE)***	***Relative suppression rate (%)***	***7 days (Mean± SE)***	***Relative suppression rate (%)***
Control	64±2.2^{a}	0.0	70±1.1^{a}	0.0	133±2.2^{a}	0.0
Anethum graveolens	45±3.1^{bcd}	29.6	55±3.5^{bc}	21.7	62±2.7^{fg}	53.6
Asphodelus aestivus	42±2.7^{d}	34.3	44±2.2^{d}	37.6	48±2.8^{i}	64.2
Eruca sativa	52±2.5^{bc}	19.0	55±2.4^{c}	36.2	89±2.8^{d}	33.1
Eucalyptus camaldulensis	46±1.6^{bcd}	28.3	67±1.3^{a}	4.6	78±1.8^{e}	41.4
Ficus carica	47±2.4^{bcd}	26.5	48±1.5^{cd}	31.6	103±4.8^{c}	22.8
Juglans regia	62±2.2^{a}	2.6	64±3.1^{ab}	9.6	107±2.5^{bc}	20.1
Laurus nobilis	52±2.0^{b}	18.2	55±2.6^{bc}	21.7	70±2.9^{ef}	47.2
Melia azedarach	60±3.3^{a}	7.0	66±4.5^{a}	6.8	113±2.8^{b}	15.2
Mentha piperita	62±2.3^{a}	3.4	64±2.6^{ab}	9.6	79±2.8^{e}	41.0
Nerium oleander	43±2.0^{d}	33.0	50±3.8^{cd}	28.4	52±3.4^{h}	61.0
Vitex agnus-castus	44±2.3^{cd}	31.1	51±2.5^{cd}	27.3	54±2.0^{gh}	59.4
Zingiber officinale	51±3.0^{bc}	19.8	55±2.2^{c}	22.0	62±2.5^{fg}	53.2

*: Means followed by the same letters within columns are not significantly different according to Duncan test at P<0.05

The interaction of time-plants, time-concentration and time-plant-concentration were statistically significant at P<0.001 and, consequently, it was found that significant relationship exist between time, plant extracts, and concentration and that they influenced each other (Supplementary File 1).

3.3. Pot experiment

Pot experiment indicated that certain aqueous extracts effectively controlled *M. chitwoodi* by reducing juvenile infection. (Table 4). The root gall index was the lowest in the soil with extracts from *F. carica, N. oleander, Eucalyptus camaldulensis, Zingiber officinale* and *L. nobilis* compared to the other plant extracts and the positive control. Most of the aqueous extracts in the soil increased the plant height and decreased the fresh and dry root weights compared to the negative control. The highest plant height, fresh and dry root weights were observed with *N. oleander, E. camaldulensis, Juglans regia Anethum graveolens,* while the lowest plant height, fresh and dry root weights were determined in *Asphodelus aestivus, Ficus carica* respectively.

Table 4- Effect of aqueous extracts on number of gall index, dry and fresh root weight, plant height in tomato plants for 8 weeks after inoculation with nematodes*

Plant species	*Root gall index (0-5)*	*Root fresh weight (g) (Mean±SE)*	*Root dry weight (g) (Mean±SE)*	*Plant height (cm) (Mean±SE)*
Anethum graveolens	3.5±0.3^{abc}	51.8±2.9^{ab}	19.8±2.9^{ab}	65.5±2.5^{cde}
Asphodelus aestivus	3.0±0.0^{bc}	50.1±10.5^{abc}	17.1±6.3^{ab}	59.5±1.3^{e}
Eruca sativa	3.3±1.1^{bc}	44.6±14.2^{abc}	13.1±6.2^{ab}	72.0±1.4^{bcd}
Eucalyptus camaldulensis	2.8±0.5^{bc}	56.1±16.5^{abc}	16.8±6.1^{ab}	83.3±3.9^{a}
Ficus carica	2.5±0.3^{bc}	23.9±2.4^{c}	7.2±1.1 ^{b}	78.5±1.9 ^{ab}
Juglans regia	4.0±0.0^{ab}	61.9±12.1^{ab}	17.2±2.8^{ab}	65.5±1.2^{cde}
Laurus nobilis	2.5±0.3^{bc}	51.7±10.5^{abc}	14.5±3.3^{ab}	67.5±2.1^{cde}
Melia azedarach	3.0±0.0^{bc}	55.9±5.4^{ab}	17.3±3.2^{ab}	71.3±2.1^{bcd}
Mentha piperita	4.3±0.5^{ab}	57.4±4.4^{ab}	16.2±4.4^{ab}	63.0±0.7^{de}
Nerium oleander	2.0±0.0^{c}	41.8±8.9^{abc}	16.0±5.6^{ab}	84.5±8.8^{a}
Vitex agnus-castus	4.0±0.4^{ab}	34.0±8.1^{bc}	16.9±2.6^{ab}	74.0±2.1^{bc}
Zingiber officinale	2.5±0.3^{bc}	47.6±4.2^{abc}	12.9±3.9^{ab}	71.3±1.3^{bcd}
Positive Control	5.0±0.3^{a}	41.7±12.8^{bc}	15.0±6.4^{ab}	68.5±0.8^{cde}
Negative Control	0.0±0.0^{d}	72.4±6.8^{a}	24.5±3.6^{a}	64.0±1.4^{de}

*: Means followed by the same letters within columns are not significantly different according to Duncan test at P<0.05

4. Gas chromatography-mass spectrometry analysis

The chemical components of the plant extracts showing the highest nematicidal effect against *M. chitwoodi* were analysed by GC-MS. Details of the chemical compositions of plants extracts are shown in Supplementary File 2, Supplementary File 3, Supplementary File 4, Supplementary File 5, and Supplementary File 6. Several plants tested in this study have previously been tested against other *Meloidogyne* species. In an earlier study, *F. carica* extracts were found to have a significant paralysis effect on J2 of *M. javanica* after 72 hours and an inhibition of egg hatching because of various alkaloids and metabolites (Alves et al. 2020). *Nerium oleander* which is known as phytotoxic caused plants and juveniles to die and suppressed the egg hatching *M. javanica* (Moosavi 2012). In the present study, *N. oleander* killed 86.8% of J2s in 24 h and inhibited 52% egg hatching of *M. chitwoodi. Mentha piperita* L. was used to nematicidal effect on nematodes. It has been reported that *Meloidogyne arenaria* (Neal 1889) and *M. javanica* died in *M. piperita* at all concentrations of the aqueous extracts tested (Aydınlı et al. 2019). In the present study, similar results were observed *M. chitwoodi*. These results can be attributed to the presence of toxic substances in the aqueous extracts. The nematicidal activity of ginger against *M. javanica* was examined in vitro and a higher concentration (100%) suppressed egg hatching and juvenile mortality (Zareen et al. 2003). In vitro results showed that the suppression of egg hatching and mortality of the J2 increased with exposure time to aqueous extracts of *Z. officinale*. Research has shown that *Eucalyptus* spp. extracts can give high mortality of *M. javanica,* J2s whereas other studies reported no such mortality (Ahmed et al. 2010). These previous findings suggest that the plants tested in this study may have broad-spectrum nematicidal activity against different *Meloidogyne* species, which is promising for their potential use in integrated pest management programs. Further research is needed to identify the specific nematicidal compounds present in these plants and to determine their efficacy against other plant-parasitic nematodes. The present study demonstrated a high mortality rate for *M. chitwoodi* with *E. camaldulensis*. Different plant parts could provide different nematicidal activity (Aviles-Gomez et al. 2022). Nematicidal activity against *M. incognita* has been reported for the essential oil from fruits of *V. agnus-castus* (Ntalli et al. 2010). *Anethum graveolens* fruits significantly reduced *M. incognita* infection (Kim et al. 2003). In the present study, leaf extracts were used against *M. chitwoodi* in vitro and in tomato plants. In this dose-response experiment, the mortality of J2s and inhibition egg hatching increase with increasing exposure time to plant extracts. Ntalli et al. (2011) investigated antagonistic and synergistic actions of *J. regia* components. *Juglans regia* was highly nematicidal with up to 100% in vitro mortality of *M. incognita* race 2 (Laxmikant 2019). Similar results were observed in vitro as well as it the pot experiment. *Eruca sativa* provides powerful natural nematicidal effects against *M. incognita* (Aissani et al. 2015). In the present study, the gall index for *M. chitwoodi* was lower than four in pots treated with extracts of *F. carica, E. camaldulensis, N. oleander, L. nobilis, A. aestivus, A. graveolens, M. azedarach, Z. officinale* and *E. sativa*. GC-MS analysis was used to identify the composition of *F. carica, N. oleander, E. camaldulensis, L. nobilis* and *Zingiber officinale* extracts.

Alves et al. (2020) report that *F. carica* leaf extracts had components that affected the developmental process of the nematode. In the present study, ketones and monoterpenoids were found in *F. carica.* Although it has been reported that eucalyptol, which is one of the compositions of *F. carica,* is not always highly effective on nematodes. Similar results were reported by Caboni et al. (2013) and Mava et al. (2013). However, active components of other higher plant components and natural products (fatty acids) may be more effective against nematodes (Chitwood 2002). The inhibitory effect of these plant extracts on the eggs of *Meloidogyne* and J2s could be related to chemical components, such as coumarins, alkaloids, saponins, ketones, aldehydes, flavonoids, benzamides, and amides (Liu et al. 2011; Ntalli et al. 2011). it was showed that is worth is worth noting that the fatty acids, dihydroedulan II, and cathinone were found in *N. oleander* extracts (Cirlini et al. 2016) which is consistent with the findings of the present study. In addition, several studies have indicated that fatty acids can have considerable nematicidal activity (Davis et al. 1997; Duschatzky et al. 2004). *L. nobilis* was also found to have a few monoterpene components with nematicidal activity, such as linalool. In a recent study, spathulenol, a tricyclic sesquiterpene with 5,10-cycloaromadendrane skeleton, eucalyptol (monoterpen) in *E. camaldulensis,* was found to have useful bioactive against nematodes (Duschatzky et al. 2004, Faria et al. 2010). *Z. officinale* is a medicinal plant and its ingredients have been shown to be effective in previous studies (Youssef et al. 2015). Overall, the present study suggests that these plant extracts, or their main or minor compositions, may serve as effective nematicides. However, further studies are needed to investigate their potential for use in controlling nematode populations in agricultural settings.

4. Conclusions

This study investigated the *F. carica, N. oleander, Zingiber officinale* and *L. nobilis* as potential sources of new nematicidal products. These plant extracts, or their main or minor compositions may serve as nematicides. Many plants are known as a source of naturally occurring nematicidal compounds. Plant species, plant part, harvest time, extraction method, nematode species and treatment conditions all affect the measured nematicidal activity of such plant components.

Plant extracts can be used as repellents, stimulants or hatching inhibitors, and as nematicide depending on the properties of the target phytoparasitic compounds and nematodes. Future studies need to further assess plant extracts for their usefulness in integrated pest management programs, particularly for the control of plant parasitic nematodes.

Finally, *F. carica, N. oleander, L. nobilis, A. aestivus, A. graveolens, M. azedarach, E. camaldulensis, Juglans regia, Zingiber officinale* and *E. sativa* were the potential plants that showed the most effective results in in vitro and pot experiments. However, further research is needed to determine the efficacy, safety, and environmental impact of using these plant extracts as nematicides. Therefore, it is recommended that a combination of different control methods are used, including cultural practices, biological control agents, and chemical nematicides, to manage plant parasitic nematodes effectively and sustainably.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The author has no competing interests to declare that are relevant to the content of this article.

Ethics approval and consent to participate. All ethical aspects are considered.

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Determination of Environmental Impacts using Life Cycle Assessment of Plants Grown for Bioenergy: Example of Sorghum x Sudan Grass Hybrid

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ABSTRACT

Renewable energy sources are the most effective and cheapest method for combating climate change. Biomass, which is one of the renewable energy sources, is also one of the raw materials for biofuels. Sorghum x Sudan grass hybrid, which is drought tolerant and has a short vegetation period, is a biomass source. This study was carried out to determine the ethanol yield of sorghum x Sudan grass hybrid plants grown in an area with a semi-humid climate and to determine the environmental impacts of biomass. Environmental impacts were assessed using the life cycle assessment method. Environmental impact categories are divided into 11 categories according to the CML-IA Baseline model. As a result, the biomass yield was 49888 kg ha^{-1} and the ethanol yield was 1674.1 l ha^{-1}. According to the life cycle impact category of sorghum x Sudan grass hybrid biomass production, the highest environmental impact was 79.21%, causing marine aquatic ecotoxicity. According to the life cycle interpretation, it caused a global effect with a rate of 83.87%. In addition, the global warming value was calculated as 0.195 kg CO_2-eq $kg_{biomass}^{-1}$ (9728.16 kg CO_2-eq ha^{-1}). The agricultural phases with the most negative impact on the environment are irrigation and fertilization.

Keywords: Global warming, Climate change, Energy crops, Biofuels, Bioethanol

1. Introduction

With rapid population growth and industrial progress, energy use in the world is also increasing. According to data from 2018, the amount of energy used in the world was calculated as 14.4 billion tons of oil equivalent. This energy comes from fossil fuels (oil, coal, natural gas, etc.) at rates of 81.1% and renewable energy sources at 18.9%. Biomass constitutes 67.5% of energy obtained from renewable energy sources. In Türkiye, the amount of energy used was calculated as 147.5 million tons of oil equivalent. Within this energy, fossil fuels provide 86.2% (44.2 million TOE of coal, 41.9 million TOE of oil and 41.0 million TOE of natural gas) and renewable energy sources provide 13.8%. Biomass constitutes 15.5% of energy obtained from renewable energy sources (IEA 2021).

Biomass is a renewable energy source obtained from plants, agricultural wastes, animal wastes and urban solid wastes with important advantages such as being clean, easily available, sustainable and environmentally friendly. Biofuels obtained from biomass are organic (bioethanol, biodiesel, biogas, biomethanol, biohydrogen) fuels derived from living organisms and obtained from carbon-based products. These fuels significantly contribute to reducing fossil fuel consumption and greenhouse gas (GHG) emissions (Eren & Öztürk 2021).

Biofuel production must be environmentally, socially, economically and energetically sustainable. Biofuels enable employment due to the presence of processing plants in rural areas. They also provide socioeconomic benefits, promote economic dynamism and have the potential to positively affect other related industries (Gilio & Moraes 2016; Moraes et al. 2016).

Sorghum x Sudan grass hybrid (*sorghum bicolor x sorghum sudanense stapf*.) is a plant species with C4 metabolism, that is annual, with wide adaptability, sugar-rich stalk and high biomass yield, and has potential as an energy plant. It can also be grown in marginal areas due to low water and fertilizer requirements. In addition to its potential as an energy plant, it can also be used as a forage plant.

Energy crops, one of the sources of biofuels, are produced in agricultural production systems. It is necessary to optimize the use of agricultural inputs in order to reduce environmental impacts and save energy in agricultural production systems. To reduce the environmental impacts of agricultural production, it is necessary to determine the environmental impacts. The agricultural life cycle assessment (LCA) method is used to determine these environmental impacts. Agricultural LCA is a method for determining the environmental impacts of inputs in the agricultural production system from the cultivation of soil to the harvesting of the product on the basis of environmental impact categories. Agricultural LCA is the application of the LCA method only from cradle to gate, not from cradle to grave, in order to determine the environmental impacts of agricultural activities. Since the agricultural product obtained is raw material for another product, LCA is carried out until the product is obtained (Eren & Öztürk 2021).

There are some agricultural life cycle assessment studies conducted to determine the environmental impacts of agricultural products during production. For example, research was carried out about energy crops (Christoforou et al. 2016), maize (Boone et al. 2016; Zhang et al. 2018; Frank et al. 2020), sunflower (Vatsanidou et al. 2020), sweet sorghum (Eren & Öztürk 2021), agricultural production (Wowra et al. 2021; Fan et al. 2022), potato (Economou et al. 2023) and barley (Stylianou et al. 2023).

Although there are many studies about LCA in the literature, studies about LCA of agricultural production in Türkiye are limited. Therefore, in this study, agricultural LCA was conducted to determine the environmental impacts of sorghum x Sudan grass hybrid biomass production.

2. Material and Methods

2.1. Materials

The research was carried out in the field at Bingöl University Agricultural Application and Research Center (38°48'46,77" N - 40°32'11,40" E) in 2020 (Figure 1). The elevation of the research area is 1100 meters above sea level.

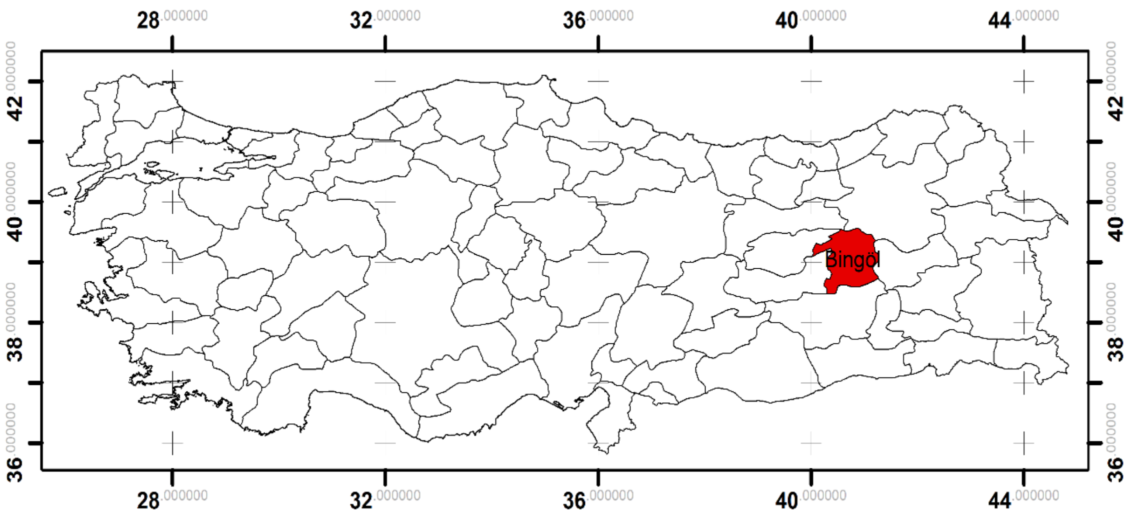


Figure 1- Location of the experimental site in Türkiye

In the experimental area, which has a semi-humid climate, the total precipitation amount in the vegetation periods (June, July, August and September) was 17.5 mm and the average temperature was 24.9 °C. During the cultivation of the sorghum plant, there is temperature demand of 20-35 °C and a water requirement of 500-600 mm (Guiying et al. 2003). During the research, the seasonal temperature in the experimental area met the temperature needed by the plant. However, since there was not enough precipitation, irrigation was needed during the vegetation period. According to the results of soil analysis carried out in the experimental area, the soil was salt-free, limeless, low in organic matter and weak in terms of N, P, K content.

2.2. Cultural practices of sorghum x Sudan grass hybrid production

Cultural practices and maintenance processes in the production of sorghum x Sudan grass hybrid were carried out as follows.

- Tillage: Deep plowing was done, followed by tillage with a cultivator.

- Sowing: In the second week of June, sowing was done at a depth of 3-4 cm with 45 cm row spacing and 5 m row length. Sowing was done so 4 kg of seeds fell per decare.

- Maintenance: 10 kg of 15-15-15 compound fertilizer per decare as base fertilizer and 22 kg of urea 46% N per decare as top fertilizer was given with planting. Hoeing was done when the plant reached 30-40 cm in height. The plant was watered by the drip irrigation method. Insecticide with 50 g/L lambda-cyhalothrin active ingredient was used once for aphids.

- Harvest: After the second week of September, the plant was harvested at full maturity with a scythe motor.

2.3. Sorghum x Sudan grass hybrid ethanol yield

Theoretical ethanol yield was calculated using the formula; [(total sugar / 5.68) x 3.78)] x 0.80 (Smith et al. 1987; Bunphan et al. 2015).

2.4. Determination of environmental impacts

The agricultural LCA method was used to determine the environmental impacts during crop production. Agricultural LCA was carried out in 4 stages of goal and scope definition, inventory analysis, environmental impact assessment and interpretation.

2.5. Goal and scope definition

According to the agricultural LCA, the system boundary in Figure 2 was defined in order to determine the environmental impacts due to cultural practices and maintenance processes during the production of sorghum x Sudan grass hybrid biomass.

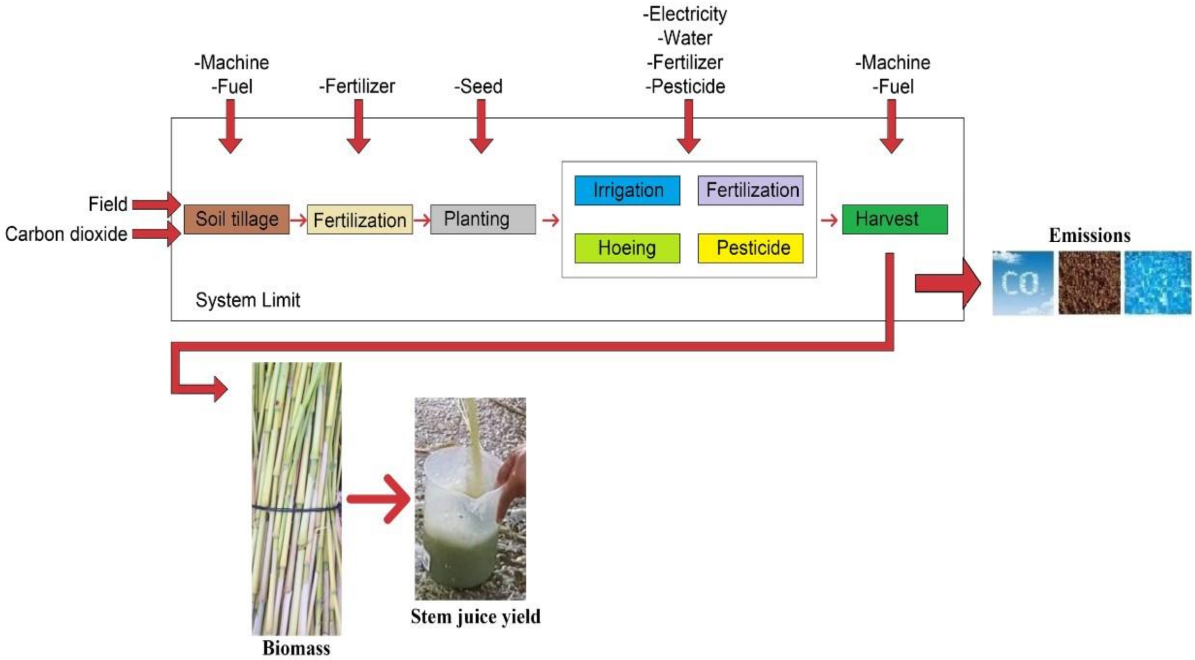


Figure 2- System boundaries of the production system

According to the defined system boundary, agricultural machinery, fuel, fertilizer, seeds, pesticides and water are considered inputs. The biomass of the harvested product and emissions (to air, soil and water) are accepted as outputs (Figure. 2).

A functional unit is a unit that provides reference by normalizing all data and impact categories in the assessment. Different functional units can be selected in agricultural life cycle assessments. In this study, sorghum x Sudan grass hybrid cultivation area (1 ha) and dry biomass amount (1 $kg_{biomass}$) were accepted as functional units.

2.6. Inventory analysis

The following assumptions were made in order to carry out the life cycle inventory analysis of the production system (Table 1).

Table 1- Assumptions

Slope of fields	No slope
Cultivability of fields	Cultivable
Type of agriculture	Irrigated agriculture
Drainage	None
Clay content of soil (%)	54
Humus content of the soil (%)	1.89
Plant potential root depth (cm)	190
Soil erosion (K) factor	Ignored
Fertilization	15-15-15 Compound and 46% N Urea
Machine to prevent ammonia losses	Not used

Then, inventories of the production system were made. The mass balance inventory (agricultural inputs and outputs used during production) values in the production system are given in Table 2 and the inventory data of the machines/tractors used are given in Table 3.

Table 2-Mass balance inventory

Inventories		*Unit*	*Amount Per Hectare (ha^{-1})*
Land Use		ha	1.00
Diesel fuel		l	92.60
Seed		kg	40
	Nitrogen		116.2
Fertilizer	Phosphorus	kg	15
	Potassium		15
Water		m^3	19871.8
Electric		kWh	2504.1
Pesticide		l	0.5
Outputs			
Biomass		kg	49888

Table 3-Agricultural machinery and tractor inventories

Machine	*Mass (kg)*	*Service life (h)*	*Working width (m)*
New Holland TD90D tractor	3700	10000	-
Plow (4 sockets)	800	2000	1.22
Cultivator	350	2000	2.70
Motorized back sprayer	10	2000	-
Motor scythe (4 blades)	7.3	2000	0.23

2.7. Environmental impact assessment

According to the results obtained from the life cycle inventory analysis, the CML-IA Baseline methodology was used in accordance with ISO 14040 standards for the evaluation of the environmental impacts of the biomass production system.
Potential environmental impacts (characterization values) were calculated with SimaPro 8.0.5.13 Analyst software based on the CML-IA Baseline methodology. This CML-IA Baseline methodology includes 11 environmental impact categories (Table 4). After calculating the characterization values, normalization values were calculated by performing normalization with the software. Normalization was done in order to evaluate the impact categories among themselves.

Table 4-Impact categories and characterization units according to the CML-IA Baseline model

Impact Category	*Characterization Unit*
Abiotic depletion	kg Sb_{eq}
Abiotic depletion (fossil fuels)	MJ
Global warming (GWP100a)	kg $CO_{2\text{-}eq}$
Ozone layer depletion (ODP)	kg $CFC11_{eq}$
Human toxicity	kg 1.4-DB_{eq}
Fresh water aquatic ecotoxicity	kg 1.4-DB_{eq}
Marine aquatic ecotoxicity	kg 1.4-DB_{eq}
Terrestrial ecotoxicity	kg 1.4-DB_{eq}
Photochemical oxidation	kg $C_2H_{4\text{-}eq}$
Acidification	kg $SO_{2\text{-}eq}$
Eutrophication	kg $PO_{4\text{-}eq}$

2.8. Interpretation

According to the normalization values, the effects of sorghum x Sudan grass hybrid biomass production system at global, regional and local effects were evaluated and interpreted. To evaluate its global impact, abiotic depletion, abiotic depletion (fossil fuels), global warming (GWP100a) potential, ozone layer depletion (ODP) and marine aquatic ecotoxicity values were considered. To evaluate the regional effects, photochemical oxidation and acidification values were considered. In order to evaluate the local effects, human toxicity, freshwater aquatic ecotoxicity, terrestrial ecotoxicity and eutrophication values were taken into consideration.

3. Results and Discussion

3.1. Theoretical ethanol yield

Biomass yield is one of the most important parameters affecting ethanol yield. The nutrima variety of sorghum x Sudan grass hybrid plant was used as biomass. The amount of ethanol obtained from this variety is 1674.1 l ha^{-1}. In previous studies, some researchers determined that the ethanol yield was between 360-1680 l ha^{-1} (Rao et al. 2013; Rutto et al. 2013; Sawargaonkar et al. 2013; Batog et al. 2020). The findings obtained in the study show that a successful result was obtained in this semi-humid region when compared with the previous studies.

3.2. Potential environmental impacts

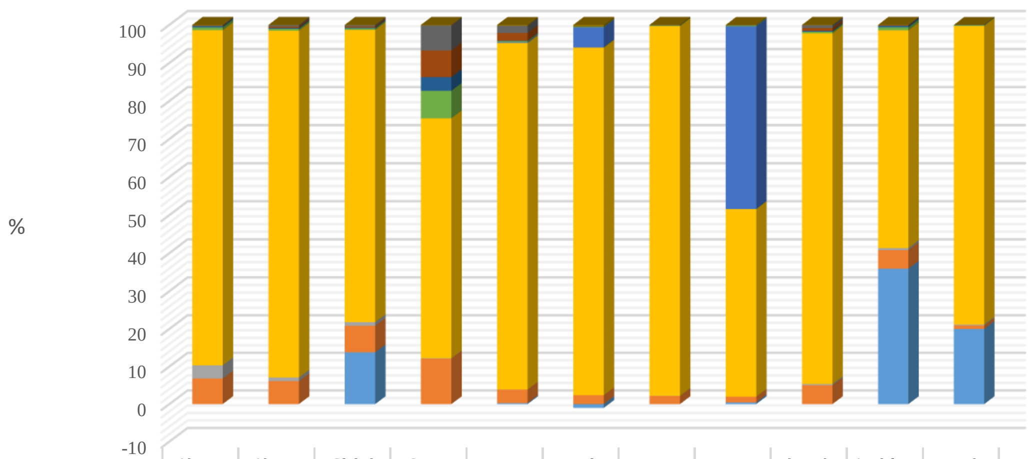
3.2.1. Evaluation of characterization results

As a result of the impact assessment of sorghum x Sudan grass hybrid biomass production, the characterization values in Table 5 and the graph in Figure 3 were obtained. Considering Table 5 and Figure 3 together;

- The abiotic depletion value was calculated as 0.00000074 kg Sb-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most depletion was irrigation (88.31%). In studies carried out on the sorghum plant, this value was obtained as 0.0001188 kg Sb-eq $kg_{biomass}^{-1}$ (Sutter & Jungbluth 2007) and 0.0003163 kg Sb-eq $kg_{biomass}^{-1}$ (Eren &Öztürk 2021).
- Abiotic depletion (fossil fuels) was calculated as 2.223 MJ $kg_{biomass}^{-1}$ and the agricultural application causing the most depletion was irrigation (91.41%).
- Global warming (GWP100a) value was calculated as 0.195 kg CO_2-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most warming was irrigation (76.85%). In previous studies, this value was reported to vary between 0.114-0.517 kg CO_2-eq $kg_{biomass}^{-1}$ (Wang et al. 2014; Eren & Öztürk 2021).
- Ozone layer depletion (ODP) value was calculated as 0.000000012 kg CFC11-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most depletion was irrigation (63.25%). Sutter and Jungbluth (2007) determined this value as 0.00000000211 kg CFC11-eq $kg_{biomass}^{-1}$.
- Human toxicity value was calculated as 0.150 kg 1.4-DB-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most toxicity was irrigation (91.39%). In previous studies, this value was reported to vary between 0.004-0.028 kg 1.4-DB-eq $kg_{biomass}^{-1}$ (Sutter & Jungbluth, 2007; Wang et al. 2014; Eren & Öztürk 2021).
- Fresh water aquatic ecotoxicity value was calculated as 0.084 kg 1.4-DB-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most fresh water ecotoxicity was irrigation (91.69%). This value was calculated as 0.015 kg 1.4-DB-eq $kg_{biomass}^{-1}$ (Sutter & Jungbluth 2007) and 0.023 kg 1.4-DB-eq $kg_{biomass}^{-1}$ (Wang et al. 2014).
- Marine aquatic ecotoxicity value was calculated as 233.792 kg 1.4-DB-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most marine ecotoxicity was irrigation (97.51%).
- Terrestrial ecotoxicity value was calculated as 0.001 kg 1.4-DB-eq $kg_{biomass}^{-1}$ and the agricultural applications causing most terrestrial ecotoxicity were irrigation (49.61%) and insecticides (48.03%). Eren and Öztürk (2021) found this value was 0.00001257 kg 1.4-DB-eq $kg_{biomass}^{-1}$.
- Photochemical oxidation value was calculated as 0.000054 kg C_2H_4-eq $kg_{biomass}^{-1}$ and the agricultural application causing the most photochemical oxidation was irrigation (92.47%). Other researchers reported photochemical oxidation of 0.00000261 kg C_2H_4-eq $kg_{biomass}^{-1}$ (Sutter & Jungbluth 2007) and 0.00000503 kg C_2H_4-eq $kg_{biomass}^{-1}$ (Eren & Öztürk 2021).
- Acidification value was calculated as 0.001 kg SO_2-eq $kg_{biomass}^{-1}$ and the agricultural applications causing the most acidification were irrigation (57.24%) and sorghum x Sudan grass hybrid biomass (35.90%). The reason for this is the increase in organic acids in soil which are produced as a result of the biological activities of the plant related to the decomposition of plant tissues by small soil creatures.
- Eutrophication value was calculated as 0.002 kg PO_4-eq $kg_{biomass}^{-1}$ and the agricultural applications causing the most eutrophication were irrigation (78.57%) and sorghum x Sudan grass hybrid biomass (19.99%). The reason for this is that the plant could not retain the nitrate from fertilization during cultivation and the nitrate that was not retained infiltrated into the soil.

Table 5- Life cycle impact indicators of sorghum x Sudan grass hybrid biomass production (per functional unit of product produced)

Impact Category	*Unit*	*Unit kg$_{biomass}^{-1}$*	*Unit ha^{-1}*
Abiotic depletion	kg Sb-eq	0.00000074	0.03691712
Abiotic depletion (fossil fuels)	MJ	2.223	110901.02
Global warming (GWP100a)	kg CO_2-eq	0.195	9728.16
Ozone layer depletion (ODP)	kg CFC11-eq	0.000000012	0.000598656
Human toxicity	kg 1.4-DB-eq	0.150	7483.20
Fresh water aquatic ecotoxicity	kg 1.4-DB-eq	0.084	4190.59
Marine aquatic ecotoxicity	kg 1.4-DB-eq	233.792	11663415.30
Terrestrial ecotoxicity	kg 1.4-DB-eq	0.001	49.88
Photochemical oxidation	kg C_2H_4-eq	0.000054	2.693952
Acidification	kg SO_2-eq	0.001	49.88
Eutrophication	kg PO_4-eq	0.002	99.77



	Abiotic depletion	Abiotic depletion (fossil fuels)	Global warming (GWP100a)	Ozone layer depletion (ODP)	Human toxicity	Fresh water aquatic ecotoxicity	Marine aquatic ecotoxicity	Terrestrial ecotoxicity	Photochemical oxidation	Acidification	Eutrophication
Electric	0.13	0.09	0.12	0.14	0.22	0.45	0.13	0.12	0.11	0.07	0.02
Pesticide application	0.09	0.35	0.30	6.56	1.91	0.01	0.01	0.01	0.71	0.07	0.01
Harvest	0.10	0.38	0.32	7.03	2.05	0.01	0.01	0.01	0.77	0.13	0.02
Tillage (Cultivatör)	0.34	0.23	0.19	3.59	0.08	0.05	0.04	0.07	0.21	0.39	0.07
Tillage (Plow)	0.74	0.47	0.41	7.23	0.26	0.14	0.11	0.17	0.43	0.77	0.15
Pesticide	0.00	0.00	0.00	0.00	0.24	5.32	0.00	48.03	0.00	0.00	0.00
Irrigation	88.31	91.41	76.85	63.25	91.39	91.69	97.51	49.61	92.47	57.24	78.57
EC Fertilizer NPK (15-15-15)	3.49	0.96	0.95	0.09	0.01	0.00	0.01	0.01	0.31	0.54	0.24
EC Fertilizer (%46 N)	6.81	6.10	7.09	12.09	3.61	2.34	2.17	1.53	4.99	4.89	0.92
Sorghum & sudangrass hybrid biomass	0.00	0.00	13.77	0.00	0.24	-1.00	0.00	0.44	0.00	35.90	19.99

Figure 3- % comparison of characterization values

3.2.2. Evaluation of normalization results

Normalization was done in order to compare the environmental effects among themselves. The normalization results and the impact categories were compared among themselves (Figure 4) and their distribution in % was evaluated (Table 6).

Table 6- % distribution of the comparison of impact categories among themselves

Impact Category	*%*
Abiotic depletion	0.35
Abiotic depletion (fossil fuels)	2.80
Global warming (GWP100a)	1.53
Ozone layer depletion (ODP)	0.01
Human toxicity	0.77
Fresh water aquatic ecotoxicity	6.42
Marine aquatic ecotoxicity	79.21
Terrestrial ecotoxicity	0.80
Photochemical oxidation	0.25
Acidification	2.02
Eutrophication	5.87
Total	*100.00*

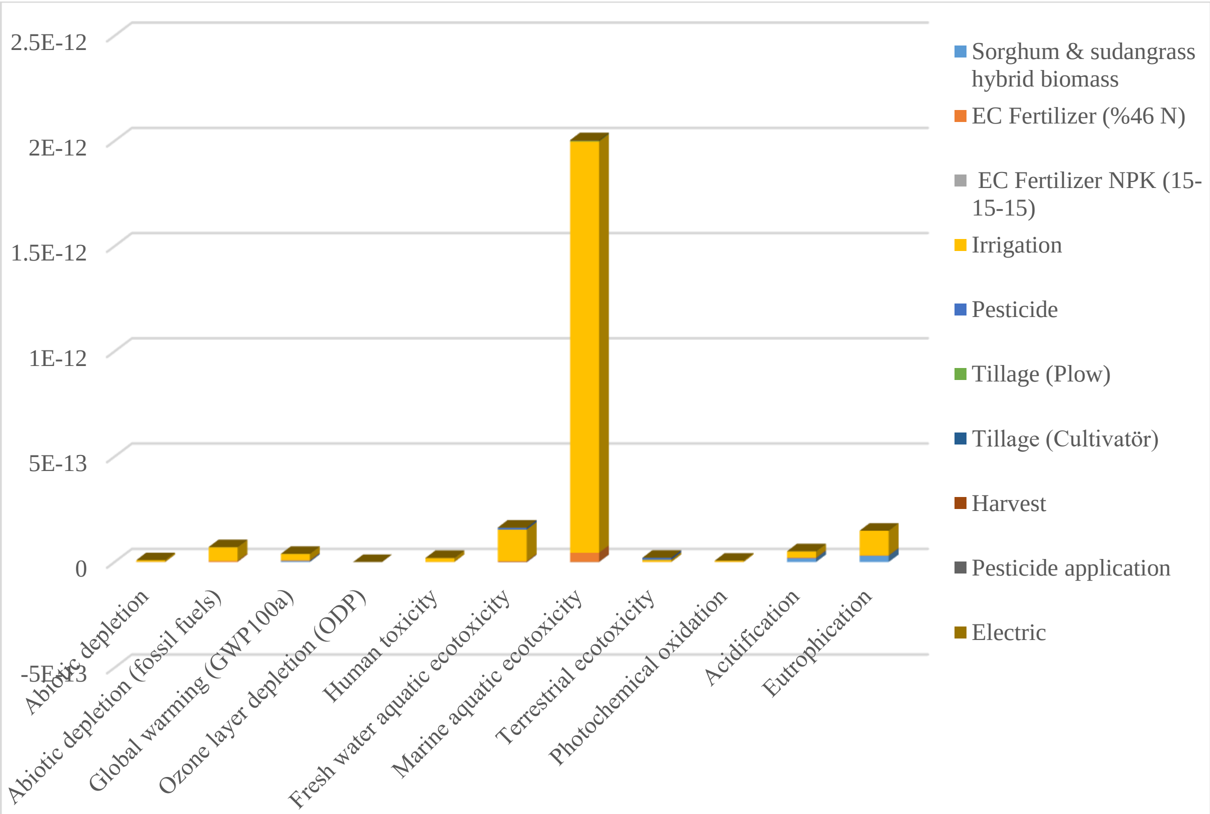


Figure 4- Comparison of normalization values on the basis of impact categories

When Table 6 and Figure 4 are examined together, the production of sorghum x Sudan grass hybrid biomass caused the most marine aquatic ecotoxicity (79.21%). Marine aquatic ecotoxicity is followed by the effects of fresh water aquatic ecotoxicity (6.42%) and eutrophication (5.87%), respectively. The effects of abiotic depletion, global warming (GWP100a), ozone layer depletion, human toxicity, terrestrial ecotoxicity and photochemical oxidation comprised less than 2% in the production system, and can be ignored.

3.3. Interpretation

3.3.1. Global influences

When the impact categories that cause global influence is evaluated among themselves (Figure 5), marine aquatic ecotoxicity (94.42%) caused the most global influence. Irrigation applications (97.51%) in the agricultural phase have the greatest impact on marine aquatic ecotoxicity (Figure 3). Irrigation studies should be carried out and practices that will minimize the effects of irrigation should be determined. Marine aquatic ecotoxicity value was followed by the effect of abiotic depletion (fossil fuels) (3.33%) (Figure 5). Irrigation applications in the agricultural phase (91.41%) were effective in increasing the effect of abiotic depletion (fossil fuels).

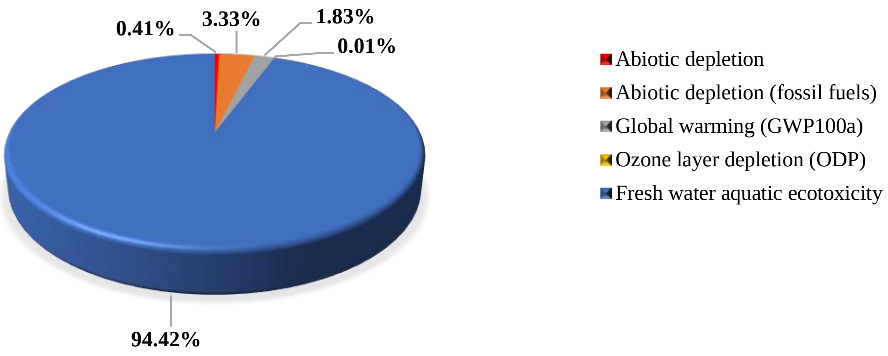


Figure 5- Distribution of impact categories that cause global influences

Due to the increasing effect of abiotic depletion (fossil fuels), it is estimated that natural resources, especially fossil fuels, will be depleted in the near future. Another factor that causes a global effect is the global warming potential (1.83%) (Figure 5). It is predicted that global warming will cause a melting of ice at the poles and a change in seasons, and thus climate change, in the next 100 years. The values for abiotic depletion and ozone layer depletion affect the global influence at very low rates.

3.3.2. Regional influences

When the impact categories causing regional influence were evaluated among themselves (Figure 6), the acidification effect (88.93%) caused the most regional influence. Irrigation applications in the agricultural phase (57.24%) caused an increase in the acidification effect (Figure 3). In addition, the effect of photochemical oxidation was determined as 11.07% on a regional scale.

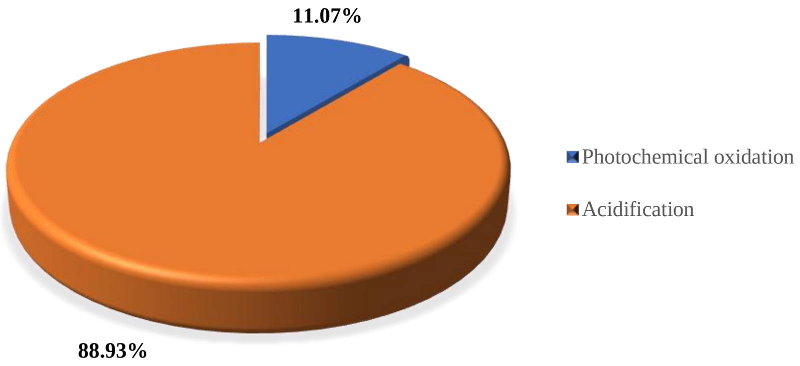


Figure 6- Distribution of impact categories that cause regional influences

The application that caused the most photochemical oxidation was irrigation (92.47%) (Figure 3). It is assumed that over-irrigation causes acidification of the soil. For this reason, acidification and corrosion may occur in soils or wetlands of the region. This may result in the restriction of other products that can be grown and the decrease in the yield of the products that can be grown.

3.3.3. Local influences

When Figure 7 is examined, the effect of fresh water aquatic ecotoxicity was 46.34%. This effect also negatively affects the environment locally. Fresh water aquatic ecotoxicity was followed by the eutrophication effect (42.38%).

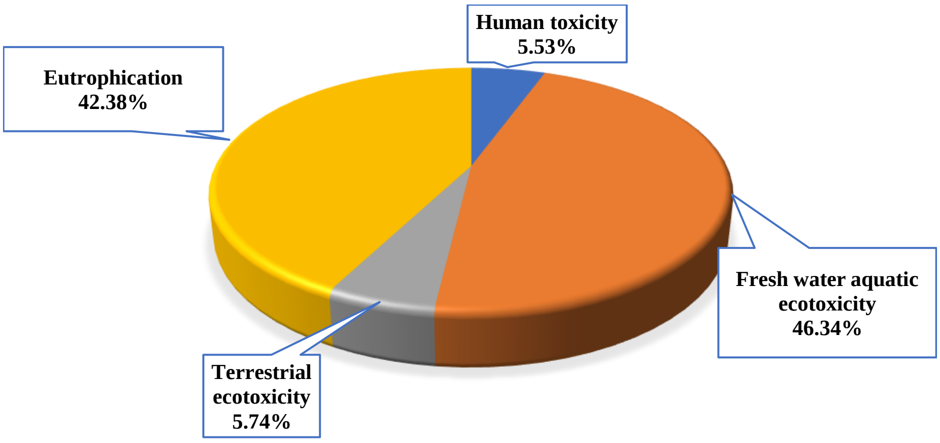


Figure 7- Distribution of impact categories that cause local influences

Irrigation practices in the agricultural phase caused an increase in fresh water aquatic ecotoxicity (91.69%) and eutrophication (78.57%) (Figure 3). Where biomass is grown, there may be a decrease in fresh water species and biodiversity.

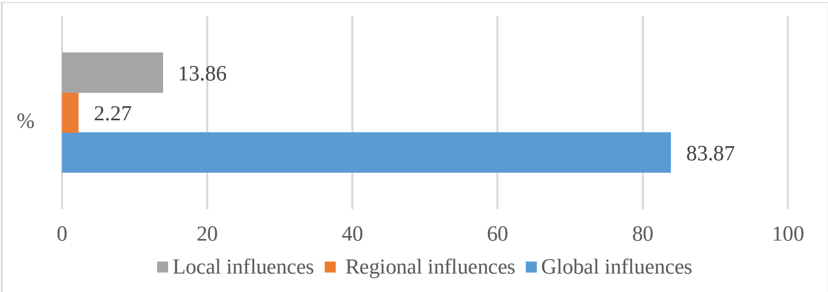


Figure 8- Comparison of impact categories with each other

When all the effects are evaluated together (Figure 8), the production of sorghum x Sudan grass hybrid biomass caused the largest impact on a global scale (83.87%). The global influence was followed by the local influence (13.86%) and the regional influence (2.27%).

4. Conclusions

According to the % distribution for normalized values of agricultural LCA of cultivating sorghum x Sudan grass hybrid plant for biomass production, the highest environmental impact with a rate of 79.21% was marine aquatic ecotoxicity. According to the agricultural life cycle assessment, production has a global influence with a rate of 83.87%. In addition, the global warming potential was calculated as 0.195 kg CO_2-eq $kg_{biomass}^{-1}$ (9728.16 kg CO_2-eq ha^{-1}).

Irrigation applications in the agricultural phase are the environmental pollutants with highest impact. Excessive water consumption causes environmental pollution. In addition, water resources in the world are decreasing due to drought resulting from climate change. Since excessive use of water in agriculture affects the environment negatively and consumes water resources, research should be increased to reduce water use by developing irrigation technologies. Agricultural life cycle assessments associated with many products should be made and the environmental impacts of the growing process of the products should be determined. Studies should be increased about the establishment of inventory databases for agricultural life cycle assessment for agricultural products around the world.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Effects of Plant-derived Smoke, Karrikin, and Salinity Stress on *Prunus armeniaca* cv. Şalak seeds and seedlings: A Morphological, Biochemical, and Molecular Approach

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ABSTRACT

The effects of plant-derived smoke on seed germination and plant growth, depending on concentration and time, are widely known. However, there are very few studies demonstrating that it provides tolerance to abiotic stresses. This study comprehensively compares the effects of SW and KAR1 on seed germination and morphological, biochemical, and molecular changes observable in the examined seeds. Moreover, the study shows that it regulates the expression of some genes encoding antioxidant enzymes in apricot seedlings (*Prunus armeniaca* L.) exposed to salinity stress (100 mM NaCl). The highest germination rate was 1:1000 DS with 60% and 1 µM KAR1 with 72%. In terms of shoot development, root and stem length, 1:100 concentration in the DS group and 1 µM concentration in the KAR1 group gave the best results. The shoot development rates were 95.83% and 87.50% in the DS and KAR1 groups, respectively. While the root length was 137.68 and 141.92 mm in the DS and KAR1 groups, respectively, the stem length was 103.78 and 102.67 mm, respectively. The data revealed that SW (1:1000 v/v) and KAR1 (1µM) increased the expression levels of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) genes in the samples taken from the apricot seedlings treated with salt at hours 3, 6 and 9. This increase varies in SW and KAR1 depending on time. When the biochemical results were examined, it was seen that the application of SW and KAR1 to the seedlings under salinity stress led to a significant decrease in the thiobarbituric acid reactive substances (TBARS) content. We can assert that SW is more effective than KAR1 on TBARS content. Morphological, molecular, and biochemical results revealed enhanced germination, growth, gene expression, and TBARS content in apricot seeds and seedlings exposed to SW and KAR1. This data may be applicable to more comprehensive trials.

Keywords: Antioxidant enzyme, Smoke water, TBARS, Salt stress, Karrikin, Gene expression

1. Introduction

Salinity, an abiotic stress, is among the main environmental factors limiting plant growth. Arid and semi-arid areas account for approximately 46% of the earth's surface. About 50% of these areas encounter salinity problems at different levels. The FAO/UNESCO World Soil Map data reports that 954 million hectares of land worldwide are affected by salt, and productivity on this land is restricted. Salinity in soil occurs in both natural and artificial ways. The former is observed in arid and semi-arid areas, in extremely hot conditions, in flat basins where soil drainage is insufficient, and when salts carried by precipitation are brought to the soil's surface. The latter occurs as a result of reckless irrigation and excessive use of fertilizers (Özbek et al. 1999; Sönmez & Sönmez 2007). Nowadays, many agricultural lands suffer from gradual degradation due to salinity. Desertification may be inevitable unless precautions are taken. According to a number of studies, of the apricot is among the group of plants sensitive to salinity stress (Bernstein et al. 1956; Bernstein 1965; Maas 1984; William 1986; Gucci & Tattini 1997; Karaoğlu & Yalçın 2018). The effects of salinity stress on plants occur as osmotic and ionic stress. Salt that becomes accumulated in the root zone of the plants and in the soil restricts water uptake by roots, which results in osmotic stress. Osmotic stress causes oxidative damage in plants as a result of the deterioration of the nutrient balance and membrane properties, and a decrease in the photosynthetic activity and stomatal conductance, and an increase in the formation of reactive oxygen species (ROS) due to decreased photosynthetic activity (Munns & Tester 2008; Rahnama et al. 2010). Ion stress occurs with the accumulation of Na^+ and Cl^- ions in plants. While the Na^+ ion prevents the uptake of K^+ and Ca^{2+} ions and the regulation of stomatal conductance, Cl^- causes chlorophyll degradation, leading to a deterioration in photosynthetic activity. Ion toxicity causes deteriorated ion balance and physiological disorders through excessive ion uptake in the roots and leaves (Tavakkoli et al. 2011). Plants have developed various adaptation mechanisms to combat the increase in ROS due to environmental stress. Plants possess enzymatic and non-enzymatic antioxidant defense systems that protect their cells from oxidative damage. Enzymatic antioxidant systems include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), etc.; while non-enzymatic antioxidant systems include ascorbic acid (ASH), glutathione (GSH), alkaloids, phenolic compounds, protein (such as proline) and non-protein amino acids, lipid peroxidation (TBARS), and α-tocopherols (Gill & Tuteja 2010).

A large body of research is available on many plant species grown in different environments where plant-derived smoke increases seed germination and seedling, shoot, and root development. These studies have shown that smoke has a stimulating or inhibitory effect on germination and plant growth in plants, depending on the concentration used and the exposure time (Baxter & Van Staden 1994; Van Staden et al. 2006; Kulkarni et al. 2008; Çatav et al. 2018; Khatoon et al. 2020). The germination-stimulating property of smoke is known to be due to KARs (KAR1-KAR6), a special type of lactone known as butenolide (3-metil-2H-furo [2,3-c] pyran-2-one) fused to a pyran ring (Flematti et al. 2015; Kemeç Hürkan 2023). In addition, nitrogen oxides and cyanohydrins have also been found to promote germination (Nelson et al., 2012). However, it is known that smoke tends to have a “bidirectional regulating” effect on germination, as higher concentrations of smoke inhibit germination, while lower concentrations have a germination-promoting property (Light et al. 2002). In addition, nitrogen oxides and cyanohydrins have been shown to promote germination (Nelson et al. 2012). Nevertheless, it is noted that smoke tends to have a "dual regulatory" effect on germination as higher concentrations of smoke inhibit germination and lower concentrations promote germination (Light et al. 2002). It has been revealed that phenolic compounds, such as cresols, dihydroxybenzenes and 2-furoic acid, naphthalene, 3,4,5-Trimethylfuran-2(5H)-one (2,3,4-trimethylbut-2-enolide), in the smoke are responsible for the germination-inhibiting activity (Light et al. 2010; Kemeç Hürkan 2023).

KARs are thought to be used by plants to protect themselves against different combinations of abiotic factors, such as oxidative stress, drought, low light intensities, high temperature, salinity, low osmotic potential (Ghebrehiwot et al. 2008; Jamil et al. 2014; Banerjee et al. 2019). Although many studies have shown that plant-derived smoke and KARs stimulate germination, there is very little research that investigates the various abiotic stress conditions. These studies suggest that the application of plant-derived smoke or KAR to plants under salt stress further increases proline accumulation while decreasing the MDA accumulation and production of H_2O_2 (Sharma et al. 2012; Vardhini & Anjum 2015). In addition, the effects of plant-derived smoke and KARs on the transcription factors and various antioxidant enzymes (CAT, SOD, and GPX) against salinity stress have been investigated (Sharifi & Shirani Bidabadi (2020); Çatav et al. 2021; Hayat et al. 2022). Previous research has indicated that plant-derived smoke and KAR increase the expression of CAT, SOD, and GPX genes in plants under salt stress (Shah et al. 2020; Sharifi & Shirani Bidabadi 2020; Çatav et al. 2021; Shah et al. 2021; Hayat et al. 2022).

The apricot (*Prunus armeniaca* L.) is in the stone fruit group belonging to the Rosaceae family. Apricots are divided into 6-8 ecological groups and 13 regional subgroups due to their of propagation by seed and growth in very different ecological conditions (Layne et al. 1996; Ledbetter 2008; Asma 2011). The apricot is considered to be an important fruit for human health as it is rich in sugar, potassium, phosphorus, calcium, iron, dietary fiber, and vitamin A (β-carotene) (Açkurt 1999). There are approximately 58 apricot varieties in Turkey. Twenty-eight of these are registered apricot varieties. Şalak is an edible apricot variety grown in the Iğdır and Kağızman regions. The fruits are oblong shaped and very large, and the average fruit weight is 50-65 g. The fruits are sweet, the skin and flesh color are yellow, the shape is symmetrical, and the abdominal line is very prominent. The Şalak apricot ripens in the last week of June under the ecological conditions of Iğdır and Malatya (Akbaba et al. 2023; Asma 2011; Aydoğdu 2016). In terms of soil requirements, apricot trees prefer deep permeable, warm, rich in organic matter and nutrients, good drainage without high ground water, pH: between 6.5-7.5, loamy or loamy-calcareous, without salinity problems (Korkmaz 2007; Asma 2011).

It is important to develop various methods for the propagation of the apricot, both for human health and because it is the most exported fruit for Türkiye. The cheapest and easiest method for apricot sapling production is graft propagation. In the graft propagation method, the seeds of cultivated apricot varieties and apricot seedling rootstocks are widely used. Additionally, almond, peach and plum rootstocks are used. Hacıhaliloğlu, Şalak, Şekerpare, Alyanak etc. are the most common apricot varieties produced by grafting on seedling rootstocks (Yıldırım 2006; Asma 2011). The present study was conducted with the aim of producing seedlings to be grafted with seeds.

Seeds of most fruit trees, especially those belonging to the Rosaceae family, either do not germinate or show an incredibly low germination rate unless they are pre-treated to break dormancy (Kaşka 1970). Apricot seeds have two different types of dormancies, both endogenous and exogenous, caused by the components of the seed itself and the endocarp, respectively. Apricot seeds require cold stratification to break dormancy. For this, it is necessary to keep the apricot seeds at 4-7 ºC degree for 90-105 days (Fadl et al. 1978; Polat 2007; Szymajda et al. 2013).

The encouraging findings in the above-mentioned research have led to the question of whether plant-derived smoke and KAR may promote plant growth more effectively under stressful circumstances. In order to answer this question, the present study aims to determine whether SW and synthetic KAR1 provide protection against salt stress and changes in the antioxidant defense system in apricots (*P. armeniaca*, cv. Şalak). The study saw the first use of SW and KAR1 in stone fruit seeds (*Prunus armeniaca*). This constitutes the originality of the study. Apricots are sensitive to salinity stress and to find a solution to this salinity stress, the apricot seeds of the Şalak cultivar were grown *in vivo* with SW and KAR1 to determine the germination rate, shoot development rate, root and stem lengths, stem diameters, number of leaves, leaf area, and lipid peroxidation (TBARS) activity. The changes in the expression of genes belonging to enzymes [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX)] were investigated by qPCR. This study sheds light on the morphological, biochemical, and molecular responses related to smoke and KAR in plants exposed to salt stress.

2. Material and Methods

2.1. Plant material, growth conditions, and treatments

In the study, mature seeds (tree age 10-12) of *P. armeniaca* (cv. Şalak), belonging to the Rosaceae family, were used as material. According to the literature review, apricot seeds need cold stratification to break their dormancy. By optimizing the cold stratification process, it was decided that keeping it at 4 ºC±1 degree for 90 days was suitable for seed germination. The sterilization process was performed adopting the method described by Kemeç Hürkan & Akı (2022). After the testa parts of the sterilized seeds were peeled, they were transferred to Petri dishes with filter paper inside. The experiment in which the seeds were sown was repeated ten times (5x10), with five seeds in each Petri dish. In order to obtain SW, 1 kg of *Medicago sativa* L. straw was burned, and the smoke was dissolved in water (1L) in the filtering flask. The *Medicago sativa* L. straw was burned at 275 ºC for 60 min. until it turned to ash and was then filtered through a sterile syringe filter with a 0.22 µm pore size (Kemeç Hürkan & Akı 2023). Afterwards, according to the treatment groups (Control: sterile distilled water, SW: 1:100; 1:500; 1:1000 (v,v), KAR1: 0.01; 0.1; 1 µM), each of petri dish containing seeds was individually wetted with the previously prepared solutions. The KAR1 material was obtained from Toronto Research Chemicals Canada. The Petri dishes were wrapped with cling film to prevent the moist filter papers from drying out. The seeds were stored under dark conditions at 4 ºC±1 (wet stratification in cold) until germination. Since apricot seeds need cold weather to germinate, the seeds in the petri dishes were kept at 4 ºC±1. The seeds were germinated after one week and transferred to vials (24 compartments) containing a mixture of perlite and peat, and the experiment was repeated four times. The plants were grown in a climatic chamber with a long day photoperiod of 16 h light/eight h dark at 24 ºC±1. The vials were watered regularly every two-three days with the solutions used in Hoagland (100%-Himedia TS1117) + treatment groups according to the dryness of the soil.

2.2. Growth measurements

One month after germination, the measurements were performed for the statistical analyses of the morphological properties of the seedling. The root and stem lengths of the developing shoots were measured with a digital caliper and recorded by calculating the average lengths. The diameters of the stems were measured with a digital caliper at 2 cm above the axis where the soil touches the stem. The leaf area was measured using ImageJ 1.53k software. We chose three mature leaves from the middle position of the stem. The measurements were done as quadruplicates.

2.3. Salt stress treatment

Each vial containing one-month-old plants was irrigated with a NaCl (Bioshop SOD004) solution prepared at a concentration of 100 mM (2000 µS cm^{-1}). The sampling was performed at hours 0, 3, 6, and 9; after the leaves were placed in 1.5 mL tubes, they were dipped in liquid nitrogen and stored at -20 ºC until examined for gene expression.

2.4. Lipid peroxidation contents

The lipid peroxidation content was determined according to the method specified by Madhava Rao & Sresty (2000). The obtained supernatants were measured at absorbances of 532 nm and 600 nm wavelengths, and the calculations were made using the extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Gene expression

The gene expression analysis was carried out with 1:1000 v/v for SW and 1 µM for KAR1 concentrations, which were morphologically more effective than the treatment groups. The DNA extraction was performed using TRIzol (Thermo, USA) according to the manufacturer's protocol, and the leaf tissues of 100 mg of the apricot plants were used in the study. The RNA concentration and quality were checked using Qubit 2 (Invitrogen, USA) and NanoDrop (Maestrogen, Taiwan) instruments, and the integrity of RNA with 2% agarose gel electrophoresis.

Before the cDNA synthesis, the RNA isolates were treated with the DNase 1 enzyme (Thermo, USA) against genomic DNA (gDNA) contamination. One microgram of RNA was used for the cDNA synthesis, which was performed according to the protocol provided by the manufacturer using the High Capacity cDNA Reverse Transcription Kit (Thermo, USA) (PCR profile set for cDNA synthesis: 25 ºC: 10 min; 37 ºC: 120 min; 85 ºC: 5 min). Actin (ACT) gene was used to confirm the success of the cDNA synthesis. The sequences, melting temperatures (T_m), and amplicon size (bp) of the gene-specific primers are given in Table 1.

Table 1- Primer specifications designed for qRT-PCR

Target enzymes	***Primer sequences (Forward/Reverse, 5′→3′)***	T_m	***Expected amplicon size (bp)***
Actin	[1]CCCTAAGGCTAACAGAGAAAAGA	59.20	212
	[1]CAGCAAGGTCCAGACGAAGAAT	57.40	
Katalase	CTCATACTGGTCTCAGGCAGA	58.62	116
	CCCACTGCTGGGAACTCAAA	60.18	
	TCTCATACTGGTCTCAGGCAG	58.62	117
	CCCACTGCTGGGAACTCAA	59.54	
	TCATACTGGTCTCAGGCAGATAAA	59.04	118
	TTGCCCACTGCTGGGAACT	61.77	
Superoxide dismutase	CCACATCGGCATAACATCCG	59.13	119
	TGGTTCAACGTGATCTCAGAA	57.26	
	GGAGATGGCCCAACTACTGT	59.10	120
	GAAATGCGGTCCAGTTGACA	58.80	
	AACCAACGGTTGCTTGTCAA	58.80	120
	CCATCGTCCCCAACAGTGAT	59.70	
Glutathione peroxidase	ACGTGGCTTCAAAATGTGGATTGA	61.76	128
	AGGCTCTTGGCCCCCAAA	61.19	
	ACGGGTTCTCTTTGAAATCACC	58.85	129
	CCTTTCCGTCAATATCCTTGACAC	59.67	
	AGGTGACCTTGTCAAGTGGAA	59.16	119
	CCAGTTTCTGGATATCCCTCTCAA	59.59	

[1] (Wang et al. 2014); T_m: Melting temperature in ºC; bp: base pair.

The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using SYBR Green dye to determine the expression of genes encoding CAT, SOD, and GPX enzymes in the apricot plant using Rotor-Gene-Q 5 Plex HRM (Qiagen, USA) with 72-well carousel. Each qRT-PCR tube (Qiagen Cat. No. 981103) included 5 µL RealQ Plus 2x Master Mix Green (Ampliqon Cat. No. A323402), 0.5 µL of 10 picomoles of each primer, 10 ng of cDNA and the reaction was completed to 10 µL with nuclease-free water. Three independent biological replicates and three technical replicates were used for each treatment group for the qRT-PCR studies. The qRT-PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 45 cycles at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. In the study, -RT control (without reverse transcriptase enzyme) and negative control (without template) reactions were also established for the control. The ACT gene was also used for normalization (Wang et al. 2014). The $2^{-\Delta\Delta CT}$ method was employed to evaluate gene expression (Livak & Schmittgen 2001). The data retrieved were analyzed using the Rotor-Gene Q Series Software 2.3.5 software.

2.6. Data analysis

According to the randomized plots trial design, all the data gathered for the purpose of this study were assessed using ANOVA on XLSTAT 2021, a statistical software. The Duncan's test was run to identify the differences between the averages once the statistically significant transactions were identified at a significance level of 5%. The obtained data were presented as mean ± standard deviation in a table.

3. Results

3.1. Morphological Growth parameters

The highest rate of germination was 1:1000 SW at 60% and 1 µM KAR1 at 72% (Table 2). It was observed that the germination increased as the concentration decreased in the SW treatment and increased as the concentration increased in the KAR1 treatment. The shoot development was observed in the sown seeds after two weeks. The shoot growth rates were assessed one month after sowing and before the salt stress application. In terms of the shoot development, a 95.83% ratio with 1:100 concentration yielded the best result in the SW treatment and an 87.50% ratio with 1 µM concentration in the KAR1 treatment. Shoot development analysis showed that there was no statistically significance observed among the control and the treatment groups of 1:500 SW, 1:1000 SW, 0.01 µM KAR1 and 1 µM KAR1.

Table 2- The effects of the SW and KAR on the growth parameters of *Prunus armeniaca* (cv. Şalak) seeds are provided in Table 2. The results are presented as mean ± SE. The values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test) [SW: smoke water (1:100, 1:500, 1:1000 v/v), KAR1: karrikin (0.01, 0.1, 1 µM)].

Treatment	*Seed germination (%)*	*Shoot development (%)*	*Root length (mm)*	*Stem length (mm)*	*Stem diameter (mm)*	*Number of leaves*	*Leaf area (cm^2)*
Control	34.00 ± 0.229^{ef}	77.27 ± 0.400^{bc}	129.06 ± 0.830^{abc}	90.90 ± 0.889^{c}	2.17 ± 0.220^{abc}	14.00 ± 2.080^{a}	3.96 ± 0.627^{ab}
1:100 SW	40.00 ± 0.387^{cdef}	95.83 ± 0.186^{a}	137.68 ± 0.701^{a}	103.78 ± 0.559^{a}	2.01 ± 0.182^{c}	14.05 ± 1.564^{a}	4.09 ± 1.257^{a}
1:500 SW	50.00 ± 0.403^{bcd}	79.17 ± 0.380^{bc}	113.77 ± 0.625^{bcd}	81.90 ± 0.561^{cd}	2.26 ± 0.283^{a}	11.35 ± 1.797^{c}	3.09 ± 0.924^{bc}
1:1000 SW	60.00 ± 0.387^{ab}	85.42 ± 0.400^{ab}	110.39 ± 0.194^{cd}	78.87 ± 0.341^{d}	2.10 ± 0.215^{abc}	12.85 ± 2.197^{ab}	3.37 ± 0.834^{abc}
0.01 µM KAR1	46.00 ± 0.229^{bcde}	83.33 ± 0.312^{abc}	104.31 ± 0.488^{d}	83.99 ± 0.226^{cd}	2.05 ± 0.234^{c}	13.35 ± 1.590^{ab}	2.57 ± 1.143^{c}
0.1 µM KAR1	54.00 ± 0.391^{bc}	68.75 ± 0.462^{c}	136.30 ± 0.862^{ab}	92.08 ± 0.200^{bc}	2.08 ± 0.291^{bc}	12.35 ± 1.424^{bc}	3.54 ± 1.015^{ab}
1 µM KAR1	72.00 ± 0.332^{a}	87.50 ± 0.250^{ab}	141.92 ± 0.143^{a}	102.67 ± 0.254^{ab}	2.26 ± 0.288^{ab}	14.10 ± 1.670^{a}	3.97 ± 0.981^{ab}
P	**P<0.002**	**P<0.025**	**P<0.002**	**P<0.0001**	**P<0.009**	**P<0.0001**	**P<0.005**

Values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test). All morphological measurements were made before salt stress application.

The root lengths were observed to have decreased as the concentration decreased in the SW treatment. In the KAR1 treatment, on the other hand, as the concentration increased, an increase in root length occurred (Figure 1). Compared to the control, the 1:100 concentration with 137.68 mm yielded the best result in the SW treatment, while the 1 µM concentration with 141.92 mm did so in the KAR1 treatment.

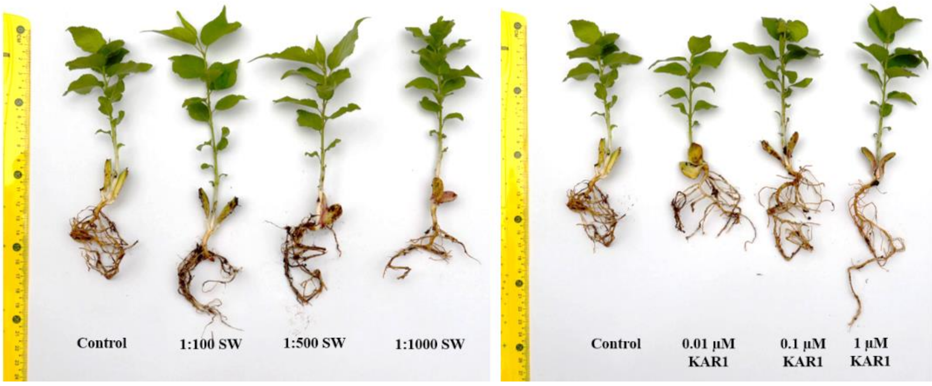

Figure 1- Effects of smoke water and karrikin on root growth

In the treatment groups after one month, a decrease in the stem length was observed in the SW treatment as the concentration decreased, and increased stem lengths were detected in the KAR1 treatment as the concentration increased (Figure 2). The 1:100 concentration with 103.78 mm in the SW treatment and the 1 µM concentration with 102.67 mm in the KAR1 treatment yielded the best results.

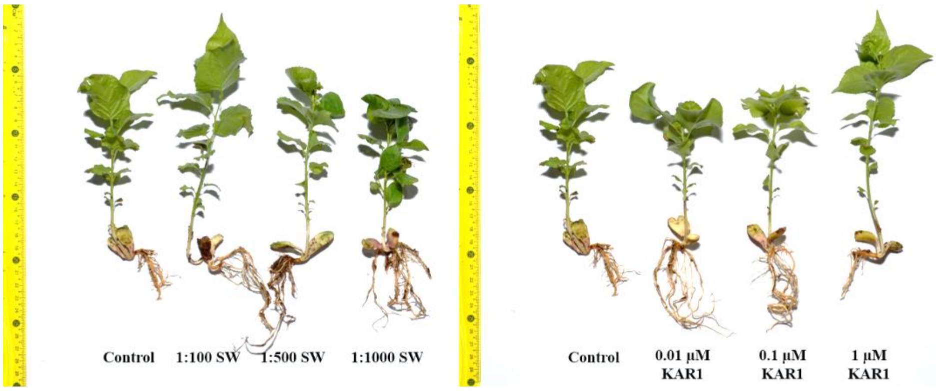

Figure 2- Effects of smoke water and karrikin on stem length

In terms of the stem diameter, 2.26 mm with 1:500 concentration yielded the best result in the SW treatment and 2.26 with 1 µM concentration in the KAR1 treatment. In terms of the number of leaves, the SW application gave the best result with 14.05 and 1:100 concentration, while the KAR1 application gave the best result with 1 µM concentration and 14.10. In terms of the number of leaves, the SW application gave the best result with 14.09 cm^2 and 1:100 concentration, while the KAR1 application gave the best result with 1 µM concentration and 3.97 cm^2. The SW and KAR1 applications gave similar results in terms of stem diameter, number of leaves and leaf area, depending on concentration.

3.2. Lipid peroxidation parameters

The effect of the treatment groups on the amount of lipid peroxidation was found to be statistically significant (P<0.05). In the TBARS level, there was a 2.31% decrease in the SW treatment and a 0.77% increase in the KAR1 treatment compared to the control (Figure 3).

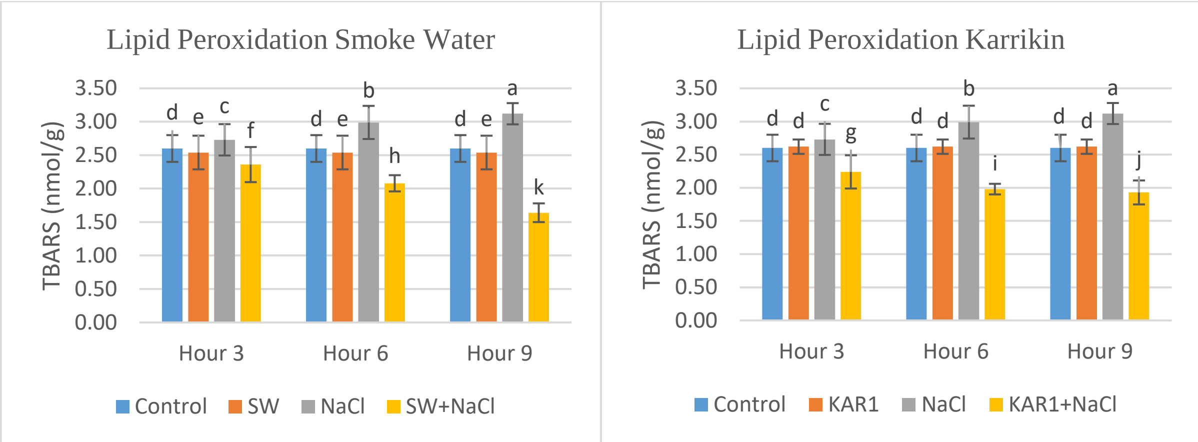

Figure 3- Effects of smoke water and karrikin on lipid peroxidation. SW: smoke water (1:1000 v/v), KAR1: karrikin (1 µM), NaCl: salt stress (100 mM). Results are presented as mean ± SE. Values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test).

At hours 3, 6, and 9 of the salt treatment, the TBARS levels increased by 5%, 15%, and 20%, respectively, in cells compared to the control, indicating that cellular membrane damage occurred a result of salt stress. It was determined that there was a decrease in the TBARS levels when salt was applied in the SW and KAR1 treatments. The TBARS levels were found to have decreased by 9.23%, 20%, and 36.92% in the SW treatment compared to the control at hours 3, 6, and 9, respectively. In the KAR1 treatment, there was a 13.85%, 23.85%, and 25.77% decrease in the TBARS levels at hours 3, 6 and 9, respectively, compared to the control. These results suggest that SW and KAR1 may mitigate salt stress-induced cellular membrane damage. However, it was concluded that SW provided a more effective improvement than KAR1.

3.3. Gene expression

3.3.1CAT gene expression parameters

The effects of the treatment groups on CAT gene expression were found to be statistically significant (*P*<0.05). The results concerning the CAT gene expression showed that the SW treatment increased gene expression by 11% compared to the control (Figure 4).

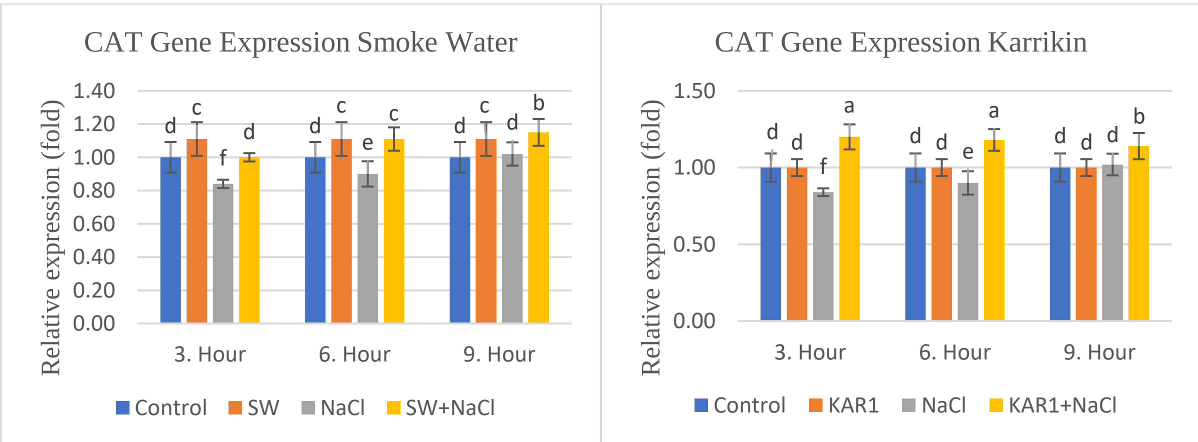

Figure 4- CAT qRT-PCR expression results. SW: smoke water (1:1000 v/v), KAR1: karrikin (1 µM), NaCl: salt stress (100 mM). Results are presented as mean ± SE. Values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test).

It was determined that there was a decrease of 16% and 10% in gene expression compared to the control at hours 3 and 6 of salt treatment, respectively and an increase by 2% at hour 9. It was found that there was an increase in the gene expression when the salt was applied in the SW and KAR1 treatments. An 11% and 15% increase was detected in the gene expression in the SW treatment compared to the control at hours 6 and 9, respectively. In the KAR1 treatment, there was an increase of 20%, 18% and 14% in gene expression at hours 3, 6 and 9, respectively, compared to the control.

The SW treatment alone was more effective in increasing gene expression than KAR1. When salt was applied in the SW and KAR1 treatments, it was understood that the KAR1 treatment was more effective in gene expression than the SW treatment. While the SW treatment showed more effects at hour 9, the KAR1 treatment started to show its effect from hour 3, which was the first step of stress. As a result, the KAR1 treatment was found to be more effective than the SW treatment when salt was applied in the SW and KAR1 treatments in CAT gene expression.

3.3.4. SOD gene expression parameters

The effects of experimental groups on SOD gene expression were found to be statistically significant (P<0.05). The results regarding SOD gene expression showed that the SW treatment increased gene expression by 5% compared to the control (Figure 5).

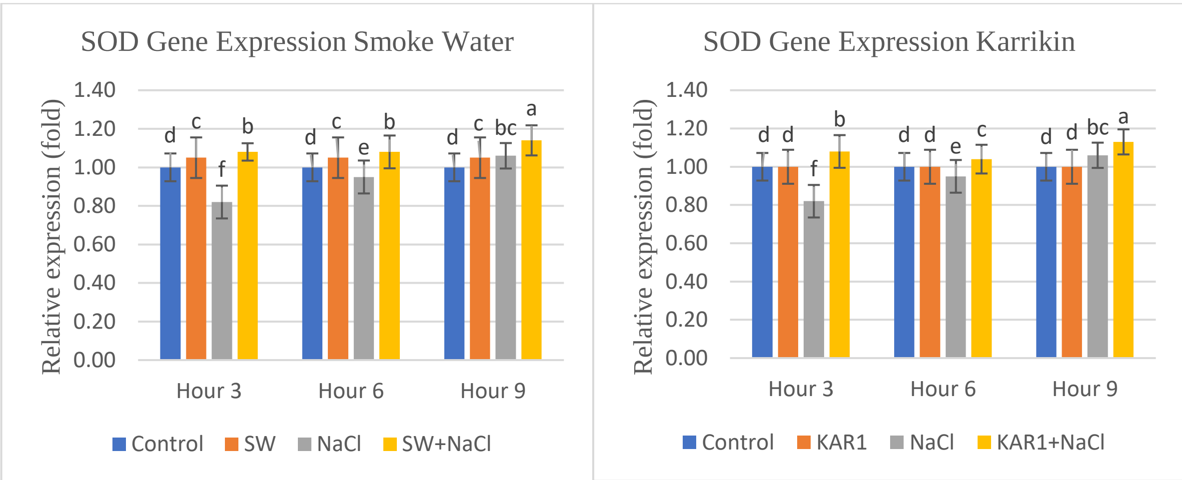

Figure 5- SOD qRT-PCR expression results. SW: smoke water (1:1000 v/v), KAR1: karrikin (1 µM), NaCl: salt stress (100 mM). Results are presented as mean ± SE. Values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test)

It was determined that there was a decrease of 18% and 5% in gene expression compared to the control at hours 3 and 6 of salt treatment, respectively and an increase by 6% at hour 9. An increase was identified in gene expression when salt was applied in the SW and KAR1 treatments. It was revealed that there was an 8%, 8% and 14% increase in gene expression in the SW treatment compared to the control at hours 3, 6 and 9, respectively. In the KAR1 treatment, an 8%, 4%, and 13% increase occurred in gene expression at hours 3, 6 and 9, respectively, compared to the control.

It was concluded that the SW and KAR1 treatments alone were not considerably effective in gene expression. When salt was applied in the SW and KAR1 treatments, both showed similar results and appeared to be more effective at hour 9 of application. As a result, SW and KAR1 treatments have similar effects on SOD gene expression.

3.3.5. GPX gene expression parameters

The effects of the experimental groups on GPX gene expression were found to be statistically significant (P<0.05). The results regarding GPX gene expression showed that the SW treatment increased gene expression by 3% compared to the control (Figure 6).

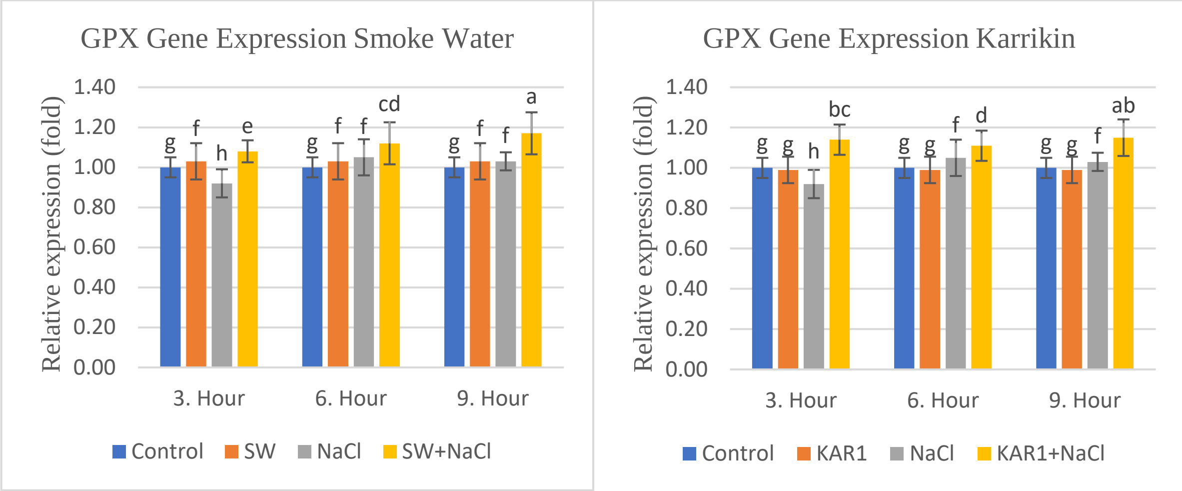

Figure 6- GPX qRT-PCR expression results. SW: smoke water (1:1000 v/v), KAR1: karrikin (1 µM), NaCl: salt stress (100 mM). Results are presented as mean ± SE. Values with different superscripts in the same row are significantly different from each other (P<0.05; Duncan's test).

It was determined that there was an 8% decrease in hour 3 of the salt treatment compared to the control, and a 5% and 3% increase in gene expression compared to the control at hours 6 and 9, respectively. It was observed that there was an increase in gene expression when salt was applied in the SW and KAR1 treatments. There was an 8%, 12%, and 17% increase in gene expression in the SW treatment compared to the control at hours 3, 6 and 9, respectively. In the KAR1 treatment, there was an increase of 14%, 11%, and 15% in gene expression compared to the control at hours 3, 6 and 9, respectively.

It was determined that the KAR1 treatment alone was more effective in gene expression than the SW treatment. When salt was applied in the SW and KAR1 treatments, both seemed to be more effective at hour 9. The SW and KAR1 treatments in GPX gene expression showed similar results when salt was applied.

4. Discussion

There are many studies suggesting the use of SW and KARs when germinating seeds as these substances increase the germination rate (Baxter & Van Staden 1994; Çatav et al. 2015; Flematti et al. 2015; Kochanek et al. 2016; Tavşanoğlu et al. 2017). KARs are water-soluble substances that have seed germination-promoting activity at incredibly low concentrations, usually below 10^{-9} mol/L (Light et al. 2009; Nelson et al. 2012). However, higher concentrations of SW inhibit germination, while lower concentrations are known to exhibit a germination-promoting property (Light et al., 2002). Therefore, in order to maximize its stimulant biological activity, SW should be diluted with water before use, usually at ratios of 1:250, 1:500, 1:1000, 1:1500, and 1:2000 (v/v), depending on the plant species (Van Staden et al. 2004). At high concentrations (1:100 or less dilution), SW inhibits germination. However, lower concentrations (1:1000 dilution) significantly increase germination compared to the control (Light et al. 2002). Consistent with the literature, this study observed that the germination of apricot seeds increased as the SW concentration decreased, whereas the germination increased as the concentration increased in the KAR1 treatment. The best germination optimization for SW was obtained at a concentration of 1:1000 (v/v) and for KAR1 at a concentration of 1 µM.

There are hypotheses that both SW and KAR1 interact with other plant growth regulators and frequently exhibit cytokinin and auxin-like activities (Chiwocha et al. 2009). Since it is thought to exhibit activities, as these phytohormones do, it has been shown that KAR and SW provide a significant increase in shoot and root length, seedling development and lengths, number of leaves, leaf area, root and shoot dry and fresh weights, stem diameters and number of fruits after germination (Baxter & Van Staden 1994; Kulkarni et al. 2006; Van Staden et al. 2006; Kulkarni et al. 2008; Chumpookam et al. 2012; Çatav et al. 2018). In a study on tomato, okra, bean, and corn seeds, SW was found to significantly increase seedling growth by increasing both root and shoot lengths. The results show that the smoke-derived compound also exhibits stimulating effects after germination and can be used as a plant growth regulator (Van Staden et al. 2006). The treatment of okra seedlings with SW has shown a significant increase in shoot/root length, shoot fresh/dry weight, number of leaves, total leaf area, and stem diameter. Thus, the application of SW to leaves is thought to be a useful and inexpensive technique to increase the seedling growth of vegetable crops (Kulkarni et al. 2007). The present study revealed that KAR1 and SW caused an increase in the shoot, root, and stem lengths. However, when compared with the available literature, no remarkable results were found in the number of leaves, stem diameters, and leaf area. Contrary to the germination results, the best concentration for SW in the morphological data was 1:100. This may be due to the decrease in the SW concentration in the soil when the soils in the vials were irrigated with the Hoagland solution, and, consequently, the decrease in the KAR concentration in its content. Because KAR in nature can be washed away by rain and decompose relatively quickly in sandy soils, their concentration is constantly decreasing (Flematti et al., 2015).

Salinity is one of the abiotic stress factors that delimit the water uptake ability of plants and cause ion toxicity (Munns & Tester 2008). It is known that these two conditions caused by salt stress negatively affect cell growth rate, leaf development, stomatal conductance, photosynthesis, ion balance, and oxidative homeostasis (Jiang & Deyholos 2006; Shabala & Munns 2012; AbdElgawad et al. 2016). Exposure to salt stress is associated with increased electrolyte leakage, proline content, H_2O_2 production, and MDA accumulation. Studies show that SW or KAR application to a plant under salt stress decreases MDA accumulation and H_2O_2 production while further increasing proline accumulation (Shah et al. 2020; Sharifi & Shirani Bidabadi 2020; Çatav et al. 2021; Shah et al. 2021; Hayat et al. 2022). In this study, it was determined that the accumulation of MDA increased only in the plants exposed to salt stress, and a decrease in MDA accumulation was observed when SW and KAR were applied to the plant under salt stress. These results suggest that SW and KAR can minimize the toxic effects of ROS in apricot plants under salt stress. Antioxidant enzymes are essential for shielding cells and macromolecules from oxidative damage brought on by ROS. It is well known that various plant species' responses to severe environmental factors and plant growth regulators alter the expression of these genes and the activity of these enzymes (Sharma et al. 2012; Vardhini & Anjum 2015). The present study investigated the independent impacts of SW and KAR on the transcription levels of several antioxidant enzymes (CAT, SOD, and GPX) under salt stress. Consistent with previous studies, our results indicated that SW and KAR increased the expression of CAT, SOD, and GPX genes under salt stress. According to a study by Sharifi & Shirani Bidabadi (2020), it was observed that KAR treatment increases the activities of CAT and SOD enzymes with salinity stress, while decreasing the activity of the GPX enzyme. Thus, it has been revealed that KAR activates the adaptation mechanism against salinity stress. Hayat et al. (2022) report that SW applied to wheat plants increases the activities of SOD, APX, and POD enzymes. In the SW treatment used in our study, an increase was observed in the SOD activity compared to the control, and it activated the protection mechanism of the plant against the damage caused by salt stress. As the study by Çatav et al. (2021) notes, salt stress with SW treatment increases the expression of Cu/Zn-SOD, Fe-SOD, and Mn-SOD genes. This finding coincides with our study and that of Hayat et al. (2022). The study also has claimed that the SOD and APX activities were greater in the treated seedlings than in the control seedlings and proposed that overexpression of the genes encoding these enzymes was crucial in enhancing salt tolerance (Hayat et al. 2022). Additionally, the above study demonstrated that while salt decreased the expression of the CAT gene in the treatment group, it had no effect on the function of the CAT gene. This decrease in the expression of the CAT gene in the above study coincides with our study. Consistent with the data of our study and of Shah et al. (2020, 2021), gene expressions of SOD, CAT, POD, and APX were found to be significantly higher in wheat seedlings treated with KAR1 under salinity stress. The related literature confirms that SW and KAR increase the activity of antioxidant enzymes and gene expression against salinity stress. It appears that KARs can control endogenous H_2O_2 production, MDA accumulation, and proline accumulation, prevent electrolyte leakage, and improve membrane integrity under abiotic stresses.

5. Conclusions

The apricot plant is a commercially important crop plants that can be used both for edible and dried purposes. Although the use of SW and KAR have been studied on many plants, there has yet to be any study of its use on fruit trees; this study will be a first in the literature. In the present research, the seeds of Şalak, were germinated with SW and KAR1, and the germinated seeds were transferred to the soil and their morphological development was evaluated. The germination results showed that SW and KAR1 have similar results with the literature by stimulating the germination with the determined concentration in this study. Our study found that germination increased as SW concentration decreased, and germination increased as KAR1 concentration increased. Literature data has shown that SW and KAR1 contribute significantly to the development of the plant after germination. In the present study, KAR1 and SW caused an increase in shoot, root and stem length.

The apricot is one of a group of plants sensitive to salinity stress and the Iğdır Plain is in the saline soil category in terms of its characteristics. While many studies have shown that SW and KAR1 contribute to plant growth in terms of germination and morphology, there are few studies showing that they increase tolerance in abiotic stress factors. In this research, it has been proven that SW and KAR1 protect the plant against the harmful effects of ROS by increasing the transcription levels of antioxidant enzymes (CAT, SOD and GPX) and reducing the amount of lipid peroxidation (MDA).

Smoke water is a cheap and simple substance that has the potential to be used in agriculture, horticulture, and laboratory studies, and can be stored and used for many years. Since it acts as a plant growth regulator on plants in many studies, it is an economic substance that supports seed germination, shoot, root and plant growth even at very low concentrations in contrast to commercially available plant growth regulators (gibberellic acid, auxin, cytokinin, abscisic acid, strigolactone, etc.), which are very expensive, cannot be stored for long, and are sensitive to heat.

We believe that the obtained data make important contributions relating to the interaction of plants with their environment, germination physiology, and discovery of new and more effective molecules in the protection of plants against abiotic stress.

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Potential of Purslane (*Portulaca oleracea* L.) in Phytoremediation: A Study on the Bioaccumulation and Bio-Transfer of Cadmium, Nickel, and Copper in Contaminated Soils

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ABSTRACT

As industrial and agricultural activities intensify and technology rapidly advances, soil pollution has escalated to alarming levels. The increasing contamination of agricultural areas and the crops cultivated therein has emerged as a significant contemporary issue. Phytoremediation, the use of plants to remove pollutants, is a promising method for mitigating soil heavy metal contamination.

This study investigates the bioaccumulation capacity of purslane (*Portulaca oleracea* L.), a potential phytoremediator, in soils artificially contaminated with cadmium (Cd), nickel (Ni), and copper (Cu). The purslane was cultivated under controlled conditions with varying doses of Cd, Ni, and Cu. After 55 days, the plants were harvested and analysed for heavy metal concentrations in their roots, stems, and leaves. The results demonstrated a direct correlation between environmental heavy metal concentration and plant heavy metal content, with the most significant accumulation occurring in the roots. The leaf chlorophyll content was adversely affected by increased Cd, Ni, and Cu applications. The highest Cu, Ni, and Cd contents were found in the roots at 140 mg kg^{-1} Cu, 80 mg kg^{-1} Ni, and 20 mg kg^{-1} Cd applications, respectively. The bio-transfer coefficient (BTC), a measure of heavy metal transport from the root region to the leaves, was calculated. The BTC values ranged from 0.84-1.09 for Cu, 0.39-0.84 for Ni, and >1 for Cd at the Control and 5 mg Cd kg^{-1} treatments.

These findings suggest that purslane has potential for phytoremediation of heavy metal-contaminated soils, although the bioaccumulation and bio-transfer of heavy metals are dependent on the specific metal and its concentration in the soil. The study also highlights the potential risks associated with the consumption of plants grown in heavy metal-contaminated soils, as heavy metals can accumulate in different plant tissues, potentially entering the food chain.

Keywords: Purslane, Heavy metal, Pollutants, Bio-transfer, Plant tissues, Chlorophyll index

1. Introduction

Soil is an indispensable source of life for every living being. However, since the mid-20th century, our soils have become increasingly polluted through ever increasing industrialization and urbanization, as well as improper agricultural practices such as excessive use of pesticides and chemical fertilizers. Consequently, the quality of soil, necessary for plant life, is degrading in quality in many parts of the world. In crop-growing areas, heavy metals are absorbed from the soil into the body of plants, affecting their physiological activities and causing yield losses. Some heavy metals, particularly cadmium (Cd), lead (Pb), and copper (Cu), can negatively affect plant growth and development even at very low concentrations. Plants under heavy metal stress due to soil pollution must adapt themselves through changes in their physiological, biochemical, anatomical, and morphological systems. In addition, heavy metals affect not only the plant but also the health of all living things that ingest these plants through the food chain. Many leafy vegetables and fruits have been found to contain varying amounts of heavy metals (Ramteke et al. 2016; Negi 2018).

Purslane is a plant resistant to negative edaphic and climatic factors and is capable of growing in fields, roadsides, and infertile areas. Purslane (*Portulaca oleracea*) is a nutritious plant with high vitamin A, vitamin C, total protein, calcium, iron, potassium, magnesium, and betacyanin in its vegetative parts (Salehi et al. 2008). It is used in soups, salads, and as a leafy vegetable in Mediterranean and tropical Asian countries, including India. Purslane, which has antibacterial, antiseptic, antidiabetic, antioxidant, antispasmodic, diuretic, antiscorbutic, and wound-healing properties, is designated by the World Health Organization as one of the most widely consumed medicinal plants (Xu et al. 2006). *Portulaca oleracea* has a high nutritional content with significant medicinal properties. Its tolerance to stress conditions during the production is as well important. Because of its resistance to unfavorable factors, it grows as weed in fields, roadsides, and infertile areas. Consequently, the heavy metal content of purslane in plants grown in industrially polluted areas has been previously identified (Yadegari 2018; Ren & White 2019). Although there is some literature on the heavy metal uptake of purslane, detailed studies on heavy metal accumulation are lacking.

For this reason, this study evaluates the bioaccumulation capacity and tendency of purslane plants growing in a soil treated with different doses of Cd (0, 5, 10, and 20 mg kg^{-1}), Cu (0, 70, 140, and 210 mg kg^{-1}) and Ni (0, 20, 40, and 80 mg kg^{-1}).

2. Material and Methods

This study was performed as a pot experiment in the greenhouses of Ege University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition according to the principles of randomized experimental design with three replications. There were 36 pots (3 different heavy metals × 4 doses × 3 replicates = 36 pots), and each pot was filled with 1 kg of soil (dry weight) sieved through 2 mm. Prior to the start of the experiment, the soil was analysed for its physical and chemical properties (Table 1). Since the soil has high fertility and is suitable for purslane cultivation, no fertilizer was applied. At the beginning, 10 purslane seeds were sown in each pot, and 4 of the plants that showed uniform development were left to be studied after the germination stage. As the treatments of the experiment, 4 different doses of $CuSO_4$ (0, 70, 140, 210 mg Cu kg^{-1}), $NiSO_4$ (0, 20, 40, 80 mg Ni kg^{-1}) and $CdSO_4$ (0, 5, 10, 20 mg Cd kg^{-1}) were mixed with irrigation water and applied to the purslane plants in the pots every 15 days. The plants were harvested after 55 days. At harvest, the purslane plants were dissected into leaf, stem, and root parts, and the heavy metal accumulation capacity of the plant parts was examined.

Table 1- Some physical and chemical properties of the experimental soil

Texture	*pH*	*EC µS/cm*	*$CaCO_3$*	*N_{total}*	*OM*	*Total (mg kg^{-1})*		
			%			*Cu*	*Ni*	*Cd*
	6.94	1015	3.32	0.022	1.48	25	5.28	0.38
Available (mg kg^{-1})								
P	**K**	**Ca**	**Mg**	**Fe**	**Zn**	**Cu**	**Ni**	**Cd**
8.40	250	672	110	31	3.40	0.60	0.60	0.12

The physical and chemical properties of the soil are provided in Table 1. The pH, water-soluble salts, $CaCO_3$, texture and organic matter contents were determined (Kacar 2016) and their plant extractable/available P, K, Ca, Mg, Na, Fe, Zn, Cu, Mn, Ni and Cd contents were measured (Alloway 2013) (Table 1). The total concentration of the non-essential heavy metals (Cd, Ni and Cu) were determined using the aqua regia ($HCl:HNO_3$, 3:1) extraction method (ISO 1995) and the heavy metal concentrations were measured by AAS analysis (Varian SpectrAA 220, FS).

The plant samples; 5 g from each stem, leaves and roots were sampled, dried and digested to measure the heavy metals (Cu, Ni, Cd), all of which were measured using an Atomic Absorption Spectrophotometer.

The results were subjected to a statistical variance of analysis, program JMP. The effect of treatments (heavy metal doses) and their interactions on purslane leaves, stem and roots were determined.

The Biological Accumulation Coefficient (BAC) was calculated according to the ratio of heavy metal concentration in the shoot divided by the metal concentration in the studied soil (Zu et al. 2005).

BAC = heavy metal concentration in shoot / heavy metal concentration in soil

Bio-Concentration Factor (BCF) was calculated as the ratio of heavy metal concentration in plant roots to that of soil (Yoon et al. 2006).

BCF = heavy metal concentration root / heavy metal concentration in soil

The Bio-Transfer coefficient (BTC) of purslane was also determined to assess the extent of the heavy metal transfer from root to leaves. It is the ratio of the heavy metal concentration in the plant stem to the heavy metal concentration in the plant root (Yoon et al. 2006).

BTC = leaf heavy metal concentration/root heavy metal concentration

3. Results and Discussion

The effect of different doses of heavy metal (Cu, Ni, and Cd) applications on the bio-absorption capacity of purslane by its different parts (root, stem, leaf) was investigated, and the tendency of it to bioaccumulation was evaluated.

3.1. Copper accumulation in the plant tissues

The Cu content of purslane leaves, stems, and roots was found to increase as a function of increasing Cu doses (Table 2). The highest Cu content (22.61 mg kg^{-1}) was in the roots, and the lowest in the stem (2 mg kg^{-1}). In this regard, if the Cu content of leaves is examined, the results are found to be statistically significant, $P \leq 0.05$, compared to that of the Control treatment. The lowest leaf Cu content (13.83 mg kg^{-1}) was determined in the Control (0), and the highest (19.73 mg kg^{-1}) in the 210 mg kg^{-1} Cu applied treatment.

As a function of increasing Cu doses, the Cu contents in the plant body also increased. The Cu content of the purslane stem was determined to be 2 mg kg^{-1} in the Control treatment (0); 3.66 mg kg^{-1} at 70 mg kg^{-1} Cu application; 4.83 mg kg^{-1} at 140 mg kg^{-1} Cu application and; and 4.80 mg kg^{-1} at 210 mg kg^{-1} Cu application. The increase in Cu content in the purslane stem as a function of the Cu doses was statistically significant. However, no statistically significant difference was found between the 140 mg kg^{-1} and 210 mg kg^{-1} Cu treatments. The Cu content in roots increased similarly to the Cu content in leaves and stems as a function of Cu treatments. The Cu content in the root was 12.71 mg kg^{-1} in the Control application (0);19.51 mg kg^{-1} at the 70 mg kg^{-1} application; 22.61 mg kg^{-1} at the 140 mg kg^{-1} application; and 20.46 mg kg^{-1} at the 210 mg kg^{-1} application. The highest Cu content (22.61 mg kg^{-1}) in the roots of the purslane plant was found at the 140 mg kg^{-1} Cu application. The plant biomass decreased as a result of the Cu accumulation in plants (Kabata & Pendias 2001). A high Cu application has been reported to affect root length, damage root cells, and membranes, and cause imbalances in plant nutrient uptake (Lothe et al. 2016). Copper toxicity is also known to disrupt physiological processes such as protein synthesis, photosynthesis, respiration, ion uptake, and cell membrane stability (Sossé et al. 2004). However, in a study investigating the effects of $CuSO_4$ applications in 9 different purslane species, Cu uptake, and plant biomass were found to change depending on the genetic variation of the purslane species (Ren & White 2019).

In our study, 4 chlorophyll indexes were measured in one-week intervals after Cu applications, and the effects of increasing Cu doses on purslane chlorophyll contents were determined (Table 2).

Table 2- Effect of different Cu doses on Cu content of purslane leaves, shoots, roots and Chlorophyll Index

Cu (mg kg^{-1})	***Leaf***	***Shoot /Stem***	***Root***	***Chlorophyll Index***			
				I	***II***	***III***	***IV***
0	13.83b	2.00c	12.71c	167	177b	218a	221a
70	19.70a	3.66b	19.51b	206	216ab	187c	174b
140	18.90a	4.83a	22.61a	211	237a	197b	166c
210	19.73a	4.80a	20.46a	181	221ab	178c	164c
Mean	18.04	3.82	18.82	191	213	195	181
	*	**	*	ns	*	**	*

*: $P \leq 0.05$ statistically significant within error limits; **: $P \leq 0.01$ statistically significant within error limits

To monitor the effects of Cu applications on leaf chlorophyll content, the effects of preharvest Cu doses on plant chlorophyll content were found to be statistically significant when measurements were taken at different time points. Particularly, in the last two pre-harvest measurements (04/06/2021 and 11/06/2021), the change in leaf chlorophyll content as a function of Cu doses was significant (Figure 1). In a study investigating the effects of Cu toxicity on cucumber growth, the response of cucumber leaves to Cu stress was found to vary depending on the growing season. Photosynthesis decreased by 52% in the mature leaves of cucumbers and 27% in young leaves compared to the Control treatment. The loss of stomata explains the further decrease in photosynthetic rate in mature leaves and thus the ability to assimilate CO_2 (Dunand et al. 2002).

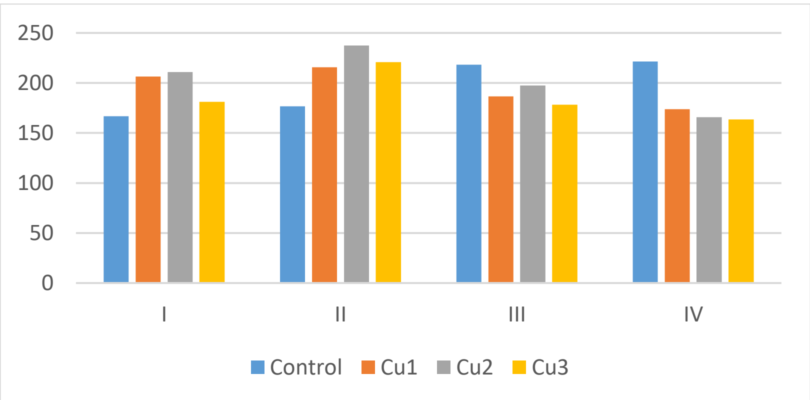

Figure 1- Chlorophyll index of purslane

The accumulation of Cu in the cells affects the photosynthetic process negatively since Cu has negative effects on plant pigment contents (Jaime -Pérez et al. 2019; Lwalaba et al. 2019). In this regard, the synthesis of photosynthetic enzymes is inhibited due to the changes in the composition of photosynthetic membrane pigments and proteins (Silva et al. 2018). It has been reported that the pigment content and photosynthetic activity of tomato plants treated with 100 mg kg^{-1} Cu for 40 days decreased (Nazir et al. 2019; Kafkasyalı 2021).

3.2. Nickel accumulation in the plant tissues

The results showed that as Ni application doses in the treatments increased, the Ni contents in the leaves, stem, and roots of the purslanes increased (Table 3). The highest Ni content was found in the roots (9.57 mg kg^{-1}) when 80 mg kg^{-1} Ni was applied and the lowest in the leaves of the Control treatment (0.85 mg kg^{-1}). If the Ni content of the purslane leaves are examined with respect to treatments, the effect of different doses of Ni applications compared to the Control treatment was found to be statistically significant at P≤0.05 and P≤0.01. Our findings showed that 0.85 mg kg^{-1} of Ni was found in the Control treatment (0); 2.15 mg kg^{-1} at 20 mg kg^{-1} Ni application dose; 3.65 mg kg^{-1} at 40 mg kg^{-1} Ni dose; and 3.71 mg kg^{-1} at 80 mg kg^{-1} Ni dose. These results indicate that Ni accumulation in purslane leaves does not exceed a certain limit. The results related to purslane stems showed that in the Control treatment the Ni content was 0.86 mg kg^{-1}; in this regard, 0.92 mg kg^{-1} at 20 mg kg^{-1} dose; 1.90 mg kg^{-1} at 40 mg kg^{-1} dose; and 2.43 mg kg^{-1} at of 80 mg kg^{-1} dose, respectively. The change in the Ni content of purslane stems due to enhancements in Ni applications is shown to be statistically significant.

The Ni content of purslane root was 1.01 mg kg^{-1} in the Control; 4.08 mg kg^{-1} in the case when 20 mg kg^{-1} Ni applied, 6.77 mg kg^{-1} at the 40 mg kg^{-1} application; and 9.57 mg kg^{-1} when 80 mg kg^{-1} Ni applied. The results showed that Ni accumulated particularly in the roots provided that there is high Ni in the soil. However, similar to Cd, the Ni transfer to leaves and stem was low.

Table 3- Effects of Ni applications on the Ni contents of purslane leaves, shoots, roots and Chlorophyll Index

Ni (mg kg^{-1})	***Leaf***	***Shoot /Stem***	***Root***	***Chlorophyll Index***			
				I	***II***	***III***	***IV***
0	0.85c	0.86c	1.01d	167b	177b	218a	221b
70	2.15b	0.92c	4.08c	212 a	217a	195b	168a
140	3.65a	1.90b	6.77b	179b	195ab	151c	151a
210	3.71a	2.43a	9.57a	203a	214ab	167c	162a
Mean	2.59	1.53	5.36	190	200	183	176
	*	**	**	*	*	**	*

*: P≤0.05 statistically significant within error limits;**: P≤0.01 statistically significant within error limits

In different parts of the purslane plant, Ni accumulation from highest to lowest was found as root > leaf > stem (Table 3). Yadegari (2018) noted that high Ni and high Cd in the soils negatively affect the shoot and root development of purslane. It has also been reported that the elemental content of *Portulaca oleracea L.* is significantly affected by the heavy metal density of the soils. Excess Ni in the plant negatively affects chlorophyll synthesis and lipid metabolism. It also damages thylakoid membranes and granules, reduces granule size, and increases the number of lamellae, negatively affecting photosynthetic activity. In addition, Ni is known to compete with essential elements such as Fe, Zn, Cu, Fe, Mn, and Mg, reducing the rate of uptake and transport of these elements by plants (Angulo-Bejarano et al. 2021). This prevents plant roots from absorbing other nutrients and leads to nutrient deficiency (Fryzova et al. 2017). For this reason, in this study 4 chlorophyll index measurements were performed at one-week intervals after applying different doses of Ni, and the effects on chlorophyll in purslane were determined (Figure 2). The effect of the last two pre-harvest measurements on plant chlorophyll content was statistically significant in monitoring the change in leaf chlorophyll content after Ni applications (Table 3).

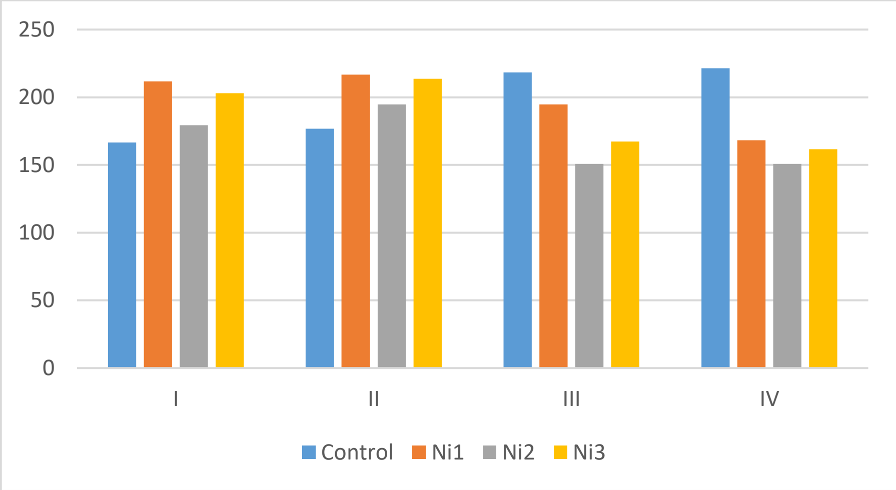

Figure 2- Chlorophyll index of purslane

If high Ni content is present in the environment where the plant grows, Ni can replace Mg, which is the building block of chlorophyll (Kumar et al. 2021). This process causes a change in the chlorophyll molecule (Angulo-Bejarano et al. 2021). In a study, the effect of increasing Ni applications (0.1, 0.3, and 0.5 mM) on beans was examined and the findings showed that there were decreases in chlorophyll a, chlorophyll b, carotenoids, total pigment I, and total pigment II contents of the beans (Macedo et al. 2016). High Ni content leading to changes in the number of photosynthetic pigments (such as chlorophyll a, chlorophyll b, and carotenoids) has also been found to cause leaf chlorosis and necrosis (Kumar et al. 2022).

3.3. Cadmium accumulation in the plant tissues

Purslanes were treated with four different doses of $CdSO_4$ (0, 5, 10, 20 mg kg^{-1}) and likewise in the case of Cu and Ni applications, each plant was dissected into leaf, stem, and root parts at harvest. The Cd accumulation capacity of these parts was investigated and the results showed that the Cd content of purslane leaves, stem, and root parts significantly increased ($P \leq 0.05$), as a function of increasing the Cd doses (Table 4). The highest Cd (1.47 mg kg^{-1}) was determined in the root, and the lowest in the stem (0.12 mg kg^{-1}). In the evaluation of leaf Cd contents, the highest value (1.12 mg kg^{-1}) was obtained at the 5 mg kg^{-1} application treatment, followed by the applications of 20 mg kg^{-1} Cd (0.79 mg kg^{-1}) and 10 mg kg^{-1} Cd (0.60 mg kg^{-1}). These results indicate that Cd accumulation in purslane leaves occurs up to a certain value i.e. increasing the Cd content in the medium does not affect Cd accumulation in purslane leaves.

The cadmium content of the stem was 0.12 mg kg^{-1} in the Control treatment (0). With respect to increasing applications of Cd (5 mg kg^{-1}, 10 mg kg^{-1}, 20 mg kg^{-1}), 0.26 mg kg^{-1}, 0.30 mg kg^{-1} and 0.28 mg kg^{-1} Cd contents were found respectively (Table 4). When the results of the stem Cd contents of purslane is evaluated, the findings indicated that enhanced Cd doses increased the Cd of stem compared to Control treatment. However, no statistical difference was found between treatments.

The effects of the different Cd doses on the Cd content of purslane roots were statistically significant (Table 4). In the Control treatment, the purslane roots had 0.15 mg kg^{-1} Cd; 0.46 mg kg^{-1} when 5 mg kg^{-1} Cd was applied; 0.93 mg kg^{-1} in the 10 mg kg^{-1} Cd treatment and when 20 mg kg^{-1} Cd was applied, it was 1.47 mg kg^{-1} Cd. The highest Cd in roots was in the highest application of Cd (20 mg kg^{-1}) but this was not so in the case of leaves and stems. Therefore, the transfer of Cd to leaves and stems is low. Cadmium applications have been reported to decrease plant biomass, but Cd accumulation in the plant occurs as a function of increasing concentration (Yadegari 2018).

Table 4- Effects of Cd applications on Cd contents of the leaves, stems, roots and Chlorophyll Index

Cd (mg kg^{-1})	*Leaf*	*Shoot /Stem*	*Root*	*Chlorophyll Index*			
				I	*II*	*III*	*IV*
0	0.30c	0.12b	0.15d	167b	177c	218a	221a
70	1.12a	0.26a	0.46c	202ab	212ab	190ab	189b
140	0.60bc	0.30a	0.93b	176ab	201b	189ab	170b
210	0.79ab	0.28a	1.47a	210a	224a	186c	179b
Mean	0.70	0.24	0.75	189	203	196	190
	*	*	**	*	*	**	**

*: $P \leq 0.05$ statistically significant within error limits, **: $P \leq 0.01$ statistically significant within error limits

Cadmium accumulation in different parts of the purslane plant is found as root > leaf > stem. Tiryakioglu et al. (2006) reported that increasing Cd applications lead to an increase in the Cd concentration of the green parts; however, much of the Cd particularly accumulated in the roots. Similarly, Pietrelli et al. (2022), in their study, exposed the plants to heavy metal pollution and found that the root parts of the plants generally accumulated higher Cd concentrations.

Cadmium, an element not essential for plant growth, negatively affects the plant uptake, transport, and utilization of plant nutrients and water. Cadmium causes physiological toxicity symptoms such as chlorosis, stunting, and leaf deformation. In addition, Cd toxicity in plants was found to cause a decrease in stomata density, respiratory and photosynthetic activities. (Rostami & Azhdarpoor 2019). High doses of Cd have been reported to affect chlorophyll biosynthesis by inhibiting protochlorophyllide reductase, which plays a role in chlorophyll biosynthesis and the synthesis of aminolaevulinic acid (Awan et al. 2020).

For this reason, four chlorophyll index measurements were performed at one-week intervals after Cd applications and the effects of increasing Cd doses on the chlorophyll of purslane were determined (Figure 3).

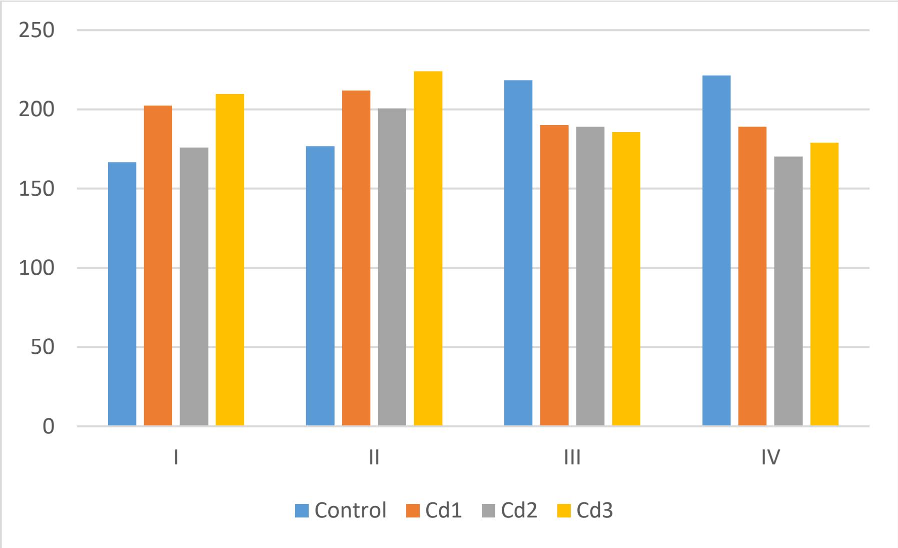

Figure 3- Chlorophyll index of purslane

No statistical difference was found in leaf chlorophyll contents when measured immediately after Cd applications (Table 4). In contrast, the effect of Cd doses on plant chlorophyll content was found statistically significant in the pre-harvest measurements. In the last two measurements, in particular, before harvest (04/06/2021 and 11/06/2021) the change in leaf chlorophyll content as a function of application doses is evident.

3.4. Bioaccumulating plant tendency of purslane

Copper and Ni hyperaccumulator plants can accumulate >1000 mg Cu and Ni kg^{-1} in their aboveground tissues (Baker et al. 2000; Sytar et al. 2021) while Cd hyperaccumulator plants can accumulate >100 mg Cd kg^{-1} in their aboveground tissues (Baker et al. 2000). In this study, >1000 mg Cu and Ni kg^{-1}, >100 mg Cd kg^{-1} were not determined in different parts of the purslane plant. However, Cu, Ni, and Cd accumulations were observed in different parts of purslane as a function of increasing Cu and Ni doses. A transfer factor greater than 1 is another important criterion for evaluating heavy metal hyperaccumulator plants (McGrath & Zhao 2003). In this study, the biological transfer coefficient (BTC) is also calculated to evaluate the transport of Cu, Ni, and Cd from the purslane root region to the leaves (Figure 4).

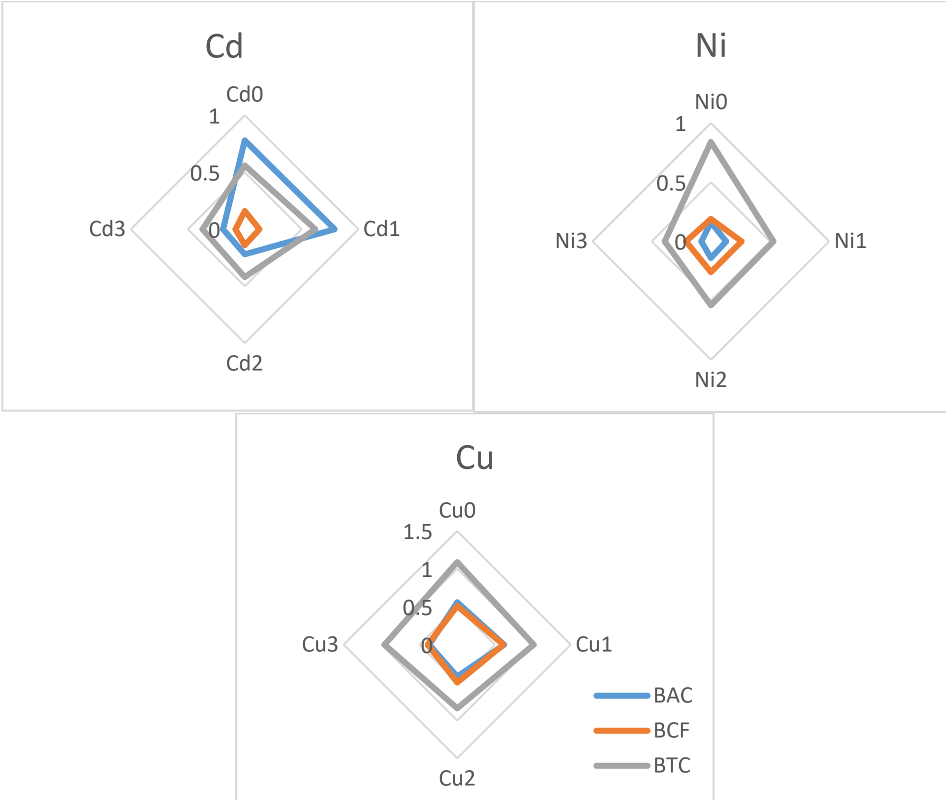

Figure 4- Effect of different doses of Cd, Ni, and Cu on the transfer coefficient of purslane

The Cu bio-transfer coefficient was determined in the range of 0.84-1.09. This coefficient, >1, is related to the Cu exposure of purslane. As the Cu content in the culture medium increases, the Cu content of purslane increases. However, the higher Cu accumulation in the purslane root region suggests that metal accumulation occurs mainly in plant roots because the transport of metals from root to shoot is slow compared to other elements. In this regard, Chandra et al. (2004) reported that many metals are higher in the roots than in the aboveground parts and that rhizofiltration affects the translocation and accumulation of metals in the root structure.

The bio-transfer coefficient for Ni was found in the range of 0.39-0.84 which is smaller than the references value (<1). Even though Ni content of the growing medium was high, the low uptake by the purslane (leaf+stem) plant shows Ni accumulation in the root zone. All of these results indicate safe consumption of the purslanes. The bio-transfer coefficient for the evaluation of Cd transport was found to be >1 for the Control and 5 mg Cd kg^{-1} treatments. The fact that this coefficient is >1 indicates that the availability of purslane is limited depending on the Cd load in the growth medium or that Cd accumulation occurs only in the root region. It is known that some elements compete with each other in plants, so their uptake and transport may change; metal ions enter plant cells via metal ion carriers or channels (Jabeen et al. 2009). Substances dissolved in water that pass through the endodermal layer in a symplastic manner, thus enter the xylem. The apoplastic transport of heavy metal ions into the xylem can occur at the root tip (Dalyan 2012; Manara et al. 2020). Microelements also use transmembrane carriers. and toxic heavy metals such as Cd compete for this transporter. Shrivastava et al, in their 2019 study, showed that Cu^{+2} and Zn^{+2} compete with Ni^{+2} and Cd^{+2} for the same transmembrane transporters (Shrivastava et al. 2019).

4. Conclusions

This study determined that the accumulation of heavy metals under consideration in different plant parts of purslane (*Portulaca oleracea*) changed with increasing Cu, Ni, and Cd doses, and that the accumulation of heavy metals was noticeably higher in the roots and leaves.

During the measurements to monitor the change in leaf chlorophyll content after the application of heavy metal, the results showed that the chlorophyll biosynthesis was disturbed, especially before harvest, and the chlorophyll content decreased accordingly.

The transfer coefficient was calculated to evaluate Cu transport from the purslane root zone to the leaves. In this respect, the transfer coefficient changed as a function of the dose increase (0.84-1.09). The fact that, this coefficient is > 1 indicates that purslane can absorb Cu when the Cu content in the medium in which the purslane grows increases.

The transportation of Ni to different parts of the purslane plant also changed in response to increasing doses, and the transfer coefficient changed in the range of 0.39-0.84 (< 1). The fact that this coefficient is <1 indicates low uptake of purslane even though the Ni content of the medium is high. This shows Ni accumulation in the roots which indicates that the above ground parts of purslane (leaf+stem) can be reliably consumed.

In relation to Cd transport, the transfer coefficient TK >1 at the Control and 5 mg Cd kg^{-1} treatments and TK<1 at the 10 and 20 mg kg^{-1}. The fact that this coefficient is >1 at the Control and 5 mg Cd kg^{-1} indicates that purslane takes up Cd to a certain concentration in the medium and that the accumulation capacity is limited with increasing Cd concentration, or that Cd accumulation occurs only in the root region.

It is concluded that purslane, which can be grown under natural conditions in the world and Turkey in general, and thus is a widely consumable plant, can accumulate Cu, Ni, and Cd in the environment up to a certain concentration, may be more in the roots. This is a very important result, especially in terms of public health. However, it is believed that heavy metal accumulation may change according to the genetic variation of purslane species. Therefore, it is recommended to study in detail the heavy metal accumulation ability of this commonly used plant, including different genetic variations.

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Improving Water Use Efficiency and Economic Benefits of Cropping System Through Intercropping in an Arid Climate

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ABSTRACT
The sustainable increase of total productivity by improving resources use efficiency in arid agricultural farming areas is crucial, and intercropping may be a good practice to be implemented in these arid regions. For this purpose, a three-year field experiment was conducted as a randomized complete block design (RCBD) with three replications at the research farm of the Agricultural and Natural Resources Research Station of Gonabad, Gonabad, Iran to assess the agronomic and economic indices of intercropping patterns. The experiment treatments included C_1: sole cotton, C_2: sole sesame, and intercropping ratios (C_3: 20:80, C_4: 40:60, C_5: 50:50, C_6: 60:40, and C_7: 80:20 cotton-sesame ratio). The results showed that the leaf chlorophyll content and leaf area index were significantly higher in the intercropped plants compared to the sole cropped plants. The yield components of both crops (such as branches per plant, capsules per plant, seeds per capsule, and 1000-seed weight for sesame, and opened bolls per plant, closed boll per plant, and seed cotton per boll for cotton) significantly improved under intercropping. However, the highest sesame seed yield (2703, 1979, and 1358 kg ha^{-1}, respectively) and seed cotton yield (3749, 2179, and 3426 kg ha^{-1}, respectively) in the three experiment years were observed in the sole cropping treatment. The implementation of intercropping significantly improved the water use efficiency of the cropping system, so that the highest values in the first to third year (0.67, 0.51, and 0.41 kg m^{-3}, respectively) were recorded in the C_4, C_3, and C_7 treatments. The intercropping evaluation indices revealed the advantage of intercropping compared to the sole cropping. The highest value of the land equivalent ration in the first year (1.28) belonged to the C_4 treatment, while in the second and third years, belonged to the C_7 treatment (1.40 and 1.10, respectively). The calculation of the aggressivity index revealed that in most of the intercropping patterns, especially in the first and second years, cotton showed greater competitive ability than sesame. The highest actual yield loss value in the first year (0.64) belonged to the C_3 treatment, while in the second and third years, belonged to the C_7 treatment (1.42 and 0.34, respectively). The highest economic advantage in terms of the monetary advantage index in the first year was obtained by the C_4 treatment (1140.5), and in the second and third years, was observed in the C_7 treatment (940.6 and 265.5, respectively). The intercropping advantage index in the three experiment years was highest (1.41, 3.38, and 0.80, respectively) for the C_7 treatment. Eventually, the results of this research show that cotton and sesame are able to adapt well to the intercropping and this cropping system can significantly improve the resources use efficiency (especially water and land) in an arid area enjoying greater economic benefit than sole cropping.

Keywords: Land equivalent ratio, Economic evaluation, Environmental resources, Plant competition, Yield

1. Introduction

Due to global population growth and the ever-increasing demand for food, fiber, and fuel, it is necessary to enhance agricultural production by decreasing the environmental footprint of farming systems. The “Green Revolution” has enhanced agricultural production over the last decades, along with reducing starvation and food poverty. However, such changes have been accompanied by hazards and risks to ecosystems and living organisms. These risks may include increased pressure on environmental resources, air and soil pollution, fluctuation in crop yields and unstable production, genetic erosion and loss of biodiversity, and disruption to agricultural productivity and sustainability. The main reasons for these challenges are the introduction of genetically modified varieties and monoculture, the application of large amounts of fertilizers and chemical pesticides, and the use of auxiliary inputs (Cassman 1999; Cook 2006; Weekley et al. 2012).

For these reasons, it is necessary to revise conventional farming, utilize traditional and indigenous knowledge, and use ecologically-oriented techniques in crop production. In the future, farmers will have to produce more agricultural products with fewer environmental resources (Schneider et al. 2011). Polyculture systems, particularly intercropping, as a crop diversification technique, is an example of this type of system that has long been in use (Liu 1994; Innis 1997). These systems have been exploited to optimize the utilization of environmental resources such as land, light, water, and nutrients by manipulating in-farm components and the interactions within the agroecosystem to ultimately improve crop productivity, stability, and sustainability of agroecosystems (Mushagalusa et al. 2008; Mao et al. 2012; Ning et al. 2017). Considering the arid and semi-arid climate of most areas of Iran and the vulnerability of agriculture to various environmental hazards, these types of agricultural systems have had a special place in Iran's traditional agriculture and have effectively helped farmers to adapt to the climate.

Despite polyculture and intercropping having a long history in Iran, less attention has been paid to these cropping systems, especially in the study area. The departure from the traditional agricultural operations to the modern ones in Iran, and the negligence of indigenous agricultural knowledge on the one hand, and the development of monoculture systems and excessive dependence on off-farm inputs, especially chemical fertilizers and pesticides, on the other hand, have all been considered as minatory factors in Iran's agriculture. Thus, the present study evaluates the agronomic and economic aspects of intercropping systems of two main field crops (i.e., cotton and sesame) cultivated in the northeast of Iran.

2. Material and Methods

2.1. The features of study area

The field experiments were conducted for three consecutive years (2016-2018) at the research farm of the Agricultural and Natural Resources Research Station of Gonabad, Khorasan Razavi province, Iran (57º45' N; 51º10' E; 1056 m a.s.l.). The area has a mean annual temperature of 11.5 °C and the total number of frost days during the cropping year is 33. Based on the Köppen climate classification, the climate of the area is arid, characterized by high temperatures during mid-spring to late summer, and low temperatures during mid-fall to late winter, with an average annual precipitation of 146 mm mostly concentrated in winter and early spring and a potential pan evaporation of 2021 mm (IRIMO 2018).

2.2. Experiment design and field management

A randomized complete block design with three replications was used with the following treatments: C_1: sole cotton, C_2: sole sesame, and intercrop patterns including C_3: 20:80, C_4: 40:60, C_5: 50:50, C_6: 60:40, and C_7: 80:20 cotton: sesame ratio, respectively.

Before implementing the design, the soil samples at the experimental field from the 0-30 cm soil depth were collected and transferred to the laboratory. Some physicochemical properties of the experimental soil are shown in Table 1. Cotton and sesame were sowed simultaneously in both intercropping and sole cropping patterns. The area of each plot was 30 m^2 (6 m × 5 m). The inter-row distance for each plant was 50 cm, and the inter-plant distance was 15 and 30 cm for sesame and cotton, respectively. Immediately after sowing, the plots were irrigated using the furrow irrigation method. To ensure uniform emergence, the second irrigation was performed three days after the first irrigation. Further irrigation was carried out according to the custom of the area at 8-day intervals. The irrigation water used was supplied from a ground water well located in the research farm. Some chemical properties of the irrigation water are presented in Table 2. To control weeds, two stages of hand weeding were conducted during each growing season. No specific pests or diseases were observed in the field during the study period.

Table 1- Physicochemical properties of the experimental soil

Soil texture	*Organic carbon*	*Total N*	*Available P*	*Available K*	*EC*	*pH*
-	%		mg kg^{-1}		dS m^{-1}	-
Sandy loam	0.18	0.045	18.8	271	1.54	7.7

Table 2- Chemical properties of the irrigation water applied

Ca+Mg	*Na*	*Cl*	*SAR*	*TDS*	*EC*	*pH*
meq L^{-1}			-	mg L^{-1}	dS m^{-1}	-
10.3	9.2	15.4	8.5	1856	2.5	7.5

2.3. Data collection and intercropping evaluations

The leaf chlorophyll content of the crops was recorded at the flowering stage using a SPAD chlorophyll meter (SPAD 502, Minolta Ltd., Japan). The leaf area of each crop was measured at the flowering stage using a leaf area meter (LI-3100C), and the leaf area index (LAI) was calculated as follows (Chimonyo et al. 2016):

$$\mathrm{LAI} = \frac{\mathrm{LA}}{\mathrm{A}}$$

Where: LA is the plant leaf area (m^2), and A is the land area occupied by the plant (m^2). Five randomly selected plants were used to record leaf chlorophyll content and leaf area index.

The yield components for each crop were measured using ten randomly selected plants in each plot. The seed cotton of fully opened and matured bolls in the four central rows of each plot was hand-harvested two times each year, and was then weighted and calculated as kg ha^{-1}. The lint percentage was measured after ginning the cotton bolls.

When the sesame plants were yellowish, and their capsules had not cracked, the plants in the four central rows were harvested and air-dried. Then, the seeds were separated from the capsules, weighted, and converted into seed yield (kg ha^{-1}).

The total volume of water used for irrigation was measured by a volumetric water meter connected to irrigation pipes so that the total amounts of water used during the first to third experiment years were 4700, 4050, and 4100 m^3 ha^{-1} for sesame, and 8400, 7750, and 7900 m^3 ha^{-1} for cotton, respectively. The water use efficiency (WUE) of both crops was calculated using the following formula (Kang et al. 2000):

$$\mathrm{WUE} = \frac{\mathrm{EY}}{\mathrm{IW}}$$

Where: EY is the economic yield (kg ha^{-1}), and IW is the irrigation water used (m^3 ha^{-1}).

The agronomic and economic indices used for the comparison between the intercropping and sole cropping systems are listed in Table 3.

Table 3- List of agronomic and economic indices used to assess the cotton-sesame intercropping

Index	*Formula*	*Reference*
Agronomic indices		
Land equivalent ratio (LER)	$\mathrm{LER} = \mathrm{LER_{cot}} + \mathrm{LER_{ses}} = \frac{Y_{\mathrm{int,cot}}}{Y_{sole,\mathrm{cot}}} + \frac{Y_{\mathrm{int,ses}}}{Y_{sole,\mathrm{ses}}}$	Willey & Rao (1980)
Aggressivity (A)	$A_{\mathrm{cot/ses}} = \frac{Y_{\mathrm{int,cot}}}{Y_{sole,\mathrm{cot}} \times F_{\mathrm{cot}}} - \frac{Y_{\mathrm{int,ses}}}{Y_{sole,\mathrm{ses}} \times F_{\mathrm{ses}}}$	Banik et al. (2000)
Actual yield loss (AYL)	$\mathrm{AYL} = \mathrm{AYL_{cot}} + \mathrm{AYL_{ses}}$ $\mathrm{AYL_{cot}} = \left[\frac{Y_{\mathrm{int,cot}}}{F_{\mathrm{int,cot}}} \Big/ \frac{Y_{sole,\mathrm{cot}}}{F_{sole,\mathrm{cot}}}\right] - 1$ $\mathrm{AYL_{ses}} = \left[\frac{Y_{\mathrm{int,ses}}}{F_{\mathrm{int,ses}}} \Big/ \frac{Y_{\mathrm{sole,ses}}}{F_{sole,\mathrm{ses}}}\right] - 1$	Banik (1996)
Economic indices		
Monetary advantage index (MAI)	$\mathrm{MAI} = \left[(Y_{\mathrm{int,cot}} \times P_{\mathrm{cot}}) + (Y_{\mathrm{int,ses}} \times P_{\mathrm{ses}})\right] \times \left[\frac{\mathrm{LER}-1}{\mathrm{LER}}\right]$	Ghosh (2004)
Intercropping advantage (IA)	$\mathrm{IA} = \mathrm{IA_{cot}} + \mathrm{IA_{ses}}$ $\mathrm{IA_{cot}} = \mathrm{AYL_{cot}} \times P_{\mathrm{cot}}$ $\mathrm{IA_{ses}} = \mathrm{AYL_{ses}} \times P_{\mathrm{ses}}$	Banik et al. (2000)

$Y_{int,cot}$ and $Y_{int,ses}$ represent the yields of cotton and sesame under intercropping, while, $Y_{sole,cot}$ and $Y_{sole,ses}$ express the respective yields under sole cropping, respectively; F_{cot} and F_{ses} are the plant proportion (%) of cotton to sesame and of sesame to cotton in the intercropping, respectively; P_{cot} and P_{ses} represent the commercial value of cotton and sesame, respectively

2.4. Statistical analysis

The data were analyzed using the analysis of variance (ANOVA) in the SAS statistical package version 9.1. The means differences were identified using the least significant difference multiple range tests (LSD) at a 5% significance level.

3. Results and Discussion

3.1. Sesame

The analysis of variance for the data from the three experiment years indicated significant differences in the traits of sesame plants grown under sole and intercropping systems. The data in Table 4 clearly show that the plants grown under the intercropping system had higher chlorophyll content than the sole-cropped plants. The highest value (47.0, 42.5, and 56.1 for the first to third experiment year, respectively) was obtained from the C_5 treatment (50:50 cotton-sesame intercropping). Weisany et al. (2015) found that growing crops under an intercropping system led to an increase in the chlorophyll content by improving nutrient availability.

The leaf area index (LAI) of the sesame plants was significantly influenced by the cropping systems. As shown in Table 4, variations in the response of the LAI to the sole and intercropping patterns were observed during the three years of the experiment, so that the highest value in the first year (4.2) belonged to the sole sesame, while in the second and third years, the C_6 treatment (60:40 cotton-sesame intercropping) resulted in the highest value (3.1 and 2.7, respectively).

The results of our experiment showed that in the three experiment years, the height of the sesame grown under sole cropping (89.9, 116.3, and 111.3 cm, respectively) or 20:80 cotton-sesame intercropping pattern (96.7, 114.7, and 109.7 cm, respectively) was higher than plants grown under other cropping systems. The height of the sesame plants increased depending on its ratio in the mixture (Table 4). The increased height of plants in the sole sesame treatment, as well as the intercropping pattern with high sesame proportion maybe due to intra-specific competition between individual plants for light. Since light does not reach the lower layer of the crop canopy, auxin does not decompose, and thus its increased concentration leads to stem elongation (Cruz & Sinoquet 2003). Basaran et al. (2017) found a plant height increase compared to sole cropping in sorghum-sudangrass hybrid intercropped with legumes.

The number of lateral branches of sesame in the experimental years (with the exception of the second year) showed a significant response to the cropping systems. The values of this trait were higher in intercropping treatments and especially in patterns with a lower proportion of sesame rather than cotton. The highest value for the three experiment years (44.3, 32.7, and 44.9, respectively) belonged to the C_7 treatment (Table 4). In addition, the intercropping of sesame with cotton significantly enhanced the number of capsules per plant so that the highest values for the three experiment years (167.4, 183.4, and 172.1, respectively) belonged to the C_7 treatment, followed by the C_6 treatment (Table 4). The ANOVA results showed that the effects of the experimental treatments on the number of seeds per capsule in the first and second years were significant, but were not significant in the third year. The best results for the three years (63.5, 69.0, and 68.0, respectively) were obtained from the C_7 treatment, followed by the C_6 treatment (Table 4). The improved sesame traits under these intercropping systems may be due to the different niches being occupied by the intercrop components, especially root distribution and aboveground architecture, which tend to enhance the use of available environmental resources (e.g., water, nutrients, and solar radiation) through complementary relationships, ultimately increasing crop productivity and resource use efficiency (Li et al. 2011; Lithourgidis et al. 2011).

As presented in Table 4, the cropping systems had no significant effect on the 1000-seed weight of sesame. Similarly, de Araújo et al. (2013) reported no significant difference between the sole and intercropping systems of sesame and cowpea for this trait.

Table 4- Physiological properties and yield components of sesame under sole and intercropping systems

Treatments / Year	*Branch per plant*			*Capsule per plant*			*Seed per capsule*			*1000-seed wt. (g)*		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
C_2	39.6bc	31.7	33.9c	152.7c	168.1c	153.9b	62.2ab	62.3c	57.9	3.1	3.3	4.0
C_3	37.0c	28.0	36.0bc	147.9c	162.3c	156.0b	55.9c	62.4c	60.5	3.1	3.3	3.8
C_4	45.4a	31.3	39.3abc	152.9c	169.2bc	164.0ab	58.0bc	64.0bc	61.7	3.1	3.3	4.1
C_5	43.1ab	31.7	43.3ab	157.2bc	173.0abc	163.7ab	58.5bc	65.5bc	63.2	3.0	3.4	3.6
C_6	44.9a	32.0	44.4a	163.8ab	179.7ab	171.0a	66.7a	68.1ab	59.3	3.2	3.3	4.2
C_7	44.3a	32.7	44.9a	167.4a	183.4a	172.1a	63.5a	69.0a	68.0	2.9	3.4	3.8
LSD 5%	4.737**	5.077ns	8.109*	10.254*	10.922**	11.422*	4.616**	4.182**	11.789ns	0.354ns	0.436ns	1.280ns

Table 4 (Continue)- Physiological properties and yield components of sesame under sole and intercropping systems

Treatments / *Year*	*Chlorophyll content (SPAD value)*			*LAI*			*Plant Height (cm)*		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
C_2	42.9	37.5b	45.2b	4.2a	2.56bc	1.7b	89.9a	116.3	111.3a
C_3	44.1	37.2b	44.1b	3.0bc	3.0ab	2.0ab	96.7a	114.7	109.7a
C_4	42.3	39.8ab	55.5a	2.8bc	3.0ab	1.7b	86.5ab	114.1	107.3ab
C_5	47.0	42.5a	56.1a	2.4c	2.9ab	2.7a	76.3b	113.0	94.3c
C_6	45.8	39.4ab	52.8a	3.7ab	3.1a	2.7a	84.5ab	113.1	99.0bc
C_7	44.8	41.3a	55.9a	3.3abc	2.3c	2.0ab	75.8b	113.0	108.0ab
LSD 5%	7.554ns	3.639*	3.979***	1.067*	0.477*	0.753*	13.44*	4.962ns	10.417*

*, **, ***: and ns indicate statistical differences at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and non-significant, respectively. The columns with the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range tests. C_2 to C_7 will be used for sole sesame, 20:80, 40:60, 50:50, 60:40, and 80:20 cotton-sesame intercropping treatments, respectively

Sesame seed yield showed a significant difference under the influence of experimental treatments. The highest seed yield was obtained when the plants were grown under sole cropping. The sesame seed yield decreased depending on its ratio in the mixture, so that its value in the C_7 treatment was 67.0%, 57.5%, and 77.5% lower than that of the sole crop in the three years of the experiment, respectively (Figure 1). This was to be expected because the density of sesame in the sole cropping pattern was the highest, and with the reduction of the proportion of sesame in intercropping, the density of the plant per unit area also decreased. Similar to these findings, Khan et al. (2017) reported a reduction in the seed yield of sesame under the intercropping system compared to monocropping in sesame-groundnut intercrop.

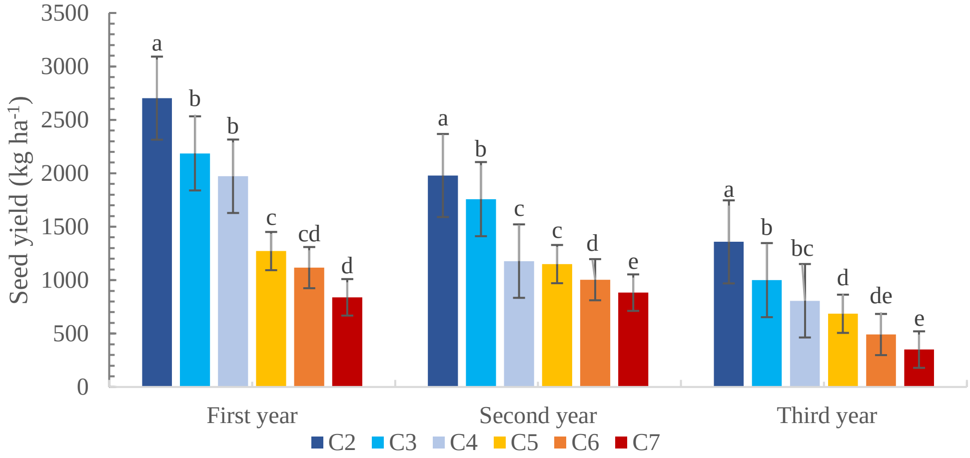

Figure 1- Sesame seed yield sesame under sole and intercropping systems
The same letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple-range tests

3.2. Cotton

The analysis of variance of the experimental data revealed that the cotton traits were significantly affected by the cropping system treatments. As presented in Table 5, the leaf chlorophyll content was lower in the plants grown under the sole cropping system in all the experiment years. However, the difference between the treatments was not significant in the first year, whereas, the SPAD values were higher in treatments with a lower proportion of cotton than sesame, especially in the C_3 treatment (51.2, 38.5, and 44.1 for the experiment years, respectively) (Table 5). This result may be due to an increased nutrient availability, uptake, and mobility in intercropping systems, which can lead to enhanced chlorophyll synthesis in leaves (Liu et al. 2014). Another reason for the higher chlorophyll content in the intercropped cotton plants compared to sole crops can be the difference in the canopy structure (spatial niche differentiation), which causes more light absorption, or in other words, increases the light use efficiency (Wang et al. 2021), and ultimately enhances the synthesis of chlorophyll (Nasar et al. 2022).

Table 5- Physiological properties and yield components of cotton under sole and intercropping systems

Treatments / *Year*	*Chlorophyll content (SPAD value)*			*LAI*			*Plant Height (cm)*		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
C_2	42.9	37.5b	45.2b	4.2a	2.56bc	1.7b	89.9a	116.3	111.3a
C_3	44.1	37.2b	44.1b	3.0bc	3.0ab	2.0ab	96.7a	114.7	109.7a
C_4	42.3	39.8ab	55.5a	2.8bc	3.0ab	1.7b	86.5ab	114.1	107.3ab
C_5	47.0	42.5a	56.1a	2.4c	2.9ab	2.7a	76.3b	113.0	94.3c
C_6	45.8	39.4ab	52.8a	3.7ab	3.1a	2.7a	84.5ab	113.1	99.0bc
C_7	44.8	41.3a	55.9a	3.3abc	2.3c	2.0ab	75.8b	113.0	108.0ab
LSD 5%	7.554ns	3.639*	3.979***	1.067*	0.477*	0.753*	13.44*	4.962ns	10.417*

Table 5 (Continue)- Physiological properties and yield components of cotton under sole and intercropping systems

Treatments / *Year*	*Chlorophyll content (SPAD value)*			*LAI*			*Earliness (%)*			*Plant Height (cm)*		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
C_1	51.2	38.5^{ab}	44.1^{bc}	3.4^{ab}	2.3^{b}	3.3^{ab}	85.6	66.6^{a}	63.7^{ab}	64.2^{a}	68.7^{a}	108.3^{a}
C_3	59.5	40.2^{a}	52.0^{a}	3.6^{a}	2.4^{ab}	3.6^{a}	83.3	57.3^{b}	55.4^{b}	46.6^{c}	41.7^{d}	$10.1.3^{a}$
C_4	59.5	38.8^{a}	49.4^{ab}	3.3^{ab}	2.7^{a}	3.4^{a}	81.1	57.6^{b}	55.6^{b}	47.4^{bc}	42.3^{cd}	98.7^{ab}
C_5	50.8	34.4^{cd}	48.6^{abc}	2.9^{bc}	2.3^{b}	2.9^{bc}	86.3	60.9^{ab}	60.3^{b}	44.9^{c}	45.3^{c}	88.3^{b}
C_6	53.1	31.6^{d}	$48.^{abc}$	3.1^{abc}	1.9^{c}	2.8^{c}	86.8	65.1^{a}	61.7^{b}	60.9^{ab}	49.7^{b}	98.0^{ab}
C_7	54.4	35.3^{bc}	43.3^{abc}	2.7^{c}	2.5^{ab}	2.8^{c}	85.9	61.1^{ab}	72.7^{a}	57.1^{abc}	66.3^{a}	101.7^{a}
LSD 5%	11.0^{ns}	3.448^{***}	5.743^{*}	0.578^{*}	0.343^{**}	0.471^{**}	7.049^{ns}	6.147^{*}	10.828^{*}	13.982^{*}	3.044^{***}	11.223^{*}

*, **, ***: and ns indicate statistical differences at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and non-significant, respectively. The columns with the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range tests. C_1 to C_7 will be used for sole cotton, 20:80, 40:60, 50:50, 60:40, and 80:20 cotton-sesame intercropping treatments, respectively

According to the results (Table 5), the leaf area index (LAI) was also higher in intercropped cottons than in sole-cropped ones. The C_3 treatment in the first and third year of the experiment resulted in the highest LAI (3.6 and 3.6, respectively), while in the second year, the C_4 treatment achieved the highest value (2.7).

The effect of the experimental treatments on the earliness of cotton in the first experiment year was not significant, while its effect in the second and third years was significant (Table 5). The C_1 and C_6 treatments in the second year resulted in the highest values of this trait (66.6 and 65.1%, respectively), while in the third year, the C_7 treatment showed the highest value (72.7%).

The cotton height varied significantly under the influence of cropping systems. Similar to sesame, the cotton plants grown under sole cropping had the highest value (64.2, 68.7, and 108.3 cm, for the first to third year, respectively), followed by the C_7 treatment. The height of the cotton increased depending on its ratio in the mixture (Table 5). These results are in agreement with the findings of Iqbal et al. (2007), who reported that the plant height of cotton under sole cropping and intercropped with sesame in high cotton density was significantly higher than cotton plants intercropped in low density. Taranenko et al. (2021) determined that the shading effect of the intercrop component with higher density and height is the main reason for plant height reduction of another crop component in intercrop.

The data presented in Table 5 indicate that the number of opened bolls per plant had the highest value in the three experiment years (10.9, 9.2, and 11.0, respectively) as affected by the C_3 treatment, followed by the C_4 treatment. The highest number of closed bolls per plant in the first year (1.13) was obtained by implementing the C_4 treatment, while in the second and third years, the highest values (1.53 and 2.13, respectively) were obtained as a result of the C_3 treatment (Table 5). These findings reveal the above and under-ground interference effects of cropping systems and that inter-specific competition between the two species was less than intra-specific competition between cotton plants in 20:80 and 40:60 mixtures compared to sole cotton and other intercropping systems (Hadejia 2011).

The seed cotton weight per boll in the first year did not show a significant variation under the cropping systems. While in the second and third years, the difference between the treatments was significant. Table 5 shows that in the second and third years, the highest seed cotton weight per boll (3.98 and 5.25 g, respectively) was observed due to the C_3 treatment.

Figure 2 shows that the seed cotton yield achieved in sole cropping (3749, 2179, and 3426 kg ha^{-1} for the first to third year, respectively) was significantly higher compared to the intercropped cotton; the C_7 treatment had the highest seed cotton yield (2703, 1980, and 2641 kg ha^{-1}, respectively) among the intercropping patterns. As the proportion of cotton decreased in the intercrop, the seed cotton yield decreased gradually. The main reason for the decrease in crop yield under intercropping compared to sole cropping was the decrease in plant density in the intercropping patterns. Other researchers have reported a reduction in crop yield under intercropping compared to sole cropping. However, the significantly lower seed cotton yield in intercropping should not overshadow the advantages of intercropping systems in total yield (Jahansooz et al. 2007).

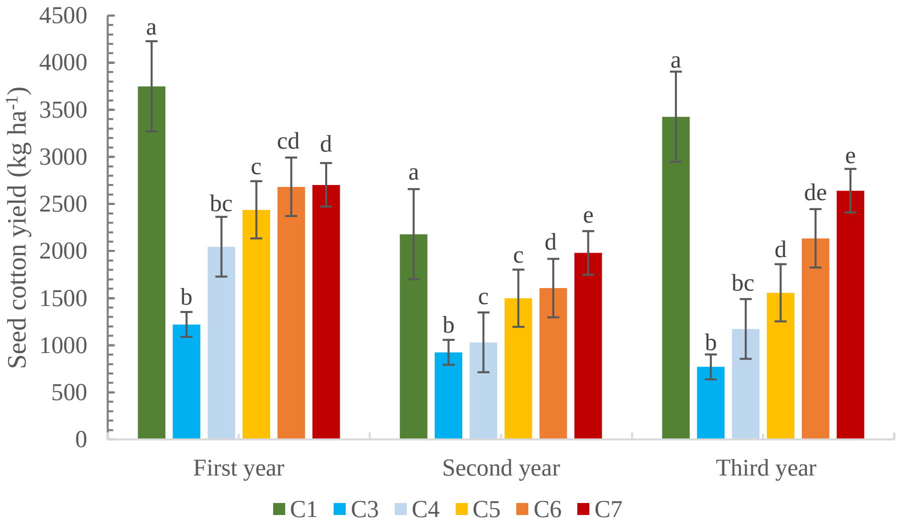

Figure 2-Seed cotton yield under sole and intercropping systems
The same letters are not significantly different at P≤0.05, according to Duncan's multiple-range tests

Based on the results (Figure 3), the lint percentage significantly increased when cotton plants were intercropped compared to those sole-cropped. The highest lint percentage in the three years of the experiment (39.2, 39.6, and 40.7%, respectively) was recorded in the C_3 treatment, while the lowest value was observed in the sole cotton (36.5, 36.0, and 36.0%, respectively). Other researchers (Wang et al. 2021) have reported an improvement in lint percentage in intercropped over sole-cropped cotton

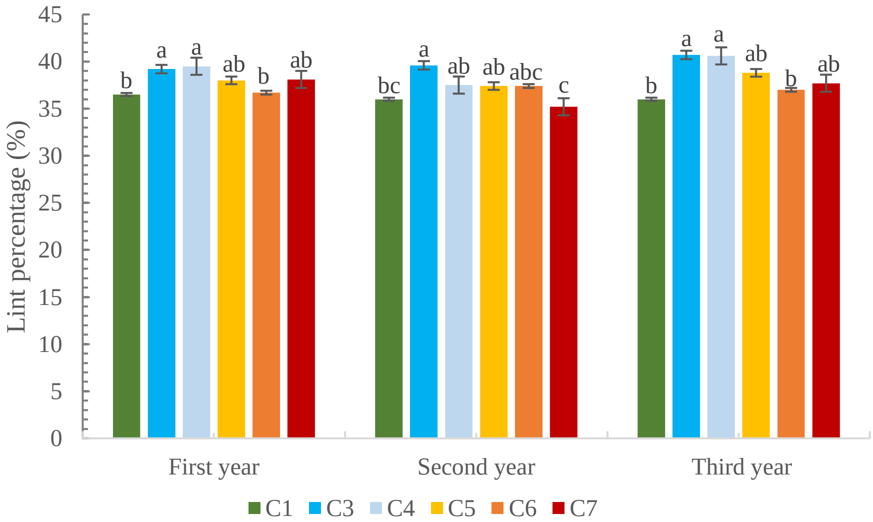

Figure 3- Cotton lint percentage under sole and intercropping systems
The same letters are not significantly different at P≤0.05, according to Duncan's multiple-range tests

3.3. *Water use efficiency (WUE)*

Figure 4 presents a comparison of means between treatments in terms of water use efficiency (WUE). Based on the results, the implementation of intercropping significantly increased the WUE of the cropping system. However, in the three experiment years, different results were obtained in terms of the highest value of WUE, and the C_4, C_3, and C_7 treatments achieved the highest value (0.67, 0.51, and 0.41 kg m^{-3}, respectively) from the first to the third year of the experiment, respectively. The different influences of intercropping systems on WUE may be due to environmental and climatic conditions, soil characteristics, and crop characteristics (Xu et al. 2008). Some researchers have noted that when component crops are properly selected in intercropping, improvements in resource use efficiency can be achieved (Dong et al. 2018). Consequently, the physiological factors at the field level, such as community structure and diversity (Above-ground and below-ground biomass), which have led to better use of resources, especially water and light, can be considered important reasons for increasing the water use efficiency of intercropping systems (de Barros et al. 2007; Li et al. 2020). Differences in the temporal and spatial water requirement of each intercrop component during the growing season may be another reason for the high WUE of intercropping (Bai et al. 2016). The mean comparison also showed that the lowest value in the first and second years was obtained from the sole-cropped cotton (0.45 and 0.27 kg m^{-3}, respectively), while in the third year, the lowest value was observed in the sole-cropped sesame (0.30 kg m^{-3}). The main reason for this result can be attributed to the significantly low yield of sesame in the third year of the experiment.

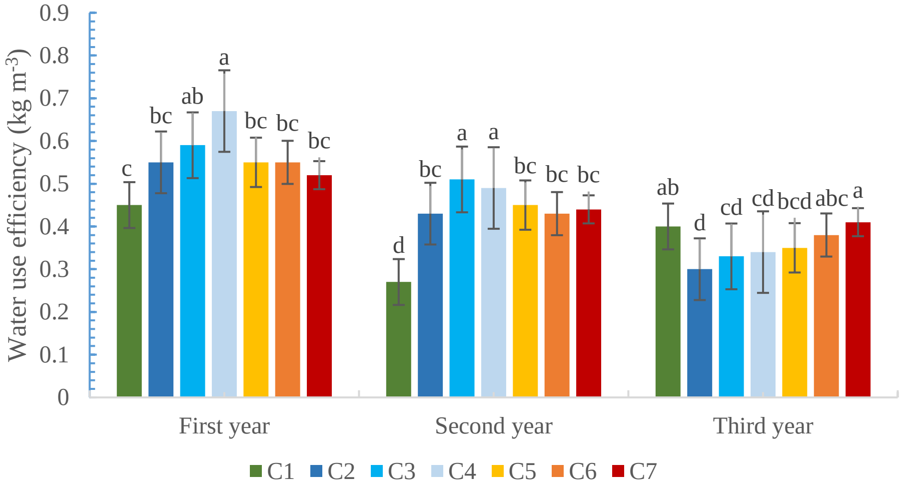

Figure 4- Water use efficiency of the cropping systems under sole and intercropping systems
The same letters are not significantly different at P≤0.05, according to Duncan's multiple-range tests

3.4. *Land equivalent ratio (LER)*

The most important index used to compare intercropping systems with sole cropping is the land equivalent ratio (LER). Calculating the partial LER for sesame indicated that the best result for the three experiment years (0.81, 0.89, and 0.74, respectively) was obtained from the C_3 treatment, while the C_7 treatment showed the highest LER for cotton (0.78, 0.95, and 0.84, respectively). The LER for each crop decreased depending on its ratio in the mixture, so the lowest LER values for sesame and cotton belonged to the 80:20 and 20:80 cotton-sesame intercrop, respectively (Table 6). According to the results (Table 6), the total LER values of all the intercropping patterns were greater than 1 (except for the C_4 treatment in the third year with a value of 0.96), revealing that implementing intercropping systems resulted in higher yield per unit area compared to sole cropping. In other words, the productivity and efficiency of the intercropping systems were higher than the sole cropping systems (Živanov et al. 2018). Table 6 shows that the LER values for different cropping patterns were almost equal, revealing that crop yield reduction of each intercrop component was compensated by another component, ultimately contributing to the constant increase of the LER. Improvements in the LER under intercropping compared to sole cropping systems have also been reported by other studies (Nandini & Chellamuthu 2004; Reddy & Mohammad 2009; Velmurugan & Ravinder 2012; Yilmaz et al. 2015; Ibrahim & Acikalin 2020). This productivity improvement maybe due to decreased competitiveness and spatially and temporally complimentary use of environmental resources such as light, water, and nutrients (Willey 1990). However, contrary to our findings, Momirović et al. (2015) reported LER values below 1 and no improvements in the land use efficiency of maize-pumpkin intercrops compared with sole crops.

Table 6- Land equivalent ratio (LER) of cotton-sesame intercropping systems

Treatments / *Year*	*Cotton*			*Sesame*			*Total*		
	1st	*2nd*	*3rd*	*1st*	*2nd*	*3rd*	*1st*	*2nd*	*3rd*
C_3	0.33	0.44	0.28	0.81	0.89	0.74	1.14	1.31	1.02
C_4	0.55	0.50	0.37	0.73	0.60	0.59	1.28	1.10	0.96
C_5	0.65	0.72	0.49	0.47	0.58	0.54	1.12	1.30	1.03
C_6	0.72	0.77	0.68	0.41	0.51	0.36	1.13	1.28	1.04
C_7	0.78	0.95	0.84	0.31	0.45	0.26	1.09	1.40	1.10

3.5. *Aggressivity (A)*

A positive aggressivity index for a species indicates its higher aggressivity and dominance over other species, while a negative index indicates the aggressivity of other species. An aggressivity value of zero indicates equilibrium between interspecific and intraspecific competition, leading to non-dominance between species (Ghosh 2004). Table 7 shows that in most intercropping systems, cotton was dominant over sesame, and this dominance was higher in the intercrop patterns with lower cotton proportions. The highest aggressivity value during the experiment years (0.62, 1.1, and 0.46, respectively) was found in the C_3 treatment, while the C_7 treatment showed negative values indicating sesame dominance over cotton. The aggressivity of cotton reflects the plant's ability for better and more efficient use of environmental resources, especially soil nutrients and light (Matusso et al. 2014; Rostaei et al. 2018).

Table 7- Cotton aggressivity (A) and actual yield loss (AYL) of cotton-sesame intercropping systems

Treatments / Year	A			AYL								
	Cotton			Cotton			Sesame			Total		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
C_3	0.62	1.11	0.46	0.63	1.22	0.38	0.01	0.11	-0.08	0.64	1.33	0.30
C_4	0.14	0.25	-0.06	0.36	0.24	-0.07	0.22	-0.01	-0.01	0.58	0.23	-0.08
C_5	0.36	0.28	-0.02	0.30	0.44	-0.01	-0.06	0.16	0.01	0.24	0.60	0.00
C_6	0.16	0.02	0.23	0.19	0.29	0.13	0.03	0.27	-0.09	0.22	0.56	0.03
C_7	-0.58	-1.04	-0.24	-0.03	0.19	0.05	0.55	1.23	0.29	0.52	1.42	0.34

3.6. Actual yield loss (AYL)

Actual yield loss is used to evaluate each species in intercropping, indicating the importance of intra-specific and inter-specific competition, and the behavior of component crops in a cropping system. AYL represents a decrease in the actual yield of any intercrop component compared to the sole crop based on crop proportion. AYL provides more comprehensive information on crop competition relative to other intercropping indices (Banik et al. 2000). The data in Table 7 show that this index was positive for both crops in most of the intercropping systems, particularly for cotton, indicating the effect of intercropping on increasing actual yield. The highest values for cotton and sesame during the first to third years were observed in the C_3 (0.63, 1.22, and 0.38, respectively) and C_7 treatment (0.55, 1.23, and 0.29, respectively). According to the average of the data obtained from our three-year experiment (Table 7), all the intercropping systems (except for the C_4 treatment in the third year) achieved positive total AYL values, revealing lower inter-specific competition than intra-specific competition as well as better adaptability of both crops under intercropping. Our results also showed that increased biodiversity in the intercropping system led to enhanced resource use efficiency compared to sole cropping (Ghosh et al. 2006). The advantage of intercropping compared to sole cropping in this experiment may also be based on the "Competitive Production Principle", revealing the possibility of better use of environmental resources through utilizing different intercropping components with different morphology, physiology, and ecology (Vandermeer 1990).

3.7. *Monetary advantage index (MAI)*

A positive MAI indicates a definite economic advantage for intercropping while negative values show a disadvantage for an intercropping system. This index was positive in all the intercropping systems (except for the C_4 treatment in the third year), which showed an economic advantage under intercropping compared to sole cropping, implying the general suitability of this polyculture production system due to the efficient use of environmental resources and the higher total crop yield achieved by intercropping. Our results show that the most profitable mixture in the first experiment year (1140.5) was the 40:60 cotton-sesame intercrop, and for the second and third years (940.6 and 265.5, respectively), it was found in the 80:20 mixture (Table 8). The higher monetary advantage in intercropping systems can be due to the higher production efficiency and crop value (Verma et al. 2013). Alabi & Esobhawan (2006) evaluated economic indices of maize-cotton intercrops and reported a 10% economic advantage for intercropping over sole cropping. The authors of that study believed that this economic advantage maybe the reason why farmers continue to grow these crops together.

Table 8- Monetary advantage index (MAI) and intercropping advantage (IA) of cotton-sesame intercropping systems

Treatments / Year	MAI			IA		
	1st	2nd	3rd	1st	2nd	3rd
C_3	594.0	908.4	45.8	0.59	1.39	0.13
C_4	1140.5	267.5	-96.3	0.90	0.19	0.09
C_5	468.2	765.0	71.8	0.11	0.82	0.01
C_6	497.4	691.9	103.3	0.25	0.96	-0.15
C_7	335.5	940.6	265.5	1.41	3.38	0.80

3.8. Intercropping advantage (IA)

Another index that shows the economic feasibility of intercropping is Intercropping advantage (IA). Positive values indicate the economic advantage of intercropping, and negative values indicate the disadvantage of intercropping over sole cropping (Banik et al. 2000). As shown in Table 8, all the intercropping treatments (except for the C_6 treatment in the third year) showed positive values, indicating the economic advantage of intercropping compared to sole cropping. The highest values for the three years of the experiment (1.41, 3.38, and 0.80, respectively) were obtained through the implementation of the 80:20 cotton-sesame pattern. The results of a 2022 study (Wang et al. 2022) conducted on the economic evaluation of intercropping of cotton with peanuts showed that intercropping reduced costs and increased resource use efficiency and finally increased the farm's net income. The authors stated that the intercropping system could not only increase the crop yield per unit area but also provide notable economic benefits, which increase farmers' tendency to favor implementing intercropping rather than sole cropping in cotton cultivation (Wang et al. 2022).

4. Conclusions

Based on our findings, intercropping led to improvements in most of the growth indices (LAI and plant height) and yield components of sesame (branch per plant, capsule per plant, seed per capsule) and cotton (opened and closed boll, seed cotton per boll). The main reason for these results may be lowered plant density and decreased intraspecific competition between individual plants. While crop yields of sesame and cotton were significantly higher in sole cropping due to the harvest of more plants per unit area, the advantage indexes for intercropping highlighted its profitability. All the intercropping patterns achieved the LER values above 1, revealing high land use efficiency and the agronomic advantage of these cropping systems. In most treatments, cotton was dominant over sesame due to its high aggressivity, which represents the competitive ability and resource use efficiency of cotton compared to sesame. The AYL index showed positive values, revealing the yield advantage of intercropping compared to sole cropping. An evaluation of the economic advantage indices of intercropping also demonstrated that in most of the intercropping patterns during the three experiment years, the MAI and IA index showed positive values, which indicate the economic preference for intercropping over sole cropping.

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Assessment of Medicinal and Aromatic Plants' Contribution to the Country's Economy by Hybrid Multi-Criteria Decision-Making Approach: The Case of Turkey

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ABSTRACT
Medicinal and aromatic plants contribute to both the national economy and the well- being of forest villagers. In addition, these plants are generally used in a variety of industries such as medicine, food, paint, and perfumery. It is gaining popularity, and individuals are turning to herbal therapy as an alternative medical treatment. These medicinal plants can be popularised and utilised to boost the economies of medicinal-growing countries and provide livelihoods for local stakeholders. Furthermore, overexploitation of these therapeutic plants should be restricted, important species with high marketing potential should be protected, and cultivation of these plants should be encouraged for future use. Against this background, this study employs a hybrid multi-criteria decision-making technique to identify the medicinal and aromatic plants that contribute the most to the Turkish economy. The medicinal and aromatic plants that contributed the most to the country's economy were found to be poppy seed, thyme, and laurel leaves. Moreover, the seed (sesame seed, flax seed, safflower seed) plants are determined as the least contributing medicinal and aromatic plants to the country's economy. Cumin, anise, and salvia have also potential in terms of economics. Hence, these findings can help the farmers and decision-makers to determine which plants are more economically beneficial. Thus, plants with high economic value can be cultivated and exported to the rest of the world.

Keywords: TOPSIS, Entropy, Medicinal and aromatic plants

1. Introduction

Non-wood forest products are defined as all kinds of herbal or animal products other than wood raw materials obtained from forests. Medicinal and aromatic plants account for the majority of non-wood forest products worldwide, including in Turkey (Kurt et al. 2016).

Mostly medicinal and aromatic plants are used to preserve human health or to cure and prevent disease. They are also utilised in nutritional supplements, herbal tea, flavour, spice, skin care, fragrance, and cosmetics (Anonymous 2005; Faydaoğlu & Sürücüoğlu 2011).

The history of medicinal and aromatic plants is as old as human history itself. The oldest book of medicinal and aromatic plants was written by Chinese Emperor Shen Nung in 3700 BC. More than 200 plants are mentioned in the book (Temel et al. 2018). For instance, the Sumerian clay medications engraved with cuneiform including agricultural and medicinal knowledge, which are roughly 5000 years old, are among the oldest recorded writings concerning the usage of plants. It comprised 12 recipes for drug preparation using variety of over 250 plants (Petrovska 2012). The Ebers papyrus, which is supposed to have been written in 1500 B.C., is the oldest and most important written resources about the medicinal and aromatic plants, including over 870 prescriptions and formulae and 700 medicinal herbs (Okigbo et al. 2009). In addition, Hippocrates, the founder of Greek medicine, and Aristotle, his student, used medicinal plants to treat diseases (Jamshidi-Kia et al. 2018).

The oldest information about these plants in Turkey can be traced to; the 11th century when IbniSina mentioned various medical substances in the second volume of his book titled ' Canon of Medicine ' (Nasser et al. 2009). Ibn al-Baytar (1197-1248) authored a work titled 'Compendium of Simple Drugs and Food'. In this study, more than 1400 medical drugs were defined (Abu-Rabia 2005).

Today, the use of plants for therapeutic purposes varies according to the development level of the countries. While 80% of the population in developing countries uses herbal products therapeutically, the rate of its use for this purpose is lower in developed countries. In some countries in Asia, Africa, Latin America and the Middle East, the rate of herbal product use is above 85%. In addition, currently, it is estimated that over half of pharmaceutical drugs are derived from medicinal plants (Acıbuca & Budak 2018; Jamshidi-Kia et al. 2018). According to FAO, about 30% of all drugs sold worldwide contain compounds derived from plant material (FAO 2005).

There are a total number of 422000 plant species in the world. 72000 of these are medicinal and aromatic plant species and the highest number of these are in China with 4941, followed by India, USA, Vietnam, Thailand, Pakistan and Malaysia (Schippman et al. 2006). In Europe, over 2000 medicinal and aromatic plants are employed for different purposes. Albania, Bulgaria, Poland, Hungary and Turkey are leading suppliers of medicinal and aromatic plants to European nations (Güney 2019). Today, medicinal plants are estimated to have an annual market value of approximately $60 billion (Faydaoğlu & Sürücüoğlu 2011).

Turkey is very abundant in flora, with approximately 12000 plant species. Over 25% of these are aromatic plants and about 8% of plant species are used in medicine purpose (Baser 2002). Approximately 400 of these plant species in Turkey are exported (Karık & Tunçtürk 2019).

Examining the foreign trade performance of medicinal and aromatic plants in the world reveals that the medicinal and aromatic plants reached a volume of 70.7 billion dollars worldwide in 2019. Turkey increased its export of medicinal and aromatic plants, which was approximately 106 million dollars in 2001, to approximately 371 million dollars in 2019 by increasing 3.5 times. Import of medicinal and aromatic plants in world trade was 71.9 billion dollars in 2019. In recent years, Turkey's medicinal and aromatic plant imports have increased and reached an import value of 656.5 million dollars in 2019 (General Directorate of Agricultural Research and Policies-TAGEM 2021).

There are several studies on the production, consumption, trade, use, market, history, ethnobotanics, and chemical and biological structure of medicinal and aromatic plants (Jamshidi-Kia et al. 2018; Göktaş & Gıdık 2019; Karık & Tunçtürk 2019; Ravi & Bharadvaja 2019; Tohidi et al. 2019; Zougagh et al. 2019; Sprea et al. 2020; Yücel & Yücel 2020). There is a limited study that attempts to identify those which contribute the most to the national economy among medicinal and aromatic plants using the Multiple Criteria Decision Making Methods in medicinal aromatic plants.

The Multi-Criteria decision making (MCDM) methods assist decision makers in making decisions in cases where there is more than one conflicting criterion. The MCDM methods divide the problems into smaller pieces, allowing handling complex problems. There are various MCDM methods used in the literature such as Analytical Hierarchy Process, Entropy, ELECTRE, TOPSIS, VIKOR (Mardani et al. 2015). In some studies, the MCDM methods have been used alone (Halicka 2020) or in combination with other methods (Singh et al. 2020). In this research, a hybrid MDCM was used, which was created by combining the Entropy and TOPSIS methods.

Among to the decision-making methods, the TOPSIS method was chosen to rank alternatives in this study because its process is simpler, it is easier to understand, it is one of the popular method, it provides good performance in different areas, it allows direct application on the obtained data, it needs less subjective input, and it has intuitive and clear logic that represents the rationale of human preference by considering both the best and worst attributes of the alternatives simultaneously (Roszkowska 2011; Vafai et al. 2018; Bahadır 2020; Emovon & Albuecefe 2020; Ersen et al. 2022). The basic principle of the TOPSIS method is based on ranking the alternatives according to the ideal solution. Starting from the alternative that is relatively close to the ideal solution, a ranking is carried out and then the relative closeness of the other alternatives is determined, respectively (Cheng-Min 2001).

One of the most important stages for MCDM methods is the determination of the criterion weights because each criterion has a different meaning and importance in a MCDM problem (Alp et al. 2015; Çatı et al. 2017). The weighting process, which shows the importance level of the criteria, is generally done in two ways: subjective weighting and objective weighting (Shemshadi et al. 2011). In subjective weighting methods, like Analytic Hierarchy Process (AHP) and Delphi, decision makers evaluate the criteria (Lofi & Fallahnejad 2010). Objective weighting methods, like entropy and CRITIC, are completely based on the characteristics of the available data (Ecer, 2020). Entropy method was chosen in this study because it is a well-known and widely used objective weighting method in decision making problems and it can prevent subjective preference assessment in the decision making process (Alp et al. 2015; Yuan et al. 2019). Entropy is a tool used to evaluate criteria in a decision matrix that contains alternative information (Nijkamp 1977).

There are many studies in which two methods were used together in the field of agriculture. Pakpour et al. (2013) attempted to identify the best of ten DNA extraction methods for agricultural soil using seven criteria. They used the entropy method for weighting the criteria and the TOPSIS method for ranking the DNA extraction methods. Wang & Hao (2016) examined the supply chain risk assessment of fresh agricultural products. They used the improved TOPSIS method, which is a combination of the improved entropy and the TOPSIS. Li et al. (2018) evaluated to the suitability of groundwater for domestic and agricultural purposes using entropy-weighted TOPSIS. Li et al. (2019) evaluated the agricultural water resources allocation plans with the entropy-based TOPSIS method. Wang et al. (2021) analyzed the impact of agricultural extension service on sustainable agricultural development using entropy and TOPSIS methods. Chen et al. (2021) focused on the agricultural investment environment and its influencing factors in the countries around the Black Sea. Criterion weights were determined by the Entropy method and the TOPSIS method was used to rank the countries. Lu et al. (2022) is designed a model on the selection of agricultural machinery with the help of the method formed by the combination of CRITIC, entropy, and GRA-TOPSIS and agricultural machines are ranked with this model. The financial performances of ten agricultural companies are examined with the help of the Entropy-TOPSIS method by Zheng & Wu (2022). Wang et al. (2022) aimed to evaluate the sustainable development level of agriculture with data obtained from 13 cities in China from 2016 to 2019 and applied the entropy and TOPSIS models. In recent years, Entropy-TOPSIS has been also successfully applied to various areas, such as transportation (Huang et al. 2018), product design (Tiwari 2019), competitiveness (Liang et al. 2019), innovation (Chen et al. 2020), construction (Dehdasht et al. 2020), energy (Sun & Yun 2021), safety (Omidi et al. 2022), optimization (Wang et al. 2022), education (Wang et al. 2022) and financial (Yen et al. 2023).

In the paper, the medicinal and aromatic plants that contribute to Turkey's economy more greatly have been attempted to be determined using a hybrid multi-criteria decision-making method. The weight values (importance levels) of the criteria were determined using the Entropy method. The ranking of alternatives was made via the TOPSIS method. Thanks to this paper, medicinal and aromatic plants that contribute the most to the economy of the country have been identified and will aid in taking the necessary measures for those products in practice.

2. Material and Methods

2.1. Material

The data were collected from the Turkey Statistical Institute and Turkey General Directorate of Forestry, and the research covers the past decade (2012-2021). The data obtained from the General Directorate of Forestry are the amount of production of laurel leaf, rosemary and linden. Another data used in the study are obtained from the Turkey Statistics Institute (TSI 2022).

2.2. Method

2.3. The Entropy method

The Entropy method was proposed by Shannon & Weaver (1949). This method is defined as the measure of uncertainty in information formulated using probability theory (Shemshadi et al. 2011; Yuan et al. 2019). The entropy method consists of 4 steps (Li et al. 2011).

Step 1: Creating the decision matrix

There are alternatives in the lines section of the decision matrix and evaluation criteria in the columns section. The decision matrix is given below:

$$X = \begin{vmatrix} x_{11} & x_{12} & \dots & x_{1j} & \dots & x_{1m} \\ x_{21} & x_{22} & \dots & x_{2j} & \dots & x_{2m} \\ \dots & \dots & \dots & \dots & \dots & \dots \\ x_{i1} & x_{i2} & \dots & x_{ij} & \dots & x_{im} \\ \dots & \dots & \dots & \dots & \dots & \dots \\ x_{n1} & x_{n2} & \dots & x_{nj} & \dots & x_{nm} \end{vmatrix}$$

Step 2: Normalization of the decision matrix

The data were subjected to normalization using the equations below.

$$a_{ij} = x_{ij} / max_{ij} \text{(i=1,….,n; j=1,….,m)} \quad (1)$$

a_{11}= 26490 / 39935 = 0.66333

$$a_{ij} = min_{ij} / x_{ij} \text{(i=1,....,n; j=1,…...,m)} \quad (2)$$

a_{13}= 5 / 1021 = 0.00490

$$P_{ij} = \frac{a_{ij}}{\sum_{i=1}^{n} a_{ij}} \quad (3)$$

$$P_{11} = \frac{0.663328}{(0.663328 + 0.395893 + 0.253512 + 0.420508 + 0.001427 + 0.058771 + \cdots + 0.499186 + 1)} = 0.14238$$

Where: i is alternative; j is criteria;P_{ij} is normalized value, and a_{ij} is benefit value.

Step 3: Calculating the entropy (e_j) value

The entropy value of each criterion is calculated by using the equation (4). The k value in the equation represents the entropy coefficient. The e_j value takes a value between 0 and 1.

$$e_j = -k \sum_{i=1}^{n} P_{ij}\, InP_{ij} \tag{4}$$

$$e_j = -0.345976 * [(0.14238 * ln0.14238) + (0.08497 * ln0.08497) + (0.05441 * ln0.05441 + (0.09026 * ln0.09026) \ldots + (0.21464 * ln0.21464)] = 0.7844$$

$$k = (\ln n)^{-1}$$

$$k = (\ln 18)^{-1} = 0.345976$$

Step 4: Calculation of weight (w_{ij}) value

The formula of w_{ij} is given in the equation (5). The sum of the weight values calculated for each criterion should be 1.

$$w_{ij} = \frac{1-e_{ij}}{\sum_{j=1}^{m}(1-e_{ij})} \tag{5}$$

$$w_{11} = \frac{0.2156}{(0.2156 + 0.3086 + 0.5985 + 0.3299 + 0.3589)} = 0.1190$$

2.3. The TOPSIS method

The Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) method was first proposed by Hwang & Yoon (1981), and later developed by Yoon (1987) and Hwang et al. (1993). This method starts with the creation of the decision matrix and consists of 6 steps. The stage of forming the decision matrix was described in the steps of the Entropy method, and the other steps were listed as follows (Tsaur 2011).

Step 2: Normalization

Normalization of the data was calculated with the help of the equation (6).

$$r_{ij} = \frac{x_{ij}}{\sqrt{\sum_{i=1}^{n} x_{ij}^2}} \tag{6}$$

$$r_{11} = \frac{26490}{\sqrt{26490^2+15810^2+10124^2+16793^2+57^2+2347+17649^2+1829^2+\cdots+19935^2+39935^2}} = 0.41026$$

Step 3: Creating a weighted and normalized decision matrix

Following normalization, weight value (w_j) is determined for each criterion. Then, the weight values are multiplied by the normalized process data ($V_{ij} = r_{ij} * w_j \rightarrow V_{11} = 0.4126 * 0.1190$), and the V matrix are obtained.

$$V = \begin{bmatrix} r_{11}w_1 & \cdots & r_{1n}w_n \\ \cdots & \cdots & \cdots \\ r_{1n}w_1 & \cdots & r_{mn}w_n \end{bmatrix}$$

Step 4: Determination of positive (v+) and negative (v-) ideal solution values

Weighted-normalized data are used to determine positive ideal solution and negative ideal solution values. Positive ideal values are determined using the equation (7), and negative ideal values are determined using the equation (8).

$$v^+ = \left\{\left(\sum_i^{max} v_{ij}/j \in J\right), \left(\sum_i^{min} v_{ij}/j \in J'\right)/i = 1,2,\ldots.n\right\} \tag{7}$$

$$v^+ = (0.0736007, \quad 0.0990801, \quad 0.0000099, \quad 0.1139570, \quad 0.0000431)$$

$v^- = \{(\sum_i^{min} v_{ij}/j \in J), (\sum_i^{max} v_{ij}/j \in J')/i = 1,2, \dots . n\}$
(8)$v^- = (0.0000018,\ 0.0000117, 0.2912629,\ 0.0000833,\ 01954206)$

Step 5: Calculation of positive (s_i^+) and negative (s_i^-) ideal solution distances

Positive and negative ideal solution distance values are calculated using Equations 9 and 10. Euclidean distances are used in the calculation.

$$s_i^+ = \left\{\sqrt{\sum(v_{ij} - v_j^+)^2}\right\} \tag{9}$$

$$s_1^+ = \sqrt{\begin{matrix}(0.0488214 - 0.0736007)^2 + (0.0783154 - 0.0736007)^2 \\ +(0.0020275 - 0.0736007)^2 + \\ (0.0766841 - 0.0736007)^2 + (0.0012634 - 0.0736007)^2\end{matrix}} = 0.0494$$

$$s_i^- = \left\{\sqrt{\sum(v_{ij} - v_j^-)^2}\right\} \tag{10}$$

$$s_1^- = \sqrt{\begin{matrix}(0.0488214 - 0.0000018)^2 + (0.0783154 - 0.0000018)^2 \\ +(0.0020275 - 0.0000018)^2 + \\ (0.0766841 - 0.0000018)^2 + (0.0012634 - 0.0000018)^2\end{matrix}} = 0.3684$$

Step 6: Calculation of the relative degree of approximation (c*) to the ideal solution and ranking

The relative degree of approximation to the ideal solution for each alternative is calculated using the equation (11). C* takes a value between 0 and 1. Alternatives are ranked according to their c* value. The alternative with the highest value is ranked first.

$$c_i^* = \frac{s_i^-}{s_i^- + s_i^+} \tag{11}$$

$$c_1^* = \frac{0.3684}{0.0494 + 0.3684} = 0.8818$$

3. Results and Discussion

The decision matrix consists of alternatives and evaluation criteria. There are 18 alternatives and 5 criteria in this research. The alternatives are: laurel leaves, thyme, anise, cumin, coriander, black cumin, sesame seed, hop, salvia, lavender, mint, rosemary, linden, fenugreek, poppy seed, flax seed, carobs, and safflower seed. Although there are many definitions of medicinal and aromatic plants in the literature, the products included in the scope of medicinal and aromatic plants are not clearly specified. In this research, the products (alternatives) used in many studies and the data of which are available were taken into consideration. The criteria in the study also were constructed by referring to the studies on forest products (Bayram 2020) and fishery and aquaculture products (Akmermer & Çelik 2021). The criteria are: production, export quantity, import quantity, export value, and import value. One of the main objectives of countries is economic growth. The role of foreign trade is very important in the economic growth process of countries. Because it activates internal dynamics. Today, economic development cannot be achieved without foreign trade. Foreign trade consists of exports and imports (Şerefli 2016). In addition, the increase in production, in the agricultural sector and manufacturing industry, positively affects all elements of sustainable development (economic, social, environmental and institutional) and therefore, increases economic growth (Behun et al. 2018). The arithmetic mean of the production, export and import quantities of each alternative in the decision matrix were determined, along with the weighted mean of the export and import values of each alternative. The decision matrix is given in Table 1. The institution that is responsible for the management of non-wood forest products in Turkey is The General Directorate of Forestry (GDF) which is affiliated with the Ministry of Agriculture and Forestry. In Turkey, non-wood forest products are generally grown in the forest and 99% of forests are owned by the state (Ok & Tengiz 2018).

Table 1- Decision matrix (TSI 2022; GDF 2022)

Alternatives	*Production (tonnes)*	*Export Quantity (tonnes)*	*Import Quantity (tonnes)*	*Export value (000$)*	*Import value (000$)*
Laurel leaves	26490	13389	1021	37730	1467
Thyme	15810	16939	1794	56069	4640
Anise	10124	2613	1743	9706	3941
Cumin	16793	5105	1753	15272	3945
Coriander	57	157	895	276	468
Black cumin seed	2347	380	3122	1066	2811
Sesame seed	17649	10373	146673	22078	226912
Hop	1829	5	167	41	2107
Salvia	586	1954	986	7451	2432
Lavender	1463	2	5	68	242
Mint	16658	471	85	1598	131
Rosemary	193	679	620	1939	844
Linden	64	95	63	1093	251
Carobs	15489	1489	3394	3214	10002
Fenugreek	633	89	253	174	184
Flax seed	1	321	62624	161	31321
Poppy seed	19935	15274	14	50698	50
Safflower seed	39935	1300	47055	686	16322

Following the decision matrix created, the data were normalized using Equations 1, 2 and 3 (P_{ij} values were obtained).

$$P_{ij} = \begin{vmatrix} 0.14238 & 0.18955 & 0.00310 & 0.18025 & 0.01440 \\ 0.08497 & 0.23981 & 0.00176 & 0.26786 & 0.00455 \\ 0.05441 & 0.03699 & 0.00182 & 0.04637 & 0.00536 \\ 0.09026 & 0.07227 & 0.00180 & 0.07296 & 0.00535 \\ 0.00031 & 0.00222 & 0.00353 & 0.00132 & 0.04513 \\ 0.01261 & 0.00538 & 0.00101 & 0.00509 & 0.00751 \\ 0.09486 & 0.14685 & 0.00002 & 0.10547 & 0.00009 \\ 0.00983 & 0.00007 & 0.01894 & 0.00020 & 0.01002 \\ 0.00315 & 0.02766 & 0.00321 & 0.03560 & 0.00868 \\ 0.00786 & 0.00003 & 0.63273 & 0.00032 & 0.08727 \\ 0.08953 & 0.00667 & 0.03722 & 0.00763 & 0.16122 \\ 0.00104 & 0.00961 & 0.00510 & 0.00926 & 0.02502 \\ 0.00034 & 0.00134 & 0.05022 & 0.00522 & 0.08414 \\ 0.08325 & 0.02108 & 0.00093 & 0.01535 & 0.00211 \\ 0.00340 & 0.00126 & 0.01250 & 0.00083 & 0.11478 \\ 0.00001 & 0.00454 & 0.00005 & 0.00077 & 0.00067 \\ 0.10715 & 0.21624 & 0.22597 & 0.24220 & 0.42239 \\ 0.21464 & 0.01840 & 0.00007 & 0.00328 & 0.00129 \end{vmatrix}$$

Using the Equations (4) and (5), the entropy value and the weight value of each criterion were determined. The entropy and weight values are given in Table 2.

Table 2-Entropy and weight values

Entropy and weight	*Production (tonnes)*	*Exportquantity (tonnes)*	*Import Quantity (tonnes)*	*Export value (000$)*	*Import value (000$)*
$\mathbf{e_j}$	0.7844	0.6914	0.4015	0.6701	0.6411
w_j	0.1190	0.1704	0.3304	0.1821	0.1981

The weight values of the criteria determined by the Entropy method were found as import quantity (0.3304), import value (0.1981), export value (0.1821), export quantity (0.1704), and production quantity (0.1190). This result represented that the import quantity is of greater significance to the ranking of the economic value of medicinal and aromatic plants in Turkey. In the study of the contribution of forest products to the national economy by Bayram (2020), the entropy weight value of the import quantity criterion was found to be the highest. Although exports are vital for a country's development and growth, the balance of imports and exports should also be observed. Imports in particular, play an important role for emerging economies such as Turkey. In the Turkish economy, imports have a larger share than exports (Çoşkun 2019). Necessary steps should be taken to reduce the dependence on imports and to increase exports (Yurdakul & Uçar 2015). In Turkey, various steps are taken to increase exports, such as creating regional export associations, and providing incentives to businesses engaged among others (Çoşkun 2019).

After determining the significance levels of the criteria using the Entropy method, the alternatives were ranked using the TOPSIS method. In the TOPSIS method, the decision matrix (Table 1) used in determining the weights of the criteria was employed. Firstly, the normalization of the data in the decision matrix was performed. Equation (6) was used for normalization. Then, the V_{ij} matrix was obtained. To get this matrix, the normalized data were multiplied by the weight values of the criteria. The V_{ij} matrix was given below.

$$V_{ij} = \begin{vmatrix} 0.0488214 & 0.0783154 & 0.0020275 & 0.0766841 & 0.0012634 \\ 0.0291380 & 0.0990801 & 0.0035625 & 0.1139570 & 0.0039961 \\ 0.0186587 & 0.0152840 & 0.0034612 & 0.0197269 & 0.0033941 \\ 0.0309497 & 0.0298603 & 0.0034811 & 0.0310395 & 0.0033975 \\ 0.0001051 & 0.0009183 & 0.0017773 & 0.0005610 & 0.0004030 \\ 0.0043256 & 0.0022227 & 0.0061997 & 0.0021666 & 0.0024209 \\ 0.0325273 & 0.0606741 & 0.2912629 & 0.0448723 & 0.1954206 \\ 0.0033709 & 0.0000292 & 0.0003316 & 0.0000833 & 0.0018146 \\ 0.0010800 & 0.0114294 & 0.0019580 & 0.0151437 & 0.0020945 \\ 0.0026963 & 0.0000117 & 0.0000099 & 0.0001382 & 0.0002084 \\ 0.0307009 & 0.0027550 & 0.0001688 & 0.0032478 & 0.0001128 \\ 0.0003557 & 0.0039716 & 0.0012312 & 0.0039409 & 0.0007269 \\ 0.0001180 & 0.0005557 & 0.0001251 & 0.0022215 & 0.0002162 \\ 0.0285464 & 0.0087095 & 0.0067398 & 0.0065323 & 0.0086139 \\ 0.0011666 & 0.0005206 & 0.0005024 & 0.0003536 & 0.0001585 \\ 0.0000018 & 0.0018776 & 0.1243586 & 0.0003272 & 0.0269742 \\ 0.0367405 & 0.0893412 & 0.0000278 & 0.1030408 & 0.0000431 \\ 0.0736007 & 0.0076040 & 0.0934417 & 0.0013943 & 0.0140568 \end{vmatrix}$$

Ideal positive (v^+) and negative solution (v^-) values were obtained with the help of the V_{ij} matrix. v^+ values were determined by choosing the highest value from each criterion (column) value, and v^- values were determined by choosing the lowest value.The positive and negative ideal solution values are given in Table 3. The assessed criteria play a decisive role in determining the positive ideal solution and the negative ideal solution. Among the criteria selected in this study, import quantity and import value are negative criteria, the smaller of which is the better, whereas the amount of production, export quantity and export value are the positive criteria, the larger of which is the better. Finally, S^+, S^-, C^* values were calculated using equations (9, 10, and 11), respectively, and the alternatives were ranked based on C^* values.

Table 3-Positive and negative ideal solution values

Ideal solution distance	*Production (tonnes)*	*Export quantity (tonnes)*	*Import Quantity (tonnes)*	*Export value (000$)*	*Import value (000$)*
v^+	0.0736007	0.0990801	0.0000099	0.1139571	0.0000431
v^-	0.0000018	0.0000117	0.2912629	0.0000833	0.1954206

When Table 4 was examined, the importance levels of medicinal and aromatic plants in terms of contribution to the country's economy were ranked as follows: poppy seed, thyme, laurel leaves, cumin, anise, salvia, carobs, mint, rosemary, linden, black cumin seed, lavender, hop, fenugreek, coriander, safflower, flax seed and sesame seed.

The reason for the poppy seed's first-place position in the ranking is that its amount of production, export quantity and export value are quite high. It is noteworthy that Turkey's poppy seed import is quite low. In terms of poppy seed trade, Turkey is among the leading countries in the world (Acıbuca & Budak 2018). Other medicinal and aromatic plants, which are significant for the Turkish economy, were found to be thyme and laurel leaves. Like poppy seeds, the production quantity and export quantities and values of thyme and laurel leaves are high. The export quantities and values of these three products are considerably higher than the others. Turkey is the leading exporter of thyme in the world (Bağdat 2006). In addition, approximately 90% of the total world market share belongs to Turkey for laurel leaves (Semerci & Çelik 2017).

Being the lowest among the products that contribute to the country's economy, it is a remarkable finding that the amount and value of sesame seed imports are high. While the production of sesame seeds may be high, the export amount and value are quite low. Flax seed production in Turkey is at a very low level, and Turkey meets its flax seed needs through imports. Another remarkable result is that although safflower seed production and import is high, its contribution to the country's economy is low. Turkey is a net importer of the seed trade (Ceylan et al. 2018). Although the production of sesame and safflower seeds is high, measures should be taken to reduce dependence on foreign markets and increase exports.

Table 4-S^+, S^-, C values and ranking

Alternatives	S^+	S^-	*C*	*Rank*
Laurel leaves	0.0494	0.3684	0.8818	3
Thyme	0.0448	0.3782	0.8941	2
Anise	0.1376	0.3474	0.7162	5
Cumin	0.1162	0.3500	0.7507	4
Coriander	0.1670	0.3490	0.6763	15
Black cumin seed	0.1635	0.3443	0.6781	11
Sesame seed	0.3619	0.0821	0.1850	18
Hop	0.1665	0.3495	0.6773	13
Salvia	0.1507	0.3485	0.6981	6
Lavender	0.1667	0.3506	0.6777	12
Mint	0.1529	0.3519	0.6971	8
Rosemary	0.1628	0.3494	0.6821	9
Linden	0.1661	0.3505	0.6785	10
Carobs	0.1478	0.3417	0.6980	7
Fenugreek	0.1669	0.3502	0.6772	14
Flax seed	0.2097	0.2371	0.5307	17
Poppy seed	0.0397	0.3780	0.9051	1
Safflower seed	0.1731	0.2784	0.6166	16

In the last part of the study, sensitivity analysis was performed to test the stability and robustness of the proposed model (Entropy-TOPSIS method). One of the approaches used in sensitivity analysis is to determine how the ranking of the alternatives changes due to the variability in the criteria (Lee & Chang 2018). The executed calculations for sensitivity analysis are based on the equations given in Yazdani et al. (2020), which can be consulted for details. First, the weight elasticity coefficients of the criteria are calculated and the results are given in Table 5. The weight elasticity coefficient of the most important criterion is always assumed to be 1. Then, the limiting bounds of weight change (Δx) for the most important criterion are calculated. This is between -0.3304 and 0.6696.After defining these limits, 9 new weights are calculated based on different values for Δx, as seen in Table 6. The rankings of medicinal and aromatic plants obtained for new criteria weight values are given in Table 7.

Table 5-Weight coefficient of elasticity used changing weights

Criteria	α_c
Import quantity (w_3)	1.0000
Production (w_1)	0.1777
Export quantity (w_2)	0.2545
Export value (w_4)	0.2720
Import value (w_5)	0.2958

Table 6-New criteria weights

Scenarios	Δx	w_1	w_2	w_3	w_4	w_5
Original value	0.0	0.1777	0.2545	0.0000	0.2720	0.2958
Scenario 1	-0.3	0.1723	0.2467	0.0304	0.2637	0.2869
Scenario 2	-0.2	0.1545	0.2213	0.1304	0.2365	0.2573
Scenario 3	-0.1	0.1368	0.1958	0.2304	0.2093	0.2277
Scenario 4	0.1	0.1012	0.145	0.4304	0.1549	0.1685
Scenario 5	0.2	0.0835	0.1195	0.5304	0.1277	0.1389
Scenario 6	0.3	0.0657	0.0941	0.6304	0.1005	0.1093
Scenario 7	0.4	0.0479	0.0686	0.7304	0.0733	0.0798
Scenario 8	0.5	0.0301	0.0432	0.8304	0.0461	0.0502
Scenario 9	0.6	0.0124	0.0177	0.9304	0.0189	0.0206

Table 7-The rankings of alternatives based on new weight criteria

Alternatives	*Original ranking*	*S1*	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*	*S9*
Laurel leaves	3	3	3	3	3	3	3	3	2	2
Thyme	2	2	2	2	2	2	2	2	3	3
Anise	5	5	5	5	5	5	5	5	5	12
Cumin	4	4	4	4	4	4	4	4	4	4
Coriander	15	15	15	15	15	15	14	14	14	13
Black cumin seed	11	11	11	10	13	14	15	15	15	14
Sesame seed	18	18	18	18	18	18	18	18	18	18
Hop	13	16	16	14	12	12	11	11	11	10
Salvia	6	8	7	6	6	6	6	6	6	6
Lavender	12	13	13	12	11	11	12	12	12	8
Mint	8	7	6	7	8	8	7	7	7	5
Rosemary	9	10	10	9	9	9	9	9	8	9
Linden	10	12	12	11	10	10	10	10	10	7
Carobs	7	9	8	8	7	7	8	8	9	15
Fenugreek	14	14	14	13	14	13	13	13	13	11
Flax seed	17	17	17	17	17	17	17	17	17	17
Poppy seed	1	1	1	1	1	1	1	1	1	1
Safflower seed	16	6	9	16	16	16	16	16	16	16

As can be seen in Table 6, setting different weights to the criteria causes a change in the ranking of some alternatives. However, for all the scenarios, while poppy seed is found as the most valuable medicinal and aromatic product for Turkey, the least valuable medicinal and aromatic product is sesame seed. This result is an indication that the proposed model is stable. Moreover, these changes are confirmed by the values of the Spearman rank coefficient correlation the rankings for different scenarios and are displayed in Figure 1.

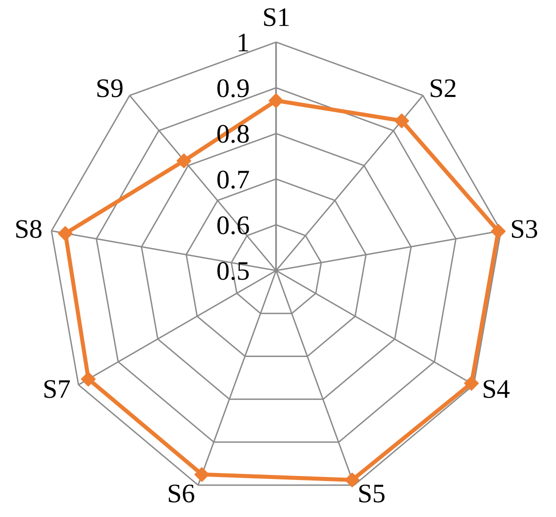

Figure 1- Spider diagram of Spearman rank coefficient correlation for 9 scenarios

According to the Spearman rank correlation analysis, it is seen that there are 87.2%, 92.8%, 99.4%, 99.4%, 98.8%, 97.5%, 97.5%, 96.9% and 81.4% similarities between the original ranking and the scenarios, respectively. The overall mean Spearman rank correlation coefficient of all the scenarios is found as 0.945, which emphasizes the reliability of the original ranking.

4. Conclusions

Medicinal and aromatic plants support the national economy as well as the well-being of forest communities. Furthermore, these plants are commonly employed in a range of sectors including medicine, food, paint, and fragrance. The value of several medicinal and aromatic plants on the national economy was investigated in this study. The following findings were obtained: Using the Entropy approach, it was discovered that the import amount was the most significant criteria, while the production quantity was the least significant. Given the requirements and alternatives, the most economically significant items among the therapeutic aromatic plants under consideration are poppy seed, thyme, and laurel leaves. Among the therapeutic aromatic plants, seed plants contribute the least to the country's economy.

Cumin, anise, and salvia have also been found to have economic potential. These products are usually exported unprocessed in Turkey and therefore they create low added value to the country's economy.

In order to test the robustness and the reliability of the method used in the study, a sensitivity analysis consisting of 9 scenarios and the Spearman rank correlation analysis were performed. Regarding 9 scenarios, supporting the study, poppy seed is found as the most valuable and sesame seed is found as the least valuable medicinal and aromatic product. Spearman rank correlation coefficient also was found higher than 0.80 for all scenarios. Thusly, the robustness and the reliability of the methodology are shown.

This study could add a new dimension to medicinal and aromatic plants research. Generally, there are studies related to the classification, usage areas, production and foreign trade of medicinal and aromatic plants. There is no known research on the economic contribution to Turkey. As a result of this research, this gap in the literature will be filled. In this respect, this research is one of the pioneering studies and provides both a theoretical and practical contribution. With this and similar studies, decision makers can determine which products are of greater economic significance.

Although this research has advantages, it also has limitations. While determining the criteria and alternatives in the study, attention was paid to whether the data were available and to receive the data from a reliable source. By accessing data from various databases, the number of criteria and the number of alternatives can be increased. In addition, different criteria weighting and alternative ranking methods can be used. Therefore, the use of only five criteria in the study can be seen as an important deficiency of the study.

According to the findings obtained from this study, suggestions for the future are as follows:

- Most of the medicinal and aromatic plants exported in our country are collected from nature. However, when the plant collectors do not have information about the harvest period, sustainability, etc., they are collected unconsciously, which causes both extinction of the plants and not the desired standards of the collected plants. Therefore, a regulation on the collection of medicinal and aromatic plants should be issued and necessary legal regulations should be made.
- Since the demand for medicinal and aromatic plants has increased with the emergence of Covid-19 disease, studies should be carried out to cultivate as many plants as possible, especially plants with high commercial value and used in Turkey. For this, an inventory of medicinal and aromatic plants in all provinces should be taken and then the most suitable places for the habitat of the species should be determined. Thus, the production of medicinal and aromatic plants will increase and country’s exports will be much more than imports.
- In particular, plants grown in nature can be used as a gene source instead of meeting the demand and exporting, and sustainability can be ensured in these plants.
- Incentive supports can be provided for the production of medicinal and aromatic products with high commercial value. For example, VAT reduction, tax exemption, support of production services such as seeds, saplings, spraying, diesel, certification, etc.
- In order to increase production, the contracted production model, which has been successfully applied in thyme production, can be applied in other medicinal and aromatic plants.
- In Turkey, certified seed production is carried out with domestic facilities of many species. Seed production is carried out especially in vegetables, field and some forage plant species. However, certified medicinal and aromatic plants seed is not sufficient. It is seen that the seed production for medicinal and aromatic plants does not meet the domestic demand and the country is foreign-dependent for these products. For this reason, it is necessary to carry out agronomic studies, breeding studies, and registration and certification procedures for species and varieties. Seed producers who want to produce seeds of medicinal and aromatic plants should be encouraged and public and private sector cooperation should be increased. Furthermore, seed subsidies and subsidized loans should be determined according to the policy of producing domestic varieties instead of imported varieties. In order to prevent seed imports, import quotas may be set and customs tax rates may be increased.

- A large part of Turkey's exports of medicinal and aromatic plants are realized in unprocessed form and the contribution of these products to the country's economy is low. The products should be processed in processing and packaging facilities and converted into high value-added products. For the establishment of such facilities, incentives, for example grants and zero-interest loan support to producers, tax exemption etc., can be provided to the producers.

- A supreme committee may be established, consisting of experts on medicinal and aromatic plants, affiliated to the Ministry of Agriculture and Forestry, operating for the purpose of determining all kinds of national policies regarding medicinal and aromatic plants.

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Toxicity of Linear Alkyl Benzene Sulfonate to Tarek (*Alburnus tarichi*) Larvae

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ABSTRACT
Linear alkyl benzene sulfonate is a group of anionic detergents widely used in domestic and industrial applications. It causes adverse effects by mixing with aquatic environments. Chemicals mixing with aquatic environments affect fauna and flora at different levels. This study was carried out to determine the toxicity of linear alkyl benzene sulfonate for the larvae of tarek (*Alburnus tarichi* Güldenstädt, 1814), a fish living in the Van Lake basin which has economic and ecological value. The chemical was administered at concentrations of 0.0, 0.6, 1.2, 1.8, 2.4, and 3.0 mg L^{-1} in the acute test and 0.0, 1.2, and 2.4 mg L^{-1} in the chronic test. Tests were carried out using 100 larvae in each group. Bioassays were carried out at mean temperature of 20.9 ± 0.4 °C. A median lethal concentration of 4.883 (4.099–6.482) mg L^{-1} at 96 hours was calculated for the larvae. Glutathione content, superoxide dismutase and glutathione S-transferase activity decreased significantly at 1.2 mg L^{-1} and increased significantly at 2.4 mg L^{-1} compared to controls ($P<0.05$). The malondialdehyde content increased significantly depending on the increase in chemical concentration ($P<0.05$). Linear alkyl benzene sulfonate is toxic to tarek larvae in terms of the measured biochemical parameters.

Keywords: Anionic surfactants, Antioxidant enzyme, Chronic test, Cyprinid fish, LC_{50}

1. Introduction

Linear alkyl benzene sulfonate (LAS) is an anionic surfactant. It has high cleaning capacity by reducing the surface tension of water. Therefore, the compound is widely used in household and industrial detergents. It enters the environment through wastewater discharge and may pose a potential risk to the receiving aquatic ecosystems (Zhang et al. 2015; Gouda et al. 2022; Filogh et al. 2023). Its microbial degradability is 6–22 days. Despite being biodegradable, conventional wastewater treatment plants are relatively inefficient at removing LAS. It can have a toxic effect on aquatic organisms (Manousaki et al. 2004; Zhang et al. 2015). Fish, an important group living in the aquatic environment, are very good bioindicators of aquatic contaminants. They react with great sensitivity to changes in water quality. Toxicity tests are performed to reveal and evaluate these reactions (Uedeme-Naa & Erondu 2014; Kankaya & Kaptaner 2016).

There are acute and chronic toxicity studies on many fish species related to LAS used in the experiment. Some of these studies examined the gill histology of *Oncorhynchus mykiss* fries by Hofer et al. (1995); the hatching of *Sparus aurata* fish eggs (Rosety et al. 2001); histopathology of LAS-absorbed sediment on different tissues of *Solea senegalensis* juveniles (Hampel et al. 2008); and histological effects on liver tissue of *Puntius ticto* fish by Varsha, Mishra & Govind (2013). In addition to these studies, Uedeme-Naa & Erondu (2014) evaluated the effects on plasma biochemical parameters of *Clarias gariepinus* juveniles, while Oyoroko & Ogamba (2017) studied the effects of detergents containing LAS on the behavior of *Heterobranchus bidorsalis*, *Clarias gariepinus* and *Heteroclarias*. Tarek (*Alburnus tarichi*, Güldenstädt 1814) is a member of the *Cyprinidae* family and is endemic to the Van Lake basin (Elp et al. 2014). It has economic value for the region with a catch of approximately 10,000 tons/year (TUIK 2022). It migrates to the rivers pouring into the lake for reproduction. Adult fish that have completed spawning return to the lake. Although the spawning calendar changes depending on the change in water temperature over the years, the spawning migration intensifies especially in May–June. Fertilization, hatching, hatching of larvae and the first feeding period after reproduction occur in the rivers. After a certain time, the fry leaves the rivers and enters the lake (Ünal et al. 1999; Kankaya & Ünal 2018). Fish are adversely affected because rivers are contaminated with different levels of pollutant load.
The aim of the study was to determine the acute and chronic toxic effects of LAS on tarek larvae.

2. Material and Methods

2.1. Collection of fish and larval production

Adult male and female tarek (*Alburnus tarichi*) were caught using a net from the Karasu river (43°13′38.46″ E, 38°35′20.41″ N) during the spawning migration period (May–June) (Figure 1). Fertilized eggs obtained by artificial insemination were hatched and larvae were obtained (Kankaya et al. 2015).

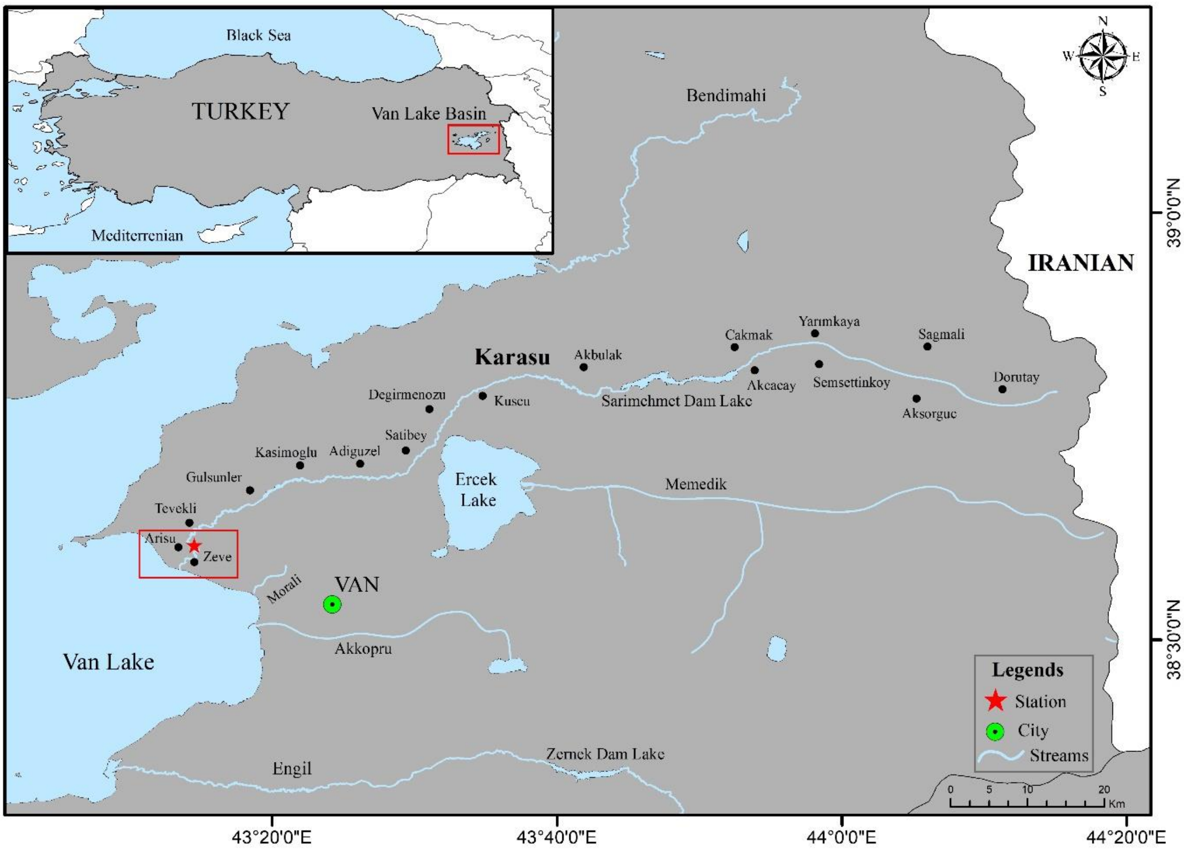

Figure 1- Fish sampling station on the Karasu river

2.2. Chemicals

LAS was purchased from Sigma-Aldrich (CAS: 25155-30-0). The superoxide dismutase (SOD) enzyme kit was obtained from Randox Laboratories Ltd. 5,5’-dithiobis 2-nitrobenzoic acid; glutathione (GSH); 1-chloro-2,4-dinitrobenzene and other chemicals were provided with analytical quality.

2.3. Experimental design

Concentrations of 0.0, 0.6, 1.2, 1.8, 2.4 and 3.0 mg L^{-1} LAS were applied in the acute toxicity test (Xu 1996; Rosety et al. 2001). In the chronic toxicity test, the amount of chemical was chosen as 1.2 and 2.4 mg L^{-1}, taking into account the median lethal concentration (LC_{50}). Applications were carried out in a glass beaker, using 100 larvae (12–24 hours) in each. Bioassays were carried out using the static test method (Wang et al. 2010). The acute test was continued for 96 hours and the chronic test for 7 days. The study was applied in natural photoperiods with 4 replications (USEPA 2002a; Çetinkaya 2010; OECD 2013; Kankaya & Ünal 2018).

2.4. Measurements and assessments

Water quality criteria (water temperature, pH, dissolved oxygen, oxygen saturation, electrical conductivity, salinity) were measured with a Hach HQ-40d multimeter regularly throughout exposure. Total hardness and total alkalinity were determined using the titrimetric method. In order to determine the actual LAS concentration of the experimental environment, water samples were analyzed using the methylene blue active substances method with Hach Lange DR 5000 UV/VIS spectrophotometer (APHA 1995; HACH 2005). During the chronic toxicity test, the morphological developmental stages of the larvae were followed (Unal et al. 2000; OECD 2013; Kankaya et al. 2015). Feed, content of which is given in Table 1, was given in the form of powder for external feeding on the 6th day after hatching of the larvae. Morphological examination and imaging of larvae were performed using a Nikon SMZ 745 T stereo light microscope with XCAM digital camera attachment. At the end of the chronic toxicity test, 15–17 larvae in all application groups were placed in Eppendorf tube and 6 Eppendorf sample tubes were created for enzyme analysis. Samples were stored at -80 °C until biochemical analysis.

Table 1- Content of feed used for external feeding of larvae in the chronic test (Maripearls trademark)

Nutrient	*Value*
Protein (%)	38
Fat (%)	4
Raw fiber (%)	4.5
Raw ash (%)	10.5
Vit E	300 IU
Vit C	200 IU
Vit A	25000 IU
Vit D3	2000 IU
Enriched algae (%)	10

2.5. Tissue homogenization

Larval samples stored at -80 °C for analysis were weighed and homogenized with tissue lysis (Tissuelyser Qiagen) in an Eppendorf tube for 3 min in 50 mM KH_2PO_4 buffer (4 °C, 1:5 w/v).

The homogenate was centrifuged at 9500 rpm for 30 min at 4 °C with a refrigerated centrifuge (Inovia, INO-HR/T16M) (Marklund 1990). The obtained supernatant was used for GSH, glutathione S-transferase (GST), SOD, malondialdehyde (MDA) and catalase (CAT) measurements.

2.6. Biochemical Analyses

GSH content was determined according to Beutler (1984) by measuring at 412 nm and GST activity was measured at 340 nm using the method of Habig et al. (1974). In addition to these, SOD activity was determined at 505 nm using the procedure of the commercial kit manufacturer (Ransod, Randox Lab., UK), MDA content was measured at 532 nm (Jain et al. 1989) and CAT activity was measured at 240 nm by the spectrophotometric method according to Aebi (1984).

2.7. Statistical analysis

LC_{50} values and 95% confidence limits were calculated using a computer package program with the probit analysis method. The data obtained as a result of biochemical analyses were evaluated using one-way analysis of variance (ANOVA) and Duncan multiple comparison test. Values with $P<0.05$ in all analyses were considered statistically significant. Values are given as mean ± standard deviation (USEPA 2002b).

3. Results and Discussion

3.1. Water quality, actual LAS concentrations and LC_{50} value

The mean values for water temperature, pH, dissolved oxygen, oxygen saturation, electrical conductivity, salinity, total hardness and total alkalinity are given in Table 2. Theoretical and actual LAS concentration results are given in Table 3. The 96-hour LC_{50} value for larvae exposed to LAS was calculated as 4.883 (4.099–6.482) mg L^{-1}.

Table 2- Quality criteria and variation of water used in bioassay

Water Quality Criteria	*Mean ± SD*
Temperature (°C)	20.9 ± 0.4
pH	8.48 ± 0.08
Dissolved oxygen (mg L^{-1})	7.4 ± 0.2
Oxygen saturation (%)	102.2 ± 1.8
Electrical conductivity (µS cm^{-1})	719 ± 28
Salinity (‰)	0.34 ± 0.02
Total hardness ($CaCO_3$ mg L^{-1})	358 ± 23
Total alkalinity ($CaCO_3$ mg L^{-1})	557 ± 17

Table 3- Theoretical and actual LAS concentrations (n=2)

Theoretical LAS concentration (mg L^{-1})	*Actual LAS concentration (mg L^{-1})*
0.6	0.53±0.08
1.2	1.11±0.07
1.8	1.63±0.12
2.4	2.27±0.10
3.0	2.88±0.09

Products containing LAS are widely used in many areas of life and they are released into the environment. Therefore, in this study, the acute and chronic toxic effects of LAS on tarek were investigated. In similar studies on the subject Gholami et al. (2010) found the 96-hour LC_{50} value of LAS in *Rutilus frisii* fries was 11.62 mg L^{-1}; Spirita Sharmili et al. (2015) reported the 96-hour LC_{50} value was 27.31 mg L^{-1} in their study determining the toxicity of alkyl benzene sulfonate in zebrafish (*Danio rerio*). Shukla & Trivedi (2018) calculated the LAS 96-hour LC_{50} value as 34.40 mg L^{-1} in *Channa punctatus* fish weighing 25 g. Gouda et al. (2022) reported the LAS 96-hour LC_{50} value was 10 mg L^{-1} for Nile tilapia (*Oreochromis niloticus*) weighing 6.6–7.8 g. In the current study, the 96-hour LC_{50} value was calculated as 4.883 (4.099–6.482) mg L^{-1} in larvae exposed to LAS. According to the calculated LC_{50} values, it is clear that LAS is moderately toxic to tarek larvae.

3.2. Morphological observations and mortality rate in larvae with the chronic test

During the test, no significant difference was found between the groups in terms of total length, yolk sac consumption, pigmentation, swim bladder formation, appearance of fins, opening of the mouth and transition to external feeding in larvae. The determinations made regarding the morphological development of the larvae during the chronic test were as follows. First day: length 7.0–7.8 mm; eyes are large; pigmentation dorsal to the yolk sac, behind the eyes, along the head and back; blood circulation in the anterior of the yolk sac and along the notochord; and 2 pairs of otoliths are seen. Second day: length 7.4–8.5 mm; intestinal development began between the yolk sac and the notochord. Third day: length 7.5–8.5 mm; pigmentation is prominent in the head region, on the back up to the tail region, on the dorsal of the yolk sac from anterior to posterior; intestinal line is seen in a structure with yellow content up to the anus. Fourth day: length 8.0–8.9 mm; the first lobe of the swim bladder is visible; pigmentation shows as round spots, spread along the dorsal from the head to the tail, along the dorsal of the yolk sac, in the region between the anus and the caudal fin; pectoral fin prominent and mouth open. Fifth day: length 8.3–8.7 mm; yolk sac is rather thin; motility was detected in the larvae. Sixth day: length 8.7–9.1 mm; increased movements; powdered feed (Table 1) was given for external feeding. Seventh day: length 8.2–8.8 mm; red-colored food given on the sixth day can be noticed as red coloration in the digestive system up to the anus; larvae are free swimming (Figure 2). At the end of the bioassay, the mean total length of the larvae was determined as 8.3 ± 0.5, 8.0 ± 0.5, and 8.0 ± 0.5 mm at 0.0, 1.2 and 2.4 mg L^{-1} concentrations of LAS, respectively. The differences between larval sizes were found to be statistically insignificantly lower in the LAS-treated groups compared to the control group. The mean length of the yolk sac in the larvae was determined as 3.8 ± 0.2, 3.8 ± 0.2, and 3.7 ± 0.2 mm for 0.0, 1.2 and 2.4 mg L^{-1} concentrations of LAS, respectively. Yolk sac length decreased insignificantly in the 2.4 mg L^{-1} group ($P>0.05$). At the end of the test, the mortality rate in larvae was 1% in the control and 2% in the LAS groups.

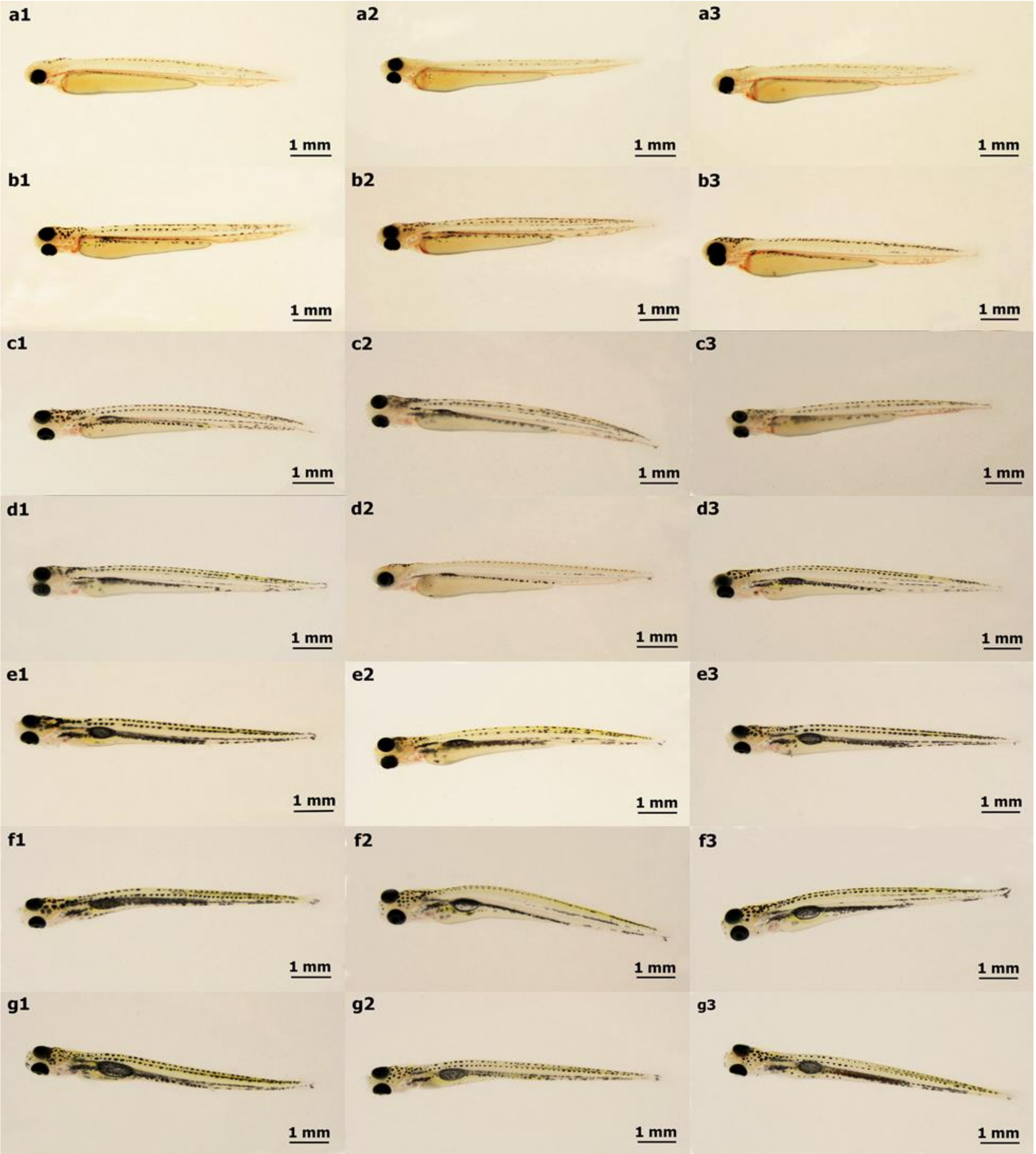

Figure 2- Microscopic images of larvae according to chemical application durations (a: 24 h, b: 48 h, c: 72 h, d: 96 h, e: 120 h, f: 144 h, g:168 h) and concentrations (0.0, 1.2 and 2.4 mg L^{-1}). The number '1' with each letter represents the control groups, '2' represents group administered 1.2 mg L^{-1} chemical and '3' represents the group administered 2.4 mg L^{-1} chemical)

Hampel et al. (2004) exposed *Sparus aurata* larvae sub lethally to LAS. They reported that the survival rate after 72 hours at 0.5 mg L^{-1} LAS concentration was 50% and severe edema could occur in the yolk sac. In the current study, the morphological development processes of the experimental group larvae during the test were similar both between the groups and to the results reported by Unal et al. (2000). No abnormality occurred in the larvae exposed to LAS for 7 days compared to the control (Sepil & Kankaya 2022). Larval length and yolk sac consumption were insignificantly different in all groups. Survival rate in LAS groups was 98%, similar to control. It was revealed that LAS did not have a significant effect in terms of yolk sac consumption, length and survival rate of tarek larvae.

3.3. Biochemical analyses

In the LAS chronic toxicity test, larvae exposed to 0.0, 1.2 and 2.4 mg L^{-1} concentrations had mean GSH content 7.59 ± 0.76, 3.79 ± 0.35, and 8.51 ± 0.97 µmol mg^{-1} protein, mean GST activity 1.23 ± 0.21, 0.44 ± 0.13, and 2.1 ± 0.64 nmol mg^{-1} protein, mean SOD activity 161.53 ± 21.24, 130.17 ± 19.28, and 203.12 ± 28.83 U g^{-1} protein, mean MDA content 7.22 ± 0.71, 25.62 ±

6.07, and 43.97 ± 3.56 nmol g^{-1} protein, and CAT activity was determined as 1.67 ± 0.19, 1.62 ± 0.30, and 3.69 ± 0.43 nmol g^{-1} protein, respectively (Figure 3). The GSH content, SOD and GST activity changed significantly between the groups. The MDA content increased significantly due to the increase in the concentration of the chemical, while CAT activity significantly increased at 2.4 mg L^{-1}.

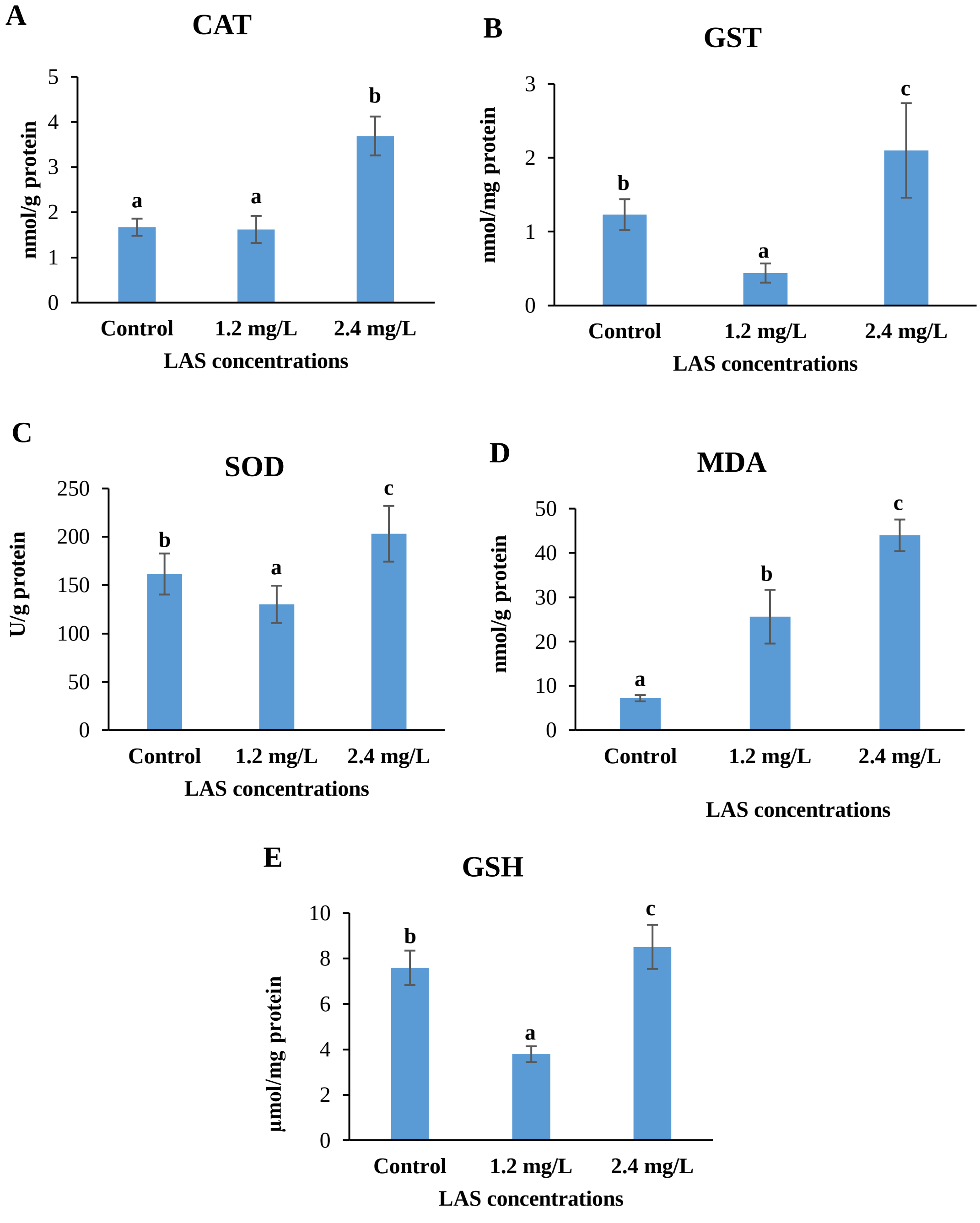

Figure 3- Changes in CAT (A), GST (B), SOD (C) activities, MDA (D) and GSH (E) contents according to the results of LAS chronic toxicity tests (0.0, 1.2 and 2.4 mg L^{-1} concentrations) in larvae

Hofer et al. (1995) reported in their study with *Oncorhynchus mykiss* that fries exposed to 0.2 mg L^{-1} concentration of LAS were significantly affected histologically and physiologically. In their study investigating LAS-induced oxidative stress and liver disorders in *Channa punctatus* fish, Shukla & Trivedi (2018) found that CAT and SOD activities determined in liver tissue increased significantly depending on the increase in concentration. Gouda et al. (2022), in their study determining the effects of LAS on the biochemical parameters of Nile tilapia (*Oreochromis niloticus*), reported that SOD and CAT activity and GSH content decreased depending on the increase in LAS concentration in serum samples. Ghosh et al. (2022) exposed *Oreochromis mossambicus* fish to sub lethal concentrations of alkyl benzene sulfonate and reported that CAT, GST, MDA, and SOD values increased significantly. In this study, no observed effect concentration (NOEC) value for CAT activity was 1.2 mg L^{-1} and the lowest observed effect concentration for GST, SOD activity and GSH, MDA content was 1.2 mg L^{-1}. The NOEC value was determined to be well above the value reported by Hofer et al. (1995) for *Oncorhynchus mykiss* fries. Since CAT has secondary importance in protecting from the formation of oxygen radicals (Doğan & Çelik 2016), the activity value was similar to the controls at 1.2 mg L^{-1}. The increase at 2.4 mg L^{-1} exposure may be the result of an adaptation response against free radical formation.

Atıcı (2020) investigated the concentration level of LAS in the Karasu river. The mean LAS value was determined as 0.018 ± 0.001 mg L^{-1}. The LAS value was reported to be class I according to the surface water quality regulation. Atici (2021) studied the seasonal changes of LAS in the surface waters of Morali, Akköprü and Kurubaş rivers, which flow into Lake Van, carry domestic and industrial wastewater, and where tarek enters to spawn. In this study, the amount of LAS changed in the range of 0.032–0.184, 0.023–0.081, and 0.170–0.401 mg L^{-1} in Morali, Akköprü and Kurubaş rivers between April and July, respectively. Although the calendar of tarek spawning migration varies depending on the increase in water temperature of the rivers, considering that it generally occurs between April–July, it is possible that tarek will not have a problem in terms of LAS amount (Atıcı 2020) in Karasu river. But if they enter the Morali, Akköprü and Kurubaş rivers, tarek will encounter an amount much higher than the safe LAS concentration predicted for larvae. This situation may adversely affect the reproduction of tarek that will enter these rivers to spawn.

4. Conclusions

In conclusion, the existence of substances containing LAS in freshwater environments in ever-increasing amounts affects all organisms in the aquatic environment at different levels. Tarek larvae were exposed to sublethal concentrations of LAS and examined in terms of biochemical parameters and problems were revealed. It is necessary to treat wastewater discharged into rivers and to monitor in terms of LAS amount. In freshwater environments where the species reproduces, the LAS concentration should not exceed 0.049 mg L^{-1} for larvae.

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Effects of Harvesting Stages and Additives on the Chemical Composition, Fermentation Quality and Relative Feed Value of Soybean Silages Varieties

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ABSTRACT

In this study, the chemical composition, silage fermentation quality and relative feed value of soybean silages varieties, namely Adasoy (A), Derry (D) and Yeşilsoy (Y), with Pioneer11C33 (I), molasses (M) and cracked wheat (W) additives at the three harvesting stages, i.e., full flowering (R2), full pod (R4) and full seed (R6) stages, were determined. It was observed that the A and D varieties were the best silage materials for the production of good quality silage in terms of both nutrient contents such as DM and CP and fermentation criteria such as LA and BA concentrations during the R4 and R6 harvest periods. Molasses and cracked wheat additives significantly improved the fermentation qualities of all soybean varieties ($P<0.05$), but the fermentation effect of the inoculant on silages was less than that of molasses and cracked wheat. The harvesting of soybean varieties in the full seed and applying molasses or cracked wheat as an additive optimally improves silage characteristics and results in well-preserved silage.

Keywords: Soybean, Lactic acid bacteria, Molasses, Fermentation quality, Interactions

1. Introduction

The soybean, as one of the most important agricultural products of Asian countries, has been the staple food of the people of that region for centuries. It was originally cultivated in northern Asia and, in recent decades, in North America and the countries of the Southern Cone of Latin America (Pratapet al. 2016). Brazil is the largest producer of soybeans, accounting for about 37.6% of world production, followed by the United States (30.7%), Argentina (12.9%), China (5%) and India (3%) (Soystats 2022). Turkish soybean production in 2021 totaled 182 thousand tons, with a 17.2% increase in production over 2020. The major provincial producers of soybeans in Turkey include Adana province constituting around 67.2%, followed by Mersin province (16.8%) (Tüik 2019). In Turkey, it can be grown as a main crop and a secondary crop since there are a variety of genotypes in terms of maturation times. With the rapid development of animal husbandry, high quality green feed demand throughout the year has been increasing (Hisham et al. 2022). Increasing the productivity of pasture and production of forage crops are of great importance in closing the current forage deficit. Silage preserves fresh grass for longer, reduces nutrient loss and is more digestible by ruminants in the absence of green forage (Kung et al. 2018). The soybean is well-adapted to dry conditions, has high grain productivity, with high protein content, and low fiber to protein ratio (Garcia 2020). Forage soybeans have an important place among legumes due to their high nutritional value and low cost (Chang et al. 2012; Zambom et al. 2012; Asekowa et al. 2014). The most important conservation methods in feed legumes are ensiling and drying. As they possess higher CP concentrations and relatively high concentrations of organic acids and cations, legumes such as alfalfa, vetch and soybean have a high buffering capacity. Moreover, they contain low levels of water-soluble carbohydrates and, therefore, limited substrate is available for fermentation by lactic acid bacteria (McAllister et al. 1998; Hartinger at al. 2019). Moisture content is vital for fermentation in the silo. For soybeans, this ideal moisture occurs just before the pods are full. Waiting until the soybean has achieved fully maturity results in higher dry matter and lower digestible feed that can lead to fermentation problems due to the high oil content of the seeds (Garcia 2020). Due to the above-mentioned factors, it resists the rapid pH decrease and storage stability of forage legumes from being ensiled and classifies them as difficult forages to ensilage. Forage soybean quality varies according to variety, growing stage and harvest losses. When legumes are harvested at the appropriate dry matter content and the appropriate additives are used, high quality silages can be produced. Therefore, the questions addressed or examined in this study include (1) what is the best period to harvest in order to produce good quality soybean silage, (2) will the inoculant, molasses and cracked wheat used as additives improve the silage fermentation quality (3) which is the best silage material to produce high quality silages among the three of soybean varieties, namely Adasoy, Derry and Yeşilsoy, grown as a second crop in the Eastern Mediterranean conditions.

2. Material and Methods

2.1. Field trial

The field experiment was carried out at the East Mediterranean Agricultural Research Institute (EMARI), ($36^0$50' N, $35^0$34' E, 12 m above sea level) in Dogankent town, trial area/location, Adana province, Turkey. The mean temperatures of the months of June, July, August and September, when the research was carried out, was determined to be between 26.5 and 27.4 ºC, and the total precipitation values were determined as 83.9- and 49.5-mm. The average relative humidity values were found to be between 66.6% and 69.0%, and the soil organic matter content in this area is at a low level of 1.07% and has a clay loam structure.

2.2. Experimental materials

In this study, carried out in the experimental field of the Eastern Mediterranean Agricultural Research Institute, Derry and Yeşilsoy were used as the soybean varieties for feed, and Adasoy was used for the grain varieties.

Adasoy (A) variety: It is a medium late soybean variety registered as grain by the Eastern Mediterranean Agricultural Research Institute. This variety is in the IV maturation group.

Derry (D) variety: It is a forage soybean variety developed in 1997 with the USDA-ARS breeding program. It is in the VI maturation group.

Yeşilsoy (Y) variety: It has been registered as silage by the Eastern Mediterranean Agricultural Research Institute. It is in the V maturation group.

Chopped forages were treated with 1) control-additive free (C); 2) Pioneer 11C33 produced by Pioneer Hi-Bred International, Des Moines, IA containing lactic acid bacteria, namely *Lactobacillus buchneri*, *Lactobacillus plantarum* and, *Enterococcus faecium*. Pioneer 11C33 inoculant was applied at the recommended rates; that is, 5 mg/kg of fresh forage; 3) Sugar beet molasses (M) containing 75% DM and 65% sucrose on a DM basis, was applied at 4% of fresh material; 4) The cracked wheat (W) which was added on the fresh material at a rate of 4%.

2.3. Experimental design

The plantings were arranged in a four-replication split plot design. The soybean varieties were the main plots and included three varieties, i.e., Adasoy (A), Derry (D) and Yeşilsoy (Y). Harvesting of soybean varieties was done in 3 different stages, i.e., the full flowering (R2), full pod (R4) and full seed (R6). Soybean varieties were ensiled with additive-free (C), Pioneer 11C33 (I), molasses (M) and cracked wheat (W) during all harvesting stages.

The soybean forage in each plot was cut at a height of 5 cm from the ground to determine their fresh weight and samples of approximately 500 g were collected and then dried in an oven at 65 °C for 48 hours to determine the DM content (Martin et al. 1990). The soybean herbage was chopped into 2 cm lengths using a forage chopper and filled into a 5 kg polyethylene bottle with a screw cap. The preparation of silages was as mentioned below: Silage treatments included control (no additives), 0.005 g/kg inoculant, 4% molasses, and 4% cracked wheat of fresh forage according to the instructions and, the same level of distilled water used during the preparation of the other groups was added to the group without additives. A total of 144 bottles of soybean silage were prepared and fermented for 60 days at a temperature of 25±2 ºC.

2.4. Determination of silage fermentation quality and chemical composition

The pH of the silage was determined with a pH meter after homogenization of 10 g of silage with 100 ml of distilled water for 1 min in a blender (Chen et al. 1994). Then, liquid was filtered using Whatman paper and the liquid was stored at -20 ºC. After the liquid of the silage was filtered through 0.22 mm membrane filter, content lactic acid and volatile fatty acids were determined with HPLC (Agilent 1100, Agilent Technologies Inc.). The chromatographic conditions were as follows: The Hi-Plex H column (7.7x300 mm, 8µm) was selected, column temperature was 50 ºC, the mobile phase was 0.004 M H_2SO_4, the flow rate was 0.6 mL/min, the detective wavelength was 210 nm, and the injection volume was 20 µl (Muck & Dickerson 1988).

Crude protein was calculated by multiplying N measurements obtained from a Kjeldahl N analyzer by 6.25 (AOAC 1990). The methods of Van Soest et al. (1991) were used for the NDF and ADF analysis. Amylase and sodium sulphite were used in the analysis of NDF, and the results were expressed on a dry matter basis, including the ash content.

The digestible dry matter (DMD), dry matter intake (DMI) and relative feed value (RFV) were calculated using the following equations (Rohweder 1978):

Digestible dry matter (DDM%) = 88.9 - (0.779 x ADF%)

Dry matter intake (DMI%) = 120 / NDF%

Relative feed value (RFV) = (DDM% x DMI%) / 1.29

2.5. Statistical analysis

SAS version 9.4 (2020) was used as a program for statistical analysis. The data analyzed included the chemical composition, fermentation quality and relative feed value of the silage. Duncan's multiple comparison test and LS-Means were applied to compare differences between means.

3. Results

The variance analysis results on the differences in chemical composition, fermentation quality and relative feed value of silage are presented in Table 1. The analysis of variance indicated that there were significant differences on the parameters in each variable within the soybean varieties, harvesting stage and additives. Therefore, in addition to the effects of the factors, the effects of the interactions are also important and should be considered together.

Table 1- Variance analysis of the differences in chemical composition, fermentation quality and relative feed value of the silage

	Chemical composition				Fermentation quality					Relative feed value		
Variable	DM	CP	ADF	NDF	pH	LA	PA	BA	AA	DMD	DMI	RFV
Within the variety	128.96**	29.83**	18.89**	28.02**	8.34**	80.49**	17.23**	12.18**	12.09**	18.78**	22.15**	21.55**
Within the stages	459.28**	17.33**	10.02**	19.74**	103.20**	9.73**	52.93**	72.31**	132.02**	9.93**	15.37**	11.56**
Within the additive	129.87**	13.28**	44.46**	50.41**	70.78**	138.29**	44.49**	156.72**	74.64**	44.45**	46.12**	51.31**
Variety x stage	10.90**	8.49**	3.73*	-	-	7.90**	-	4.73**	-	3.70*	-	-
Variety x additive	4.39**	-	-	2.92*	2.56*	6.48**	4.46**	-	4.71**	-	-	-
Stage x additive	3.04**	5.13**	-	-	-	5.88**	10.24**	8.40**	4.12**	-	-	-
Variety x stage x additive	-	-	-	-	2.12*	-	-	-	3.17**	-	-	-

*: indicates significant differences at the 0.05 level; **: indicates significant differences at the 0.01 level and "-" indicates no interaction. DM: dry matter; CP: crude protein; ADF:acid detergent fiber; NDF:neutral detergent fiber; LA: lactic acid; PA: propionic acid; BA: butyric acid; AA: acetic acid; DMD: digestible dry matter; DMI: dry matter intake; RFV: relative feed value.

Table 2- Differences in the chemical composition, silage fermentation quality and relative feed quality within the soybean varieties, harvesting stage and additives

		Chemical composition				Fermentation quality					Relative feed quality		
Factor	Ex	DM, %	CP, %	ADF, %	NDF, %	pH value	LA	PA	BA	AA	DMD,%	DMI	RFV
Variety	A	27.38±0.18b	14.33±0.22a	40.89±0.48c	47.32±0.47c	4.66±0.03a	3.70±0.05a	0.44±0.01b	0.39±0.01b	0.51±0.02a	57.04±0.38a	2.56±0.02a	113.88±1.68a
	D	28.30±0.18a	12.11±0.22b	45.03±0.48a	52.22±0.47a	4.47±0.03b	3.42±0.05b	0.44±0.01b	0.38±0.01b	0.44±0.02b	53.83±0.38c	2.34±0.02c	95.54±1.68c
	Y	24.38±0.18c	13.95±0.22a	42.31±0.48b	49.09±0.47b	4.68±0.03a	2.89±0.05c	0.52±0.01a	0.45±0.01a	0.55±0.02a	55.94±0.38b	2.49±0.02b	108.62±1.68b
Stage	R2	22.59±0.18c	12.93±0.22b	44.35±0.48a	50.31±0.47a	5.01±0.04a	3.23±0.05b	0.56±0.01a	0.51±0.01a	0.70±0.02a	54.36±0.38b	2.43±0.02b	103.22±1.68b
	R4	27.19±0.18b	14.51±0.22a	42.59±0.48b	51.12±0.47a	4.58±0.04b	3.28±0.05b	0.42±0.01b	0.38±0.01b	0.43±0.02b	55.72±0.38a	2.39±0.02b	104.24±1.68b
	R6	30.29±0.18a	12.96±0.22b	41.29±0.48c	47.18±0.47b	4.22±0.04c	3.50±0.05a	0.42±0.01b	0.34±0.01c	0.37±0.02c	56.73±0.38a	2.57±0.02a	113.57±1.68a
Additive	C	24.75±0.21b	12.74±0.25b	44.95±0.56b	52.02±0.54a	4.84±0.04a	2.52±0.05d	0.58±0.01a	0.60±0.01a	0.69±0.02a	53.89±0.44b	2.33±0.03c	97.97±1.94c
	M	29.01±0.21a	14.30±0.25a	39.07±0.56c	45.35±0.54c	4.09±0.04c	3.91±0.05a	0.40±0.01c	0.28±0.01c	0.33±0.02d	58.47±0.44a	2.68±0.03a	122.22±1.94a
	W	28.47±0.21a	14.21±0.25a	40.15±0.56c	47.30±0.54b	4.56±0.04b	3.74±0.05b	0.40±0.01c	0.30±0.01c	0.43±0.02c	57.62±0.44a	2.57±0.03b	115.01±1.94b
	I	24.53±0.21b	12.60±0.25b	46.81±0.56a	53.48±0.54a	4.93±0.04a	3.18±0.05c	0.50±0.01b	0.46±0.01b	0.55±0.02b	52.44±0.44c	2.28±0.03c	92.83±1.94c

Different letters in the same column mean significantly differences at P<0.05. DM: dry matter; CP crude protein: ADF:Acid detergent fiber, NDF: Neutral detergent fiber, LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; DMD: Digestible dry matter; DMI: Dry matter intake; RFV: Relative feed value, A:Adasoy; D: Derry; Y:Yeşilsoy; R2:Full flowering; R4:Full pod; R6:Full seed; C: Additive free (Control); M:Molasses, W:Cracked wheat; I:Pioneer11C33(*Lactobacillus buchneri, Lactobacillus plantarum, Enterococcus faecium*)

Table 3- Differences in the chemical composition, silage fermentation quality and relative feed quality for the interaction of soybean variety and additives

		Chemical composition				Fermentation quality					Relative feed quality		
V	A	DM, %	CP, %	ADF, %	NDF, %	pH value	LA	PA	BA	AA	DMD,%	DMI	RFV
A	C	25.79±0.36d	13.66±0.44	42.45±0.97	49.23±0.94de	4.94±0.08a	3.12±0.09f	0.50±0.02c	0.60±0.02	0.69±0.03b	55.83±0.75	2.45±0.05	106.88±3.36
	M	29.79±0.36b	15.18±0.44	36.85±0.97	43.77±0.94g	4.04±0.08e	4.28±0.09a	0.39±0.02d	0.27±0.02	0.32±0.03g	60.18±0.75	2.77±0.05	129.33±3.36
	W	28.85±0.36b	14.58±0.44	40.04±0.97	46.66±0.94ef	4.68±0.08bc	3.77±0.09c	0.38±0.02d	0.30±0.02	0.51±0.03cd	57.70±0.75	2.59±0.05	115.96±3.36
	I	25.10±0.36e	13.92±0.44	44.22±0.97	49.60±0.94de	4.98±0.08a	3.64±0.09cd	0.52±0.02bc	0.43±0.02	0.52±0.03cd	54.45±0.75	2.45±0.05	103.33±3.36
D	C	26.39±0.36d	11.70±0.44	48.10±0.97	55.40±0.94ab	4.60±0.08c	2.53±0.09g	0.57±0.02b	0.58±0.02	0.57±0.03c	51.45±0.75	2.20±0.05	87.67±3.36
	M	29.65±0.36b	12.65±0.44	41.40±0.97	47.93±0.94e	4.14±0.08de	3.83±0.09bc	0.38±0.02d	0.27±0.02	0.34±0.03fg	56.65±0.75	2.53±0.05	111.96±3.36
	W	31.05±0.36a	13.12±0.44	40.47±0.97	47.69±0.94e	4.31±0.08d	4.05±0.09ab	0.40±0.02d	0.24±0.02	0.38±0.03fg	57.38±0.75	2.55±0.05	113.83±3.36
	I	26.15±0.36d	10.97±0.44	50.15±0.97	57.84±0.94a	4.85±0.08ab	3.27±0.09ef	0.40±0.02d	0.43±0.02	0.49±0.03de	49.84±0.75	2.09±0.05	80.71±3.36
Y	C	22.06±0.36f	12.87±0.44	44.30±0.97	51.44±0.94cd	4.96±0.08a	1.92±0.09h	0.67±0.02a	0.64±0.02	0.80±0.03a	54.39±0.75	2.35±0.05	99.38±3.36
	M	27.58±0.36c	15.06±0.44	38.94±0.97	44.34±0.94fg	4.09±0.08e	3.60±0.09cd	0.43±0.02d	0.30±0.02	0.35±0.03fg	58.56±0.75	2.73±0.05	125.38±3.36
	W	25.53±0.36de	14.94±0.44	39.93±0.97	47.55±0.94e	4.70±0.08bc	3.41±0.09de	0.41±0.02d	0.34±0.02	0.41±0.03ef	57.79±0.75	2.56±0.05	115.25±3.36
	I	22.34±0.36f	12.92±0.44	46.06±0.97	53.01±0.94bc	4.95±0.08a	2.63±0.09g	0.57±0.02b	0.52±0.02	0.64±0.03b	53.02±0.75	2.29±0.05	94.46±3.36

Different letters in the same column mean significantly differences at P<0.05. DM: dry matter; CP crude protein: ADF:Acid detergent fiber, NDF: Neutral detergent fiber, LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; DMD: Digestible dry matter; DMI: Dry matter intake; RFV: Relative feed value; A:Adasoy; D: Derry; Y:Yeşilsoy; R2:Full flowering; R4:Full pod; R6:Full seed; C: Additve free (Control); M:Molasses, W:Cracked wheat; I:Pioneer11C33 (*Lactobacillus buchneri, Lactobacillus plantarum, Enterococcus faecium*)

3.1. Differences in the chemical composition, fermentation quality and relative feed value of silages for the single factor

3.1.1. Soybean varieties (V)

The mean DM, ADF and NDF of the D variety at different harvesting stages were significantly higher than that of the A and Y varieties. Compared with the D variety, the average content the CP of the A and Y varieties was significantly greater. Both the low DM and high HP content of the Y variety negatively affected the fermentation quality compared to the other two varieties. The mean pH of the D variety was significantly lower than the A and Y varieties at different harvesting stages and additives, but the average LA levels of the A varieties were significantly higher than that of the D and Y variety. Compared with the Y variety, the A variety had higher LA content and lower BA and PA content, indicating that the fermentation quality of A was better than that of the Y variety. The mean DMD, DMI and RFV of the A variety at different harvesting stages was significantly higher than that of the D and Y varieties (P<0.05; Table 2).

3.1.2. Harvesting stage (S)

With the advancing maturity, the mean dry matter content of the soybean varieties gradually increased and reached the optimum dry matter content in order to produce quality silage at the R6 stage. The highest average CP content was observed in the R4 stage during the harvesting stages (P<0.05). The mean pH of soybean varieties ensiled with different additives had the lowest value at the R6 stage, followed by R4, both significantly lower than pH values at the R2 harvest stage (P<0.05). With the delay of the harvesting period and with the increase of maturing, the average LA content increased, while the concentration of PA, BA and AA significantly decreased. In terms of DMD and other relative feed value parameters, the best harvesting stage for the three silages was the R6 stage (Table 2).

3.1.3. Additives (A)

In relation to the different additive treatments, significant differences were observed for the mean nutrient composition, fermentation quality and relative feed value parameters among the different varieties harvested at different stages. While the M and W additives increased the DM content of the silages, they decreased the ADF and NDF content. The mean pH value, PA, BA and AA concentrations of the M and W treatments were significantly lower than the ones in C and I treatments. Compared with C treatment, the mean concentration for LA of I treatment was significantly higher. The opposite was observed for PA, BA, and AA; this indicated that the ensiling with additive I had a better fermentation quality than ensiling without additives (P<0.05). Table 2 showcases that the mean silage fermentation quality of the M treatment was the best, followed by W, and the fermentation quality of W treatment was significantly higher than that of I and C (P<0.05). The addition of M and W additives to the silages increased the DMD, DMI and RFV values (P<0.05) but these values of the silages were not affected by the addition of I additive in comparison to the control silage.

3.2. The interactions

3.2.1. Soybean varieties (V) x harvesting stages (S)

The V x S interaction was not significant (P>0.05) for most of the measured components, with the exception of the DM, CP, ADF, LA, BA concentrations and DMD% (Table 1 and Figure 1). With the advancing maturity, the DM of the soybean varieties increased gradually, and at the R6 stage the DM contents ranged from 27.71% to 32.53%, the most suitable DM to produce a quality silage. The CP content of silage harvested at the R4 stage was significantly higher than that of R2, because the R4 stage soybeans possessed well-developed leaves and pods. Regarding the Vx S interaction, the ADF concentration decreased from the early to the late stage of the harvest in all soybean varieties.

For the three silages with different additives at stages R2, R4 and R6, no differences were found for the average pH, AA and PA concentrations. Silage LA and BA concentrations were affected by the V x S interaction. The LA of the all-soybean varieties increased with the stage of harvest, although the increase was higher with the D and Y varieties in R6 harvesting stage than with the D and Y varieties in R4 harvesting stage (P<0.05). The mean BA concentration of the Y variety with different additives at the R2 and R4 stages was significantly higher than that of the A and D varieties.

Silages digestible dry matter ranged from 51.92% to 58.38%. The highest value was calculated for the silage from the A variety at the R4 and R6 harvesting stage. The lowest value was calculated for the silage from the D variety for all the harvesting stages. No significant differences were found in the dry matter intakes and relative nutritive values of the silages of the three soybean varieties at stages R2, R4 and R6.

3.2.2. Soybean varieties (V) x additives (A)

The differences in the chemical composition, silage fermentation quality and relative feed quality for the interaction of soybean variety and additives are shown in Table 3. Interactions were detected between variety and additives for DM (P<0.01), NDF (P<0.05), pH (P<0.05), LA (P<0.01), PA (P<0.01) and AA (P<0.01). The DM was the highest in the D variety cracked wheat additives (31.05%) followed by the A variety with cracked wheat (28.85%) and molasses (29.79%) additives in silages. Furthermore, the effect of cracked wheat was greater in the D variety than that of the A and Y varieties.

Treatment with M resulted in lower pH (4.14 for D variety) compared to C, it was even lower (P<0.05) in other varieties (4.04 for A and 4.09 for Y). Treatment with all additives resulted in significantly higher (P<0.001) LA concentrations than C silages (Table 3). In addition, among the three varieties, when the A variety was ensiled with molasses and the D variety with wheat cracked, the LA concentration was found to be higher compared to other silages. PA concentrations in soybean varieties were also significantly (P<0.001) reduced by M and W additives; treatment with M and W was more effective (P<0.001) in reducing PA concentration in the Y variety than in the A and D varieties. Compared to AxC (0.69%), DxC (0.57%) and YxC (0.80%), concentrations of AA were lower (P<0.001) in additive-treated silages (range 0.32% to 0.52%).

3.2.3. Harvesting stage (S) x additives (A)

The interaction of harvesting stage and type of additives on silage chemical composition and quality is presented in Figure 1. Their interaction significantly affected silage DM, CP, LA, PA, BA and AA concentrations (P<0.001; Table 1). DM recorded in silage with no additives and, I additives in the R2 harvesting stage was significantly lower than others. With the progress of the harvest period, the DM content of all silages with additives increased compared to the silages without additives. CP content was significantly higher in M added silage in R2 stage and W added in R4 stage than others. The lowest CP was recorded in I added silage R2 harvesting stage (10.92%; Figure 2).

The effect of M and W additives on silage LA, PA, BA and AA content was stronger than that of I additive, and this also depended on the harvesting stage.

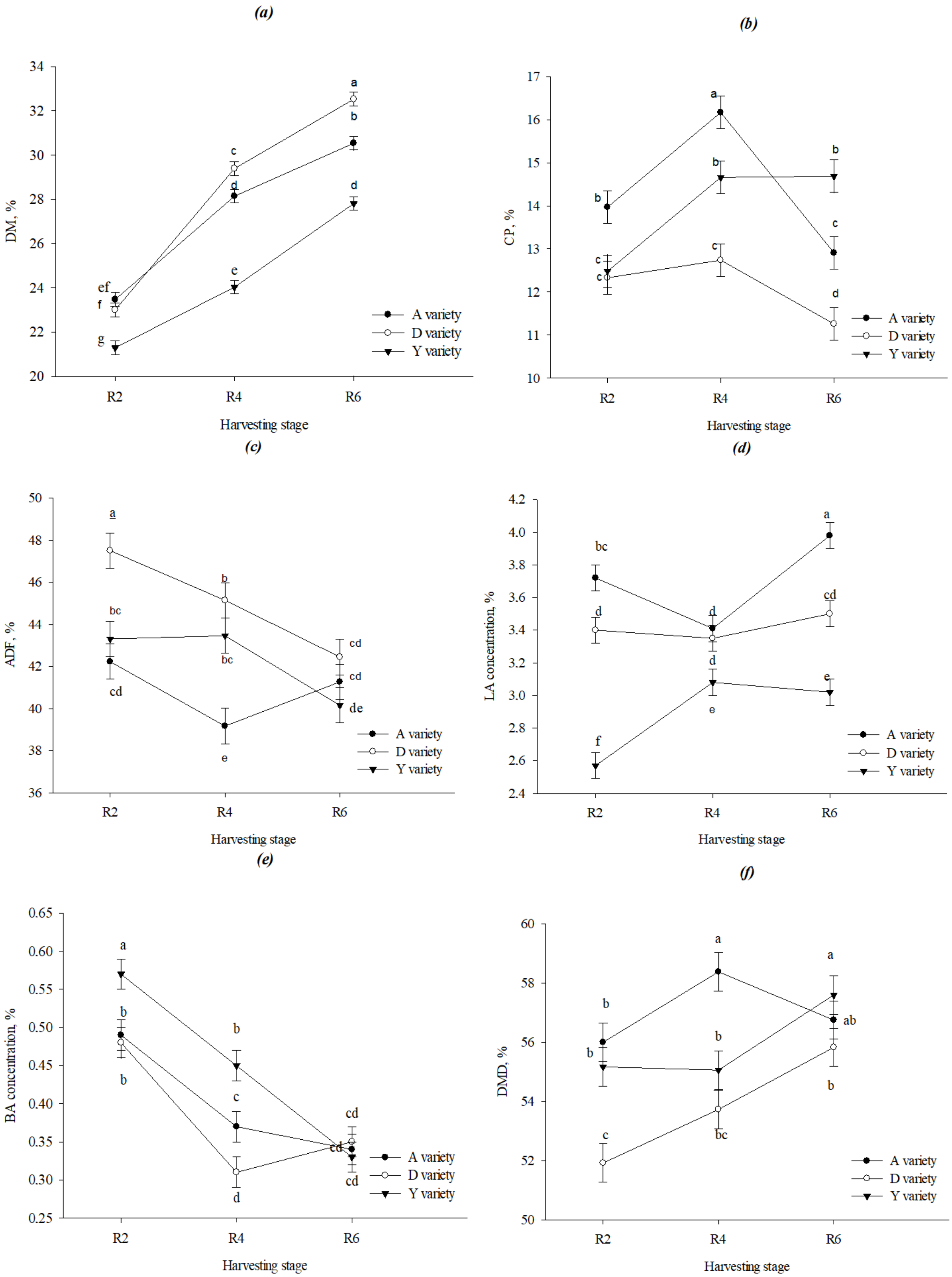


Figure 1- Differences in the chemical composition, silage fermentation quality and relative feed quality for the interaction of soybean variety and harvesting stage. Different letters in each figure mean significantly differences at P<0.05. A: Adasoy; D: Derry; Y: Yesilsoy; R2: full flowering; R4: full pod; R6: full seed

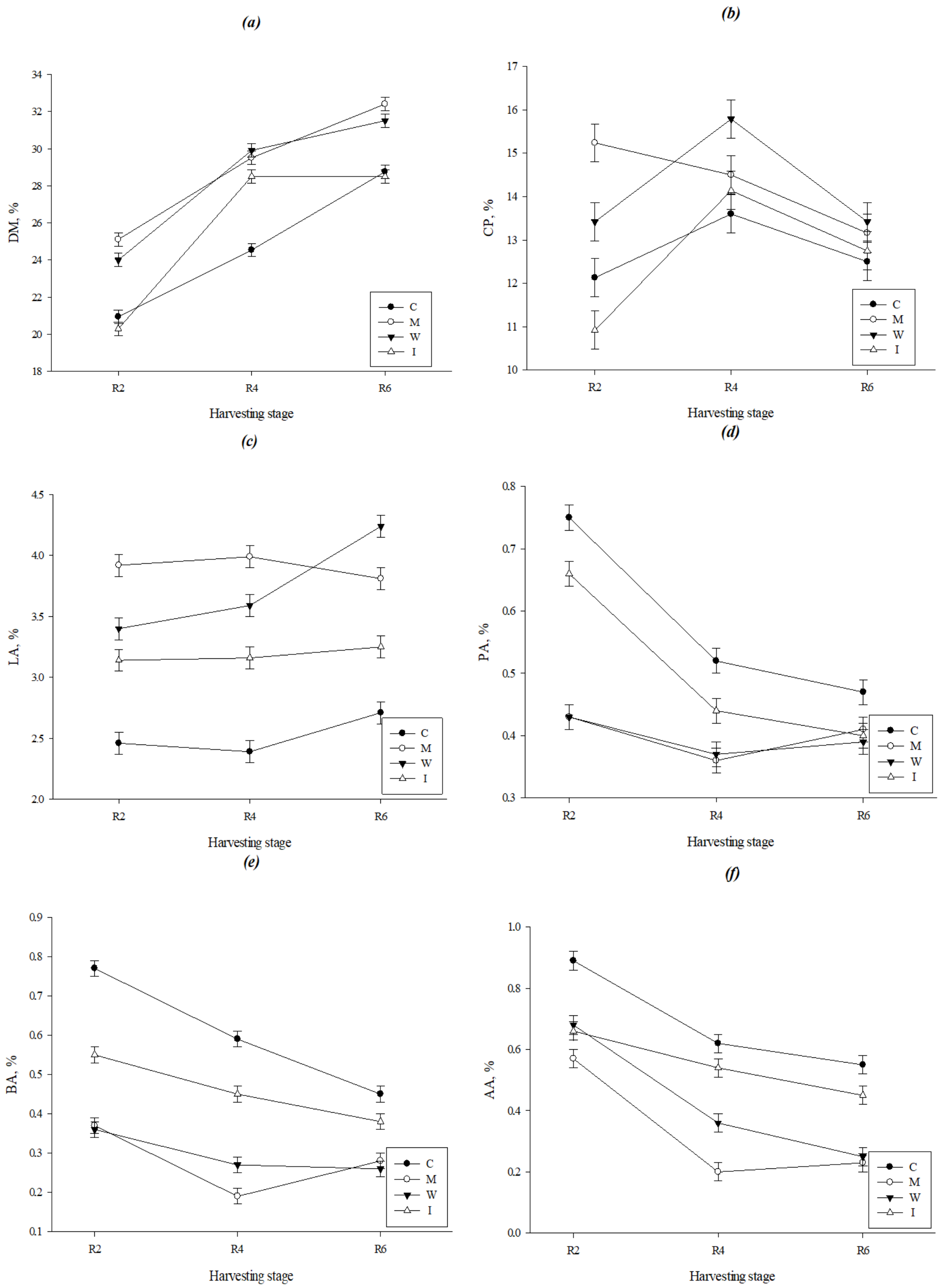


Figure 2- Differences in the chemical composition (a and b) and silage fermentation quality (c, d, e and f) for the interaction between harvesting stage and additives. R2: full flowering; R4: full pod; R6: full seed; C: control-additive-free treatment; M: molasses; W: cracked wheat; I: Pioneer11C33

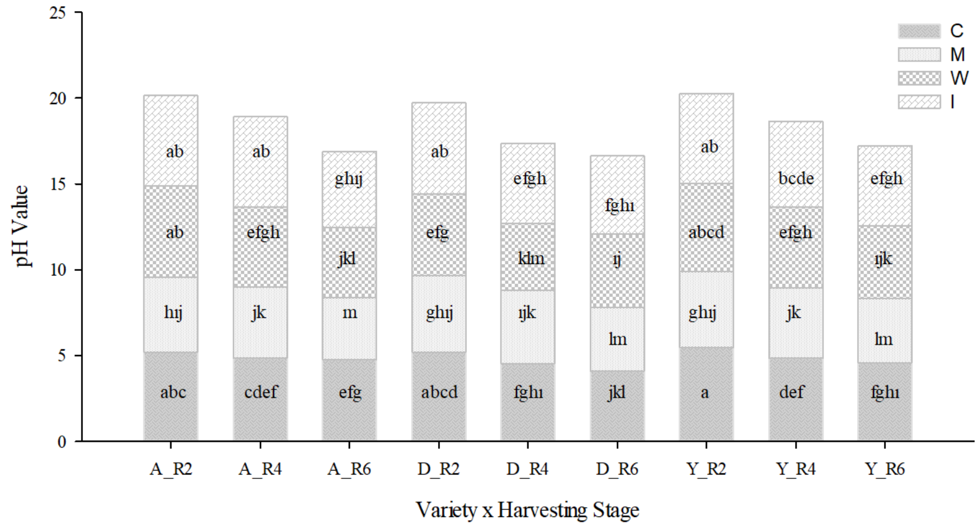
3.2.4. The interactions of the soybean variety, harvesting stages and additives

At the R6 stages, the silage pH of the A, D and Y soybean silages with molasses additives was significantly lower than that of other harvesting stages and additives. In all varieties and at the R2 harvest stage, the addition of inoculant caused a significant increase in pH, one of the silage fermentation criteria, followed by silage without additives. Molasses and cracked wheat used as additives exerted a significant effect in reducing pH and acetic acid concentration in all soybean varieties and in the R6 harvest period ($P<0.05$) (Figure 3a, b). In conclusion, the addition of molasses to all soybean silages, harvested at the R6 stage, improved the silage fermentation characteristics of silages, but had no positive effects on digestible dry matter.

3.2.5. Correlations between different soybean silage compositions

Silage pH was negatively correlated with DM content and LA concentration, but was positively correlated with AA, BA and PA concentrations. Among all the soybean silage composition parameters determined, DM concentration had the greatest correlation with silage pH (r=-0.68, P<0.001, Figure 4), and AA, BA, and PA concentrations were moderately correlated with pH (r=0.54, r=0.54, and r=0.41, respectively, P<0.001). CP content had positive correlations with DMD, DMI and RFV, but negative correlations with ADF and NDF content. A strong negative correlation was observed between ADF and NDF content of silages and DMD, DMI and RFV (r=-1.00, r=-0.81, r=-0.92; r=-0.82, r=-0.81, r=-0.98, r=-0.96, respectively, P<0.001, Figure 4).

(a)



(b)

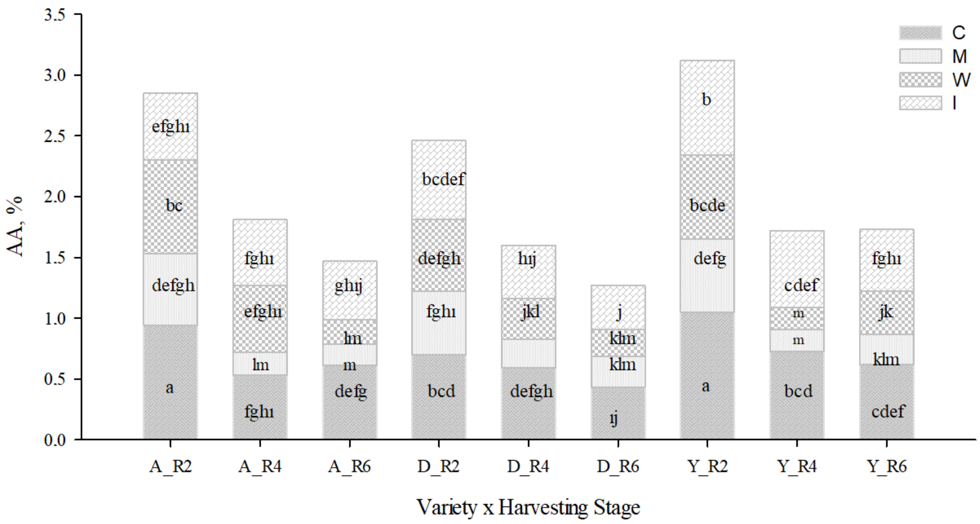


Figure 3- Differences in pH value (a) and acetic acid concentration (b) for the interaction between variety, harvesting stage and additives. Different letters in each figure mean significantly differences at P<0.05. A: Adasoy; D: Derry; Y: Yesilsoy; R2: full flowering; R4: full pod; R6: full seed; C: control-additive-free treatment; M: molasses; W: cracked wheat; I: Pioneer11C33 (*Lactobacillus buchneri, Lactobacillus plantarum, Enterococcus faecium*)

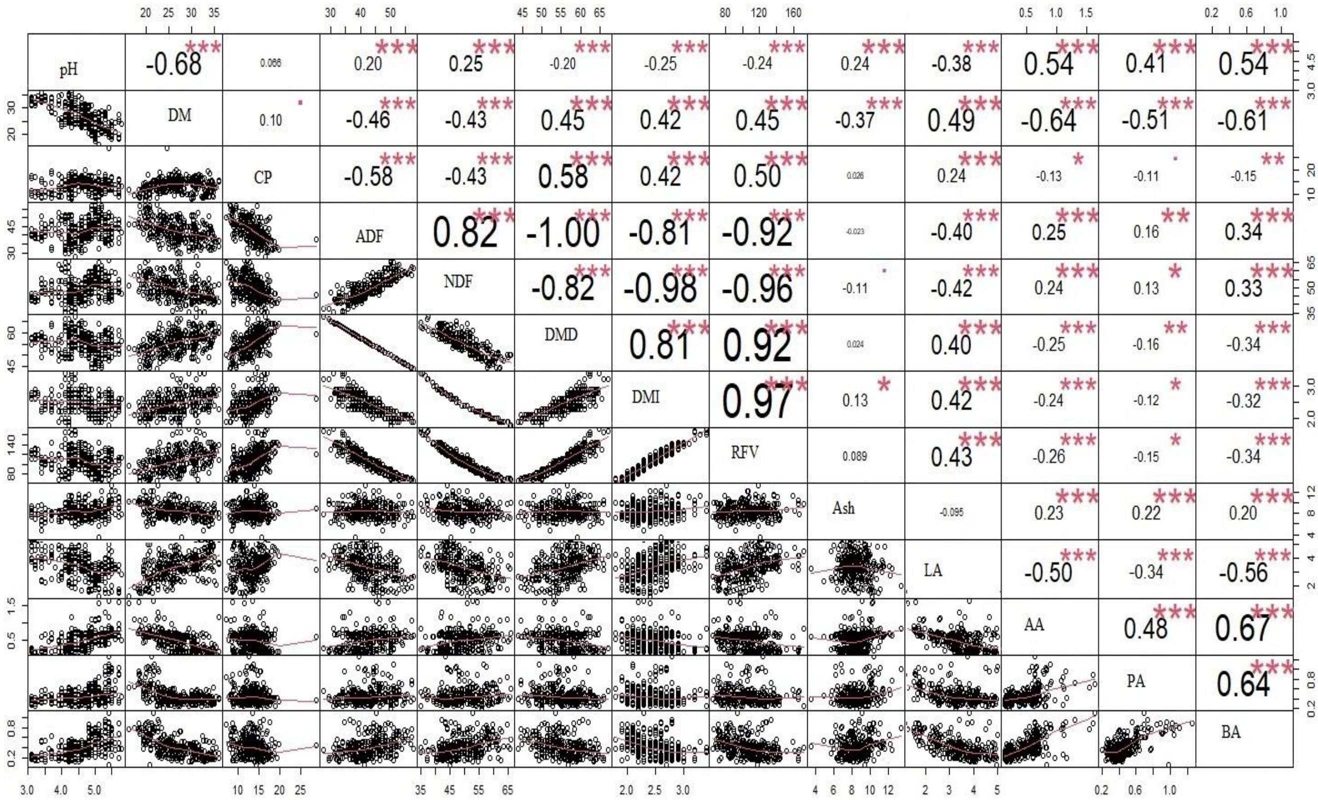


Figure 4- Correlation matrix of soybean silage nutrient composition and fermentation quality. "*, ** and *" indicate significant levels of P<0.05, P<0.01 and P<0.001, respectively**

4. Discussion

The chemical composition and fermentation quality of the silage was significantly influenced by soybean variety, harvesting stage and additives. The dry matter content of forage is an important parameter for optimal silage quality (Borreani et al. 2018). At dry matter contents below 30-35%, high moisture forages (especially legumes) are more susceptible to clostridial fermentation (high levels of butyric acid and/or ammonia-N) (Vissers et al. 2007). Conversely, the use of forage with a dry matter content >45-50% DM in silage should also be avoided, as ensiling and packaging is more difficult and can trap air in the silage mass, causing overheating and nutrient loss. Where conditions allow, the 30-35% DM content of forages makes it difficult for clostridia to dominate ensiling and limits total fermentation acids (Borreani et al. 2007; Kung 2009). In this study, the DM contents of soybean varieties harvested at the full pod and full seed varied from 27.19% to 30.29% (Table 2), which makes it possible to optimize silage quality. It is noteworthy that legume silages often show higher aerobic stability than maize silages, which are prone to aerobic degradation after feeding. However, the high buffering capacity and low soluble carbohydrate concentration in legumes are limiting factors for lactic acid synthesis and rapid pH drop, which we refer to as good fermentation (Bernardi et al. 2019). Silage pH is an indicator of the suitability of optimal fermentation. The higher the lactic acid content of the silage, the lower the pH of the silage. However, this decrease is related to the dry matter content of the silage (Coffey et al. 1995; Li et al. 2022). The composition of silage has a significant impact on the silage fermentation quality and relative feed value. Additionally, acetic and lactic acid concentrations were negatively related to DM content, as reported by Kung et al. (2018). For legume silages with 30-35% DM content, the silage pH is reported to be between 4.2-4.8. In addition, silages at the R6 harvest stage had the highest LA concentration, and the lowest BA concentration and pH value. It is seen that the A and D varieties are the best silage materials to produce good quality silage in terms of both nutrient contents such as DM and CP and fermentation criteria such as LA and BA concentrations during the R4 and R6 harvest periods (Figure 1). Spanghero et al. (2015), in their study on the effect of harvesting stages of soybeans on the chemical composition of silage, showed that the favourable stages for harvesting whole plants are from R4 to R6, as they have a high nutritional value for ensiling.

The addition of fermentable nutrients such as molasses and broken grains, and inoculants during ensiling lowers the pH, increases the LAB count and lactic acid content, and competitively inhibits harmful bacteria (Xia et al. 2018; Li et al. 2021; Wang et al.2021). The content of water-soluble carbohydrates required for proper fermentation in legume plants is low (Blount et al 2006). Therefore, the addition of a readily fermentable source of sugars, such as molasses, and/or the use of microbial inoculants can help to ensure adequate fermentation during the ensiling of soybeans. In this study, similar results were obtained, showing that the addition of molasses, cracked wheat and inoculant significantly increased the LA content and decreased the pH and AA content in silages of soybean varieties (Table 3), which agrees with Mahana and Chase (2003) and Rosa et al. (2018).

In the present study, when soybean varieties, harvest stages and additives were considered as single factors, differences in relative feed values were found to be significant and, in terms of quality class, variety A, R6 harvest stage and molasses application were found to be higher quality than other varieties, harvest and additives. Compared to other varieties, harvest period and additives, ruminants will have higher voluntary feed intake with the highest DMI in silages made with variety A, R6 harvest stage and molasses application. It was found that the RFV values of soybean silages were similar to the RFV values of corn and corn-soybean silages reported by Kızılşimşek et al. (2020), but lower than the RFV value of soybean and cowpea silages reported by Gülümser et al. (2021).

5. Conclusions

Different soybean varieties, harvesting stages, and additives had significant effects on the nutritional composition, and silage fermentation quality. The optimal harvesting stage for soybean varieties to produce quality silages was the full seed. Almost all additives improved the silage fermentation qualities of the soybean varieties. Molasses and cracked wheat additives were the best silages in terms of silage fermentation quality criteria. The A and D varieties of soybean are the best raw material to produce quality silages. Overall, a good quality silage could be produced by using the A and D varieties as silage material, harvesting at R6 stage and adding molasses and cracked wheat as additives.

Funding

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Influence of Solid and Liquid Red California Vermicompost (*Eisenia foetida*) on Growth and Yield of Lettuce (*Lactuca sativa* var. *crispa* L.)

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ABSTRACT
The intensive use of chemical inputs in the agricultural field has reduced soil fertility as well as affected human health and the environment. To overcome these problems, environmentally friendly alternatives such as vermicompost applications should be used. Vermicomposting is an eco-friendly way in which earthworms convert organic residues into compost and contribute to plant growth and development. This is the first study of two forms of vermicompost applied as solid and liquid on plant growth and fungicide tolerance in lettuce. In this study, barnyard manure was composted with the Red California earthworm *Eisenia foetida* (Lumbricidae). The effects of different doses (0, 10, 20 and 30 %) of solid vermicompost and commercial liquid vermicompost were investigated on some growth and yield parameters (leaf length, leaf width, SPAD chlorophyll amount, number of marketable leaves, number of discarded leaves, leaf pH, leaf nitrogen content and fungicide residue values) of lettuce (*Lactuca sativa* var. *crispa* L.) grown under greenhouse conditions. The parameters were statistically significant for vermicompost type and dose for all results except leaf pH, number of discarded leaves and fungicide residue analysis. The results of solid vermicompost application were higher than control and commercial liquid vermicompost in terms of all parameters. The highest leaf length and width values were determined at a 20% dose of solid vermicompost, while the highest SPAD chlorophyll value and leaf nitrogen values were determined at a 30% dose. The number of marketable leaves was higher in all doses of solid vermicompost application. However, there was no statistically significant difference in fungicide residue analyses in terms of vermicompost types, dose, and vermicompost type-dose interactions. Fungicide residue levels were detected above the MRL (maximum residue limits) in all samples.

Keywords: Earthworm, Growth, Fungicide residue, Fungicide tolerance, Sustainable agriculture

1. Introduction

Rapid human population growth leads to increased demand for food (Nauman et al. 2020). To meet this demand, the use of fertilization and pesticide in agriculture are among the most widely used methods. It is known that mindless and excessive chemical fertilization can *adversely affect* pH, organic matter, nutrients, and structure of soil (Gill & Garg 2014; Bisen et al. 2015). However, it is necessary to add the substances to the soil to ensure the continuity of the development of the plants (Li & Marschner 2019). This concern has increased research on reducing the use of chemicals in agriculture and developing alternative methods. Vermicompost process is a biological oxidation method in which fermented waste is converted into a peat-like product with beneficial microbial activity, which provides high aeration, a high-water retention rate (Dominguez & Edwards 2011). Vermicomposting is an important production system that avoids the use of inorganic fertilizers and pesticides (Yatoo et al. 2021). Different forms of vermicompost have the potential to be used for plant growth, yield, disease, and pest control (Öztürkci & Akköprü 2021). Many studies about solid and liquid forms of vermicompost have indicated that this application can convert plant nutrients into forms beneficial to the plant (Olle 2019; Gül et al. 2021; Öztürkci & Akköprü 2021; Ducasse at al. 2022).

It has been reported that the application of organic fertilizers to the soil at regular intervals will improve the physical and chemical properties of the soil (Al-Amin et al. 2017). Compost applications obtained from organic residues/wastes of plant and animal origin in agricultural production have become widespread in recent years (Hussain et al. 2017). Fermenting organic wastes and turning them into compost, vermicompost, which is formed by adding earthworms to this processing, is an important soil conditioner (Wang et al. 2001; Garg & Gupta 2009; Sharma & Garg 2018). Earthworms have very useful tasks for natural ecosystems and improve the quality of soil by breaking down organic matter into inorganic (Nurhidayati et al. 2018). Vermicompost has an important place in eliminating the negative effects of organic wastes, recycling wastes, and sustainable agriculture methods (Ludibeth et al. 2012; Manyuchi et al. 2013; Bhat et al. 2018). Vermicomposting with a high potential for crop production has been widely used in solid and liquid forms as soil conditioners for the last two decades (Panth et al. 2009;

Yatoo et al. 2021). However, differences in the chemical and microbial properties of the liquid and solid forms of vermicompost may affect its effectiveness (Bademkıran et al. 2018; Franke-Whittle et al. 2019).

Arancon et al. (2020) reported that vermicompost application has shown plant disease suppression. It has been reported that fungal diseases such as *Rhizoctonia, Pythium* and *Verticillium* are significantly reduced in plants growing in environments with vermicompost extracts. It has been stated that this may be related to the presence of biological suppressive agents in vermicompost (Edwards et al. 2004; Datta et al. 2016; Öztürkci & Akköprü 2021). However, it has also been reported that inhibition can be caused by the activation of mechanisms such as competition, antibiosis, hyperparasitism and induced plant resistance (Sarma et al. 2010; Simsek-Ersahin 2011).

Lettuce (*Lactuca sativa* L.), a temperate climate plant of the Asteraceae family, is produced in many countries of the world. Fresh lettuce leaves are an important product in trade (Jimenez-Arias et al. 2019). Lettuce grows quickly in soils rich in organic matter (Zandvakili et al. 2019). Because the intensive application of nitrogen-containing fertilizers can cause harmful effects on humans by causing nitrate accumulation in the plant, it is necessary to pay attention to fertilization when growing lettuce (Santamari 2006).

To our knowledge, although there are separate studies on the effects of solid and liquid vermicompost on the yield of lettuce plants grown in greenhouses, there is no study comparing them together. Moreover, the influence of fungicides on lettuce of solid and liquid worm application has not been explored. Therefore, we compared the effect of solid and liquid vermicompost application on the growth and development of lettuce plant and the amount of fungicide residue in this study. We thought that the resistance that vermicompost application may provide to the plant may influence the amount of fungicide residue.

2. Material and Methods

2.1. Materials

Plant

The Maritima lettuce (*Lactuca sativa* L. var. *crispa*) variety produced by Istanbul Seed Inc. was used in the study. Lettuce is an annual temperate zone plant belonging to the genus *Lactuca* of the family Compositae (Asteraceae). The plant reaches maturity in 2–3 months and grows rapidly in soils rich in organic matter.

Vermicompost

In the study, solid vermicompost was obtained from *Eisenia foetida* (Red California earthworm) from cow manure (separator) placed in 2 × 1.20 m cases (Figure 1a, b). The humidity requirement of the medium was kept between 65-75% with meter device. The solid vermicompost, harvested after about 6 months, was made ready for use after drying and sieving. (Figure 1c). Solid vermicompost analyses were carried out at the Laboratory of Central Research Institute of Soil, Fertilizer and Water Resources of the Ministry of Agriculture and Forestry. Soil analysis was performed at the Tekirdağ Commodity Exchange Analysis Laboratory.

Analysis results of solid vermicompost are given Table 1. According to the analysis of vermicompost formed by composting cow manure, it was determined that solid vermicompost was rich in organic matter, nitrogen, phosphorus, and potassium. The solid vermicompost used in this study had neutral pH, 6% humidity, 50.94% organic matter, 5.94 EC (dS/m) electrical conductivity, 2.02% nitrogen, 1.56% potassium, 1.10% phosphorus, 736.00 ppm calcium, 138.00 ppm magnesium and 196.00 ppm iron. The liquid vermicompost used in the experiment was purchased from Solomcan organic fertilizer company. It was stated that liquid vermicompost had a pH range of 8–9, 8% organic matter, and 1% nitrogen.

Table 1- Analysis results of solid vermicompost

Solid vermicompost	***Results***
pH	7.05
Humidity (%)	60.12
Organic matter (%)	50.94
EC (dS/m)	5.94
Total nitrogen (N)(%)	2.02
Total potassium (K)(%)	1.56
Total phosphorus (P)(%)	1.10
Total calcium (Ca)(ppm)	736.00
Total magnesium (Mg) (ppm)	138.00
Total iron (Fe) (ppm)	196.00

a b c

Figure 1- a) Solid vermicompost case, b) *E. foetida* (Red California Worm) c) Vermicompost

Fungicide

Commercially purchased Captan 50 WP (Wettable Powder) fungicide, which is a form of wettable powder used in lettuce mildew and belongs to the phthalimide class of pesticides, was used in the present study. It is a trichloromethyl sulfonyl-containing fungicide. The recommended dose of fungicide is 300 grams per 100 liters of water.

2.2. Experimental design

Lettuce seeds were sown with 2–3 seeds in each section of the viols prepared by mixing peat and perlite in a ratio 3:1 ratio. The first germination was seen 3–4 days after the seeds were sown. After about 30 days, the healthy seedlings were planted to 3-kg pots filled with soil. The study was carried out in a plastic greenhouse. The floor of the greenhouse is covered with a tarp to prevent the viol and pots from contact with the soil. 48 pots were used, and a seedling was planted in each pot. No fertilization was applied in the study other than vermicompost application. Vermicompost was applied to 48 pots except the control group (0%). 24 of the 48 pots were used to examine the yield characteristics of the plant, and the other 24 were used to determine the fungicide effect. Three different doses (10, 20 and 30) of solid and liquid vermicompost were applied to all pots. At the beginning of the experiment, 300-600-900 g of solid vermicompost was added to 24 pots of 3 kg used in the study at doses of 10, 20 and 30 % and completed to 3 kg. Liquid vermicompost was prepared by adding 0.1 mL to 900 mL water, 0.2 mL to 800 ml water, 0.3 mL to 700 mL water in accordance with the company's recommendation. Liquid vermicompost was applied two times in total, with an interval of one months. The study was carried out in three replications.

All pots were watered two times a week. Watering was performed by spraying five times using a spray bottle. Common tap water was used for irrigation. Lettuce was harvested 60 days after planting in pots. Fungicide (Captan 50 WP) was applied three times by using spray, according to the dosage recommended by the manufacturer. The plants were harvested 7 days after fungicide application.

2.3. Plant measure and analysis

Leaf length and width were measured with a ruler on the harvested plants. The number of discarded leaves was determined by counting the outermost yellowed, spoiled, and rotten leaves. The number of marketable leaves was determined by removing the discarded leaves from the harvested plants and counting the edible leaves. After the plant leaves were ground, the pH of the water obtained from the leaves was measured with a Hachlange HQ40d Multimeter. The chlorophyll amount was measured with a chlorophyll meter (Minolta SPAD-502, Osaka, Japan). Leaf nitrogen was determined by the Kjeldahl method with a Kjectec Auto 1030 Analyser (Tecator, Sweden). Fungicide residue analyses were evaluated by the AOAC 2007.01 method using liquid chromatography-mass spectrophotometer (LC-MS; mg/kg) and gas chromatography-mass spectrometry (GC-MS; mg/kg).

2.4. Statistical analysis

The assumptions of the data were tested with Kolmogorov-Smirnov tests. The homogeneity of the variances of the groups was determined using the Levene test statistic. The differences between the group means were revealed by using independent samples t-test or Mann Whitney U test for paired groups and two-way ANOVA and Welch's ANOVA, or Kruskal-Wallis H test statistics for more than two groups. Tukey post-hoc and Games-Howell test statistics were used to reveal possible differences. Existing differences are presented by the lettering method. The results were evaluated at the 5% significance level. All calculations were performed with SPSS v. 24 statistical software.

3. Results

The descriptive statistics results of all parameters were presented Table 2. According to the results of variance, it was observed that different doses of liquid vermicompost application were statistically significant on the leaf length averages at the 0.01 significance level.

The different doses of solid and liquid vermicompost were found statistically significant at 0.01 significance level on leaf width averages. When the sources of the differences were examined, it was observed that the averages of the leaf length and leaf width variables obtained from the control pots were statistically significant and lower compared to the liquid vermicompost added groups.

The number of marketable leaves was evaluated under the two-way Anova model in terms of vermicompost type and doses (Table 2). Vermicompost type, dose level, and vermicompost type*dose interaction were found to be statistically significant in the number of marketable leaves ($P<0.01$). Accordingly, it was observed that the solid vermicompost application averages (18.45±6.39) were more effective than the liquid averages (11±2.39), and the solid and liquid vermicompost usage averages were statistically significant compared to the control group averages (7.5±2.64 cm).

Chlorophyll amount was higher than control group (13.97±3.64) and statistically significant in 10% (17.83±3.39, $P<0.05$), 20% (22.68±5.38, $P<0.01$) and 30% (18.98±6.01, $P<0.05$) doses of solid vermicompost applications. However, 20% dose (22.68±5.38) of liquid worm compost application was higher and statistically significant than 10% (17.83±3.39) application.

The leaf nitrogen content analysis results have shown that while the interaction between vermicompost type and dose was not significant statistically ($P>0.05$), it was observed that both vermicompost type and dose alone had a statistically significant effect on nitrogen content ($P<0.01$). The nitrogen content of solid vermicompost application (7.01±2.21) was found to be statistically significant and higher than liquid vermicompost (4.03±1.33%) (Table 2). On the other hand, when the statistical differences in dose levels were examined regardless of the vermicompost type, it was observed that the average nitrogen content increased regularly as the dose increased. One source of this difference was the lower nitrogen mean of the control group (2.75±0.07) compared to the 20% and 30% dose mean (5.92±1.97 and 7.5±2.19). The other difference is since the average (7.5±2.19) at the 30% dose of any vermicompost type is higher than the 10% nitrogen average (4.2±1.61).

Two-way Anova results showing descriptive statistics and the effect of vermicompost type and dose on pesticide residue analysis results are presented in Table 2. The amount of fungicide was found no significant differences in terms of vermicompost type, dose and vermicompost type-dose interactions ($P>0.05$). It was no found statistical difference in terms of vermicompost type and doses in discarded leaves and pH ($P>0.05$).

Table 2- The descriptive statistics and two-way ANOVA results

Vermicompost	*Dose*	*Leaf length (cm)*			*Leaf width (cm)*			*Marketable leaf number (pieces/plant)*			*Chlorophyll amount (SPAD)*			*Leaf N Content (%)*			*Fungicide Residue Analysis Results (mg/kg)*		
		n	*M ± Sd*	*CV*	*n*	*M ± Sd*	*CV*	*n*	*M ± Sd*	*CV*	*n*	*M ± Sd*	*CV*	*n*	*M ± Sd*	*CV*	*n*	*M ± Sd*	*CV*
Solid	0%	19	6.54±3.47Aa	1.89	19	3.74±2.08Aa	1.8	2	6.50±2.12Aa	3.06	19	13.19±4.93Aa	2.68	1	2.80±0.00Aa	-	2	0.35±0.26	1.36
	10%	77	14.99±4.09Ba	3.66	77	11.11±3.09Ba	3.59	3	23.67±2.52Ba	9.4	77	23.67±6.86Ba	3.45	2	5.55±0.64ABa	8.72	3	10.51±8.22	1.28
	20%	68	15.73±4.16Ba	3.78	68	11.22±2.85Ba	3.93	3	19.67±1.53Ba	12.87	68	24.45±6.99BCa	3.5	3	7.43±1.46BCa	5.08	3	15.25±10.28	1.48
	30%	67	15.11±4.07Ba	3.72	67	11.21±2.67Ba	4.2	3	20.00±2.00Ba	10	67	26.43±6.31Ca	4.19	3	8.97±0.32Ca	27.89	3	10.01±4.7	2.13
Liquid	0%	15	6.71±3.56Aa	1.89	15	4.85±2.47Aa	1.96	2	8.50±3.54Aa	2.4	15	13.97±3.64Aa	3.84	1	2.70±0.00Aa	-	1	0.35±0.00	-
	10%	20	11.19±3.01Bb	3.71	20	7.72±2.19Bb	3.52	2	9.50±0.71Ab	13.43	20	17.83±3.39Ab	5.26	2	2.85±0.21Ab	13.44	1	3.25±0.00	-
	20%	40	10.42±2.34Bb	4.45	40	8.46±1.70Bb	4.97	3	12.67±1.15Ab	10.97	40	22.68±5.38Ba	4.21	3	4.40±0.78Ab	5.63	2	6.52±2.32	2.81
	30%	25	11.10±3.34Bb	3.32	25	8.22±2.24Bb	3.67	2	12.50±0.71Ab	17.68	25	18.98±6.01ABb	3.16	2	5.30±1.70Ab	3.12	3	12.08±4.99	2.42
F stats. and p-values	V	F(1,323)=42.83, P<0.001			F(1,323)=33.68, P <0.001			F(1,12)=55.65, P<0.001			F(1,323)=19.38, P<0.001			F(1,9)=19.96, P<0.01			F(1,10)=1.01, P=0.340		
	D	F(3,323)=28.872, P<0.001			F(3,323)=41.577, P<0.001			F(3,12)=21.582, P<0.001			F(3,323)=23.376, P<0.001			F(3,9)=11.908, P<0.01			F(3,10)=1.88, P=0.198		
	V * D	F(3,323)=4.471, P<0.01			F(3,323)=6.196, P<0.001			F(3,12)=12.428, P<0.001			F(3,323)=5.123, P<0.01			F(3,9)=1.538, P=0.271			F(3,10)=0.74, P=0.558		

Different capital (/lower case) letters indicate significant difference at means for dose (/vermicompost) by multiple comparisons with Bonferroni adjustment

4. Discussion

Lettuce can grow in a short period of time in soils rich in organic matter. Therefore, fertilization is one of the most important factors affecting the yield and quality of lettuce. Excessive and unconscious use of nitrogenous chemical fertilizers increases the accumulation of nitrate, which is harmful to plant health. The use of materials of organic origin improves the physical, chemical, and biological properties of soils and ensures healthy and high-quality products. Therefore, it is reported that the use of organic soil conditioners in agriculture should be widespread (Wu et al. 2020; Ye et al. 2020). The earthworm vermicompost enriches the content of the soil, increases its fertility, and improves soils contaminated with chemicals (Chew et al. 2019). It has also been reported that vermicompost applied in soil contaminated with pesticides restricts the movement of pesticides (Romero et al. 2006; Fernandez-Bayo et al. 2009). The intensive and unconscious use of chemical inputs in crop production creates an increasing pollution burden on soil, groundwater, and the atmosphere. This situation has become a serious threat to the health, wildlife, and environment in the world. It is observed that soil pollution, especially in crop production, has also become a global problem and this situation poses great threats to sustainable agriculture and food security.

The growth, development parameters and fungicide resistance of lettuce plants treated with different doses of solid and liquid vermicompost were compared in this study. It is seen that studies on vermicompost have started to increase especially in recent years, and there are many studies, especially on solid vermicompost applications (Srivastava et al. 2020; Yuvaraj et al. 2021, Ducasse et al. 2022). However, there is no detailed study comparing the effects of solid and liquid vermicompost on plant growth and development and tolerance to pesticide applications on the same plant.

The leaf length, leaf width, chlorophyll amount (SPAD), number of marketable leaves, leaf nitrogen values were found statistically significant in terms of vermicompost type and dose ($P<0.01$ and $P<0.05$). It was found no statistically significant number of discarded leaves and, leaf pH values.

The leaf width and length values of the lettuce plant, which is one of the important indicators of vegetative development of the plant and a vegetable whose leaves are eaten, were found to be higher and statistically significant in both vermicompost types compared to the control. Different forms of vermicompost applications are observed to affect plant growth parameters at different levels (Öztürkci & Akköprü 2021). We observed that solid vermicompost application results were better than liquid vermicompost application in this study. Some researchers have reported that solid vermicompost had a significant and positive effect on plant growth. Karademir & Kibar (2022) detected that vermicompost had positive effects on plant growth, quality properties and element contents in curly lettuce. We determined that leaf growth parameters showed a positive effect, especially at low doses of solid vermicompost. Our results are consistent with the findings of some previous studies (Yourtchi et al. 2013; Öztürkci & Akköprü 2021).

Solid vermicompost has plant growth-promoting properties such as high organic matter, macro- and micronutrient content and enhanced beneficial microbial activity and diversity in the soil (Şimşek-Erşahin 2011). However, the slow release of solid vermicompost can be an important advantage. In addition, it was stated that the solomic fluid in the digestive system of earthworms contains many enzymes, namely proteases, lysozymes, fibrinolytic enzymes, polysaccharides, antimicrobial proteins, and nutrients (Wang et al. 2010; Kocakurt 2022). At the same time, unlike chemical fertilizers, vermicompost stays in the soil for a long time due to the solomic fluid it contains (Samal et al. 2019). It has also been stated that cow manure is the best material for vermicompost production (Xie 2016). An increase in plant biomass was reported in a meta-analysis study evaluating the effects of earthworms on plant growth. It has been reported that Asteraceae family is one of the most sensitive families to vermicompost application (Blouin et al. 2019).

It was found that solid vermicompost applications had significant differences in the leaf size of *Plectranthus amboinicus* (Lour.) Spreng (Cuban oregano) (Yüksek et al. 2020). Many studies have reported that vermicomposting positively affected leaf size in onion (Srivastava et al. 2012), eggplant (Kumari et al. 2017) and garlic (Kenea & Gedamu 2018) compared to the control. Arancon et al. (2003) found that vermicompost increased the leaf area of pepper and tomato. Ducasse et al. (2022) was determined that solid vermicompost application higher than liquid vermicompost application on the yield of tomato. The low effectiveness of liquid vermicompost may be that the frequency of application is insufficient for the plant. Contrary to our results, a study using sunflower plant (*Helianthus annuus* L.), in which solid and liquid vermicompost applications were performed at similar rates to our study, showed better results with liquid vermicompost application. This difference may be due to the difference in plant species and genetic characteristics of the species (Gül et al. 2021).

The number of marketable leaves showed statistical significance according to vermicompost type and dose. The higher marketable leaves were determined from the solid vermicompost applications than the control and liquid application. Similar results were found in the study carried out by Maloisane & Kayombo (2022) on the lettuce. Karademir & Kibar (2022) reported that vermicompost applications increased the number of marketable leaves in lettuce compared to the control. It has been stated that the cow manure vermicompost application significantly increased the marketable yield of Chinese cabbage (Wang et al. 2010). The increases in yield may be due to the production of plant growth regulators by microorganisms.

In the present study, the leaf SPAD chlorophyll amount was found to be higher and statistically significant in both vermicompost types compared to the control groups. Solid vermicompost chlorophyll values were higher than liquid fertilizer values as in other parameters. Theunissen et al. (2010) reported that vermicompost application has a positive effect on SPAD chlorophyll amount. Narkhede et al. (2011) in pepper, Srivastava et al. (2012) in onion, and Altunlu (2021) in lettuce and Karademir & Kibar (2022) in lettuce reported that the amount of SPAD chlorophyll in vermicomposted plants was higher than in the control group. However, Luján-Hidalgo et al. (2016) reported that the amount of chlorophyll in Mexican Pepper leaf (*Piper auritum* Kunth) was not affected by vermicompost applications, contrary to our results. Leaf nitrogen (N) values were found to be statistically significant according to vermicompost type and doses. Solid vermicompost applications have higher values in terms of leaf nitrogen (N) values than liquid vermicompost applications. Leaf nitrogen values found higher than the control was between 5.55-8.97. Similar results were found in previous studies (Aslam & Ahmad 2020; Gül et al. 2021). A parallelism was observed between nitrogen and SPAD chlorophyll content values determined depending on different vermicompost and dose applications in the present study. Since nitrogen is one of the important elements in photosynthesis, leaf chlorophyll content is considered an indicator of nitrogen uptake by plants.

Fungicide residue analysis results were not statistically significant. However, residues above the MRL (Maximum Residue Limit) were determined in the control group (0.3510 mg/kg), solid vermicompost (10.0143 mg/kg -15.249 mg/kg) and liquid vermicompost applicated samples (3.25 mg/kg -12.0783 mg/kg). This limit is 0.03 for Captan fungicide for lettuce according to the Maximum Acceptable Residue Limits for Turkish Food Codex and the European Union Food Codex (Yaşa 2011). Fungicide residue amounts were higher in solid vermicompost applications compared to liquid vermicompost applications in the present study. The highest value was determined at 20% (15.2490 mg/kg) dose and the lowest value was determined at 30% (10.0143 mg/kg) dose in the solid vermicompost applications, while a dose-dependent increase in fungicide residue amount was determined in the liquid vermicompost application. A review of pesticide residues in food samples published in 2010 and later in Turkey showed that the Maximum Residue Levels (MRL) were exceeded in 20 of 120 studies on lettuce (Tözün & Akar 2022). Since the fungicide was applied in the greenhouse, airborne contamination may be the reason why fungicide residues were found in the control samples, although no direct application was performed (Table 7). In addition, pesticides can be carried by air currents passing through the applied surface by passing into the vapor phase. Pesticides can be transported to different areas where they are not sprayed by the wind. The reason for the pesticide residues to be found in the samples applied vermicompost may be that there is a period of approximately one week between the application and the harvest time.

Leaf samples were collected according to the recommended harvest time and analyzed for fungicide residue in the present study. It has been stated that the time between fungicide application and harvest should be considered to avoid residues (Stensvand 2000). It has been reported that washing and cold storage significantly affect pesticide dissipation (Cengiz et al. 2007). However, in fresh vegetables such as lettuce the washing process will have a limited impact on the pesticide residue content (Eştürk et al. 2014). Although pesticide resistance of vermicompost applications was tried to be determined in this study, it is known that vermicompost application increases plant resistance against various pests and diseases. It has been reported that vermicompost application reduces pest attacks and prevents them from harmful pests (Olle 2019).

5. Conclusions

We examined the effects of different types and doses of vermicompost applications on the product and quality characteristics of lettuce plants and fungicide application in the present study. We found that solid vermicompost is better than both the control and commercial liquid vermicompost in all parameters examined in terms of yield and quality. The highest leaf length and width values were determined at 20% dose of solid vermicompost, while chlorophyll amount and leaf nitrogen values were determined at 30% dose. The number of marketable leaves was the same in all doses of solid vermicompost application. However, fungicide residues above the limits were found in all the solid and liquid vermicompost doses studied. The residue amounts were less in liquid vermicompost. It can be concluded from the present study that a much longer period of seven days will be needed between application and harvest. Alternative techniques can also be developed to reduce the amount of pesticide residues after harvest. Therefore, different studies can be carried out in liquid vermicompost applications to reduce the amount of fungicide residue amount. In addition, it would be useful to investigate the resistance of vermicompost-applied plants against different harmful pests.

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An Analysis of Predatory Bugs (*Orius* spp., Hemiptera: Anthocoridae) and Pest Insects on Some Crop Plants: Their Distributions, Abundance and Population Developments

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ABSTRACT
The seasonal distributions of predatory bugs, specifically species of *Orius* (Hemiptera: Anthocoridae), and the population dynamics of some sucking pests including aphids, leafhoppers, and thrips in crops plants (i.e. winter and summer vegetables, fruit trees and field crops) in Balcalı, Adana Province, Turkey, were investigated during 2019–2020. A total of four *Orius* species were identified, which are: *Orius laevigatus* (Fieber), *Orius niger* (Wolff), *Orius albidipennis* (Reuter) and *Orius vicinus* (Ribaut). The most common species was *O. laevigatus*, constituting 78.36% of the total adult individual insects. Both *O. laevigatus* and *O. niger* were relatively more abundant on broad beans, beans and potatoes compared to fruit trees and open field crops, such as cotton, sesame and soybean. Very few *Orius* individuals were recorded on fruit trees. Those that were recorded were found mostly on apple trees. *Orius* and thrips individuals were collected mainly from the flowers of the plants, and their population patterns depended on the flowering phenology patterns of the plants. Significant correlations were detected between the population patterns of *Orius* individuals and thrips populations in winter and summer vegetables, fruit trees and field crops. This may indicate that thrips are the primary prey of *Orius* individuals throughout the year in agricultural ecosystems with rich plant diversity.

Keywords: Density, Broad bean, Distribution, *Orius laevigatus*, Pest thrips

1. Introduction

To maintain the population of harmful insects/mites at a rate below the economic damage threshold in plant production and increase yields, insecticides/acaricides have been widely used due to their biological effectiveness and rapid suppression of pests (De Waard et al. 1993). Pesticides were first introduced in the 1940s and, while their use has increased, as have their negative effects, the widespread commercial use of pesticides initially did not raise concerns about residue problems. Harmful organisms such as insects and mites have developed resistance to pesticides over time, potentially rendering them less effective in the future (Bass et al. 2015; Gould et al. 2018). The indiscriminate and intensive use of pesticides in modern agriculture has adverse effects on human and environmental health. It leads to pesticide resistance, a reduction in beneficial organisms, decreased biodiversity in agricultural ecosystems and crop residue problems (Carson 1962; Brammall & Higgins 1988; Dreistadt et al. 1994; Rosell et al. 2008; Pereira et al. 2016; Silva et al. 2019; Alengebawy et al. 2021). As an alternative to chemical control, there have been efforts to adopt more economical and environmentally friendly methods that promote the health of living organisms and minimise the unnecessary use of pesticides. Studies on biological control methods have been conducted for this purpose (Clausen 1958; Howarth 1991; Bueno et al. 2006).

Some predatory insect species, which belong to the Anthocoridae (Hemiptera) family, are commercially reared and used primarily to control harmful thrips in greenhouses (Riudavets & Castena 1994; Topakçi & Keçeci 2017; Van Lenteren et al. 2020). The genus *Orius* in the family Anthocoridae is common in nature, particularly in areas with high biodiversity, and six species of this genus have been recorded in Turkey (Önder 1982). Both nymphs and adults of the *Orius* species exhibit zoophagous behaviour, feeding on various insects and mites that are detrimental to plants, including whiteflies, aphids, spider mites, psyllids, Lepidoptera and Coleoptera, with a particular focus on species of thrips (order Thysanoptera) (Riudavets and Castane 1994; Önder 1982; Lodos 1986; Lattin 1999; Pehlivan & Atakan 2020). *O. laevigatus*, *Orius insidiosus* (Say), and *Orius tristicolor* (White) have been commercially used for controlling *Frankliniella occidentalis* (Pergande) in protected crops since 1990 (Chambers et al. 1993; Ferragut & Zamora 1994; Disselvet et al. 1995; Pozzebon et al. 2015). *Orius niger* (Wolff) has been found together with *F. occidentalis* on weeds in the Adana and Mersin Provinces, located in the Eastern Mediterranean region of Turkey (Atakan & Tunç 2010). In the Adana Province, *O. niger* was the most common species in fields where broad beans, clovers and strawberries were cultivated (Atakan &Tunç 2004; Atakan 2010a; Atakan 2011).

While polyphagous *Orius* species are generally associated with thrips, understanding their interactions with different types of prey in various crop plants throughout the season can provide valuable ecological information about their role in suppressing crop pests. This study aimed to examine the composition of predatory *Orius* species and the distribution of common pests, such as aphids, thrips and leafhoppers, in various agricultural crop plants products within a designated polyculture ecosystem. Additionally, it investigated the population changes and interactions among these pest insects. The results obtained about the importance of the *Orius* species as biological control agents of crop pests will be discussed. Overall, the study provides valuable insights into the seasonal dynamics of the *Orius* species and pest insects across various plant groups in different ecological areas, enhancing our understanding of their interactions and population fluctuations in agricultural areas.

2. Material and Methods

2.1. Definition of sampling area

The location Balcalı is a neighbourhood located in Adana Province, Turkey. It houses the Çukurova University, Faculty of Agriculture Research and Application Farm, situated approximately seven kilometers away from the main campus. This research area spans around 700 hectares where citrus, stone fruits, subtropical fruits and both cool and warm climate cereals are cultivated. In addition, wheat is grown in an area of approximately 400 hectares that is not irrigated. Flood irrigation is the predominant method used in irrigable sections. It is worth mentioning that the region also serves as a nature reserve, ensuring the protection of wildlife. The location Balcalı experiences a typical Mediterranean climate, characterised by warm and dry summers, mild and wet winters and overall moderate temperatures throughout the year.

2.2. Sampling of insects

Seasonal abundance and distribution of the *Orius* species, and its relationships with different types of prey (thrips, aphids and leafhoppers) were investigated in Balcalı at the Çukurova University Farm, in 2019–2020. Three ecological areas have been identified where different plant species are grown for both production and research. For this purpose, winter and summer vegetables and citrus were sampled in the Research and Implementation Area of the Plant Protection Department to check for numbers of pest insects such as aphids, leafhoppers and thrips, as well as predatory bugs. In the Field Crops Research and Implementation Area, various field crops, including cotton, soybean, sesame, peanuts, and temperate climate fruit trees such as apple, nectarine, and loquat, were sampled as part of the research conducted in Research and Implementation Area of the Horticulture Department. The sampling sites are about two or three kilometers apart from each other. Insect pests have been recorded on all these plants. Species-level distinctions were not done for the pest groups: Aphids, thrips and leafhoppers. Common species were found in vegetables and field crops. These included the cotton aphid, *Aphis gossypii* (Glover) (Hemiptera: Aphididae), western flower thrips, *Frankliniella occidentalis* (Pergande), cotton thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) and cotton leafhopper, *Empoasca decipiens* Paoli (Hemiptera: Cicadellidae).

For winter vegetable plants, plots were created in four territorial units using a randomised block trial design. The plants included spinach (*Spinacia oleracea* L.), lettuce (*Lactuca sativa* L.), cauliflower (*Brassica oleracea var. botrytis* L.), red cabbage (*Brassica oleracea var. appitate f. rubra* L.), cabbage (*Brassica oleracea* L.), rocket (*Eruca vesicaria* L.), peppergrass (*Lepidium sativum* L.), radishes (*Raphanus sativus* L.) and broad beans (*Vicia faba* L.). Each plot consisted of four rows, with fifteen plants in each row. A free space of 1.5 meters was left between plots, and there were five meters between the blocks. A similar trial model was created for the following summer vegetable plant species: cucumber (*Cucumis sativus* L.), squash (*Cucurbita moschata* L.), beans (*Phaseolus vulgaris* L.), peppers (*Capsicum annuum* L.), tomatoes (*Solanum lycopersicum* L.), potatoes (*Solanum tuberosum* L.) and eggplants (*Solanum melongena* L.). To investigate the population density of thrips and predatory insects in some field crops, plots containing cotton (*Gossypium hirsutum* L.), sesame (*Sesamum indicum* L.) and soybean plants (*Glycine max* L.) were established according to the randomised block design with four replicates. The plot size for each plant species was designed as 96 square meters with a row length of ten meters, consisting of ten rows, with a distance of 0.80 meter between rows. A free space of two meters was left between the plots and five meters between the blocks. Nectarines (*Pyrus persicae* L.) and pomegranates (*Punica granatum* L.) were planted with 3 x 5 spacing, apples (*Malus domestica* L.) with 1 x 4 spacing, pears (*Pyrus communis* L.) with 4 x 6 spacing, plums (*Prunus persica* L.) lemons (*Citrus lemon* L.) and almonds (*Prunus dulcis* Mill. D. A. Webb) with 5 x 5 spacing and loquats [(*Eriobotrya japonica* (Thunb.) Lindl.)] with 7 x 7 spacing. Ten rows of each fruit tree species were planted, with twenty trees in each row. There was a 20-meter gap between the large plots representing each fruit species, and they were lined up next to each other. Each tree species planted was nearly fifteen years old. In the samplings, 20 plants from annual herbaceous crops (vegetable species and field crops) and 30 cm-long flowering or fruit-bearing shoots were randomly selected from each of the 20 fruit trees, positioned in the four cardinal directions. In general, the plants with flowers were taken into consideration in the samplings, which started with the beginning of the flowering period of the different plant groups. This decision was made because *Orius* and their primary prey, thrips, are found mostly on flowers, as these predatory species feed on the nectars and pollens in the flowers when there is no prey (Riudavets & Castane 1994; Hansen et al. 2003; Funderburk et al. 2000; Funderburk et al. 2018). Flowering or fruiting shoots measuring 30 cm-long in four different directions on the trees were randomly selected for sampling. In herbaceous plants, the upper halves of the plants were considered in the sample. In the surveyed areas, shoots or plants were shaken for 5–10 seconds into a white container measuring 34 × 23 × 7 centimeters, and the *Orius* species and herbivorous insects that fell into the container were lifted out by a suction tube and/or fine brush and placed in Eppendorf tubes containing 70% ethyl alcohol. Insect samples collected from each plant species on each sampling date were placed in a tube. The information about the samplings of insects in various plant groups is summarised in Table 1. Sampling was carried out in three different ecological areas on the same day between 08:00 and 12:00. Insect samples (*Orius* and herbivorous pest insects) were brought to the laboratory and placed in Petri dishes to be counted under a stereo binocular microscope, to classify them as thrips, aphids or leafhoppers without making any distinction between species. While making the diagnosis, nymphs of *Orius* and larvae of thrips were sampled from the most common broad bean plants but were not evaluated because they were found only in very small numbers. *Orius* adults were separated according to genital organ and morphological characteristics, after which they were identified.

Table 1- Basic information on the insect samplings in various plant groups in Balcalı during the years 2019-2020

Plant group	*Number of sampling date*	*Number of plant species sampled*	*Total number of sample*	*Sampling unit*	*Sampling period*
Winter vegetables	15	9	135	Leaves+flowers	Oct. 2, 2019 – Mar. 18, 2020
Fruit trees	20	8	160	Leaves+flowers	Oct. 2, 2019 – May 27, 2020
Summer vegetables	23	7	161	Leaves+flowers	Oct. 2, 2019 – Dec. 18, 2019 and Apr. 15, 2020 – Oct. 21, 2020
Field crops	15	4	60	Leaves+flowers	Oct, 2, 2019 – Nov. 20, 2019 and Jul. 1, 2020 – Oct. 21, 2020

2.3. Identification of Orius

The samples collected in the survey were first placed into Eppendorf tubes containing ethyl alcohol and their setae in the pronotum were examined under a stereo binocular microscope. Then the tips of the abdomens were cut off and transferred to glass containers containing 10% potassium hydroxide (KOH) solution and left at room temperature for one day. At the end of a day, it was put into clove oil for about five minutes, after which, using a needle, it was transferred to a slide prepared with Hoyer's medium and covered with a coverslip. In addition, nail polish was applied around the slide to prevent air from getting in. The preparation was dried in an oven heated to 45°C, where it remained for fifteen days (Silveira et al. 2003), after which the mating tubes of the females and the genital organs of the males of the *Orius* species were examined under a light microscope and a diagnosis was made according to the key identifications published by Péricart (1972) and Önder (1982).

2.4. Statistical analysis

The seasonal distributions of harmful insects and *Orius* species in various cultivated plants were calculated separately following the method of Karman (1971). The distributions, representing the ratios of total adult individuals, were calculated using the Microsoft Excel spreadsheet program. To analyse the seasonal averages of both *Orius* spp. and prey pests in the plant groups within the same ecological area, one-way analysis of variance (ANOVA) and the Tukey test were applied with a significance level of $P<0.05$ (SPSS 2016). The comparison of averages considered the same sampling dates for each plant group, including both winter and summer vegetables, fruit trees and field crops. To evaluate the population fluctuations of the predator and pest groups, as indicated in the methods section because the number of predatory and pest individuals in certain plant species within the plant group was very low or could not be found on some sampling dates. We calculated the average number of individuals collected from these plant species on each sampling date by adding up the counts and dividing by the number of samples. The combined data were then presented as total mean values for summer/winter vegetables, fruit trees and field crops. Since the cultivated plants sampled from different plant groups were grown in different ecological areas and had varying growing periods, the seasonal averages of beneficial insects (*Orius* spp.) and harmful insects on crop plants were not statistically compared. The prey/predator ratios were determined based on the number of harmful insects and *Orius* obtained from the plant groups. The total values obtained from 20 plants in each plant category (vegetables, field crops and fruit trees) were entered into the Excel program, according to the respective sampling dates. To assess the correlations between the mean numbers of predators (*Orius* spp.) and the numbers of pest insects (aphids, leafhoppers and thrips) within each plant group, a simple correlation analysis (Pearson correlation) was conducted at a significance level of $P<0.05$ (SPSS 2016). Specifically, we considered 21 sampling dates for winter vegetables, 19 for summer vegetables, 20 for field crops and 31 for fruit trees. The study's statistical analysis and tables provide information about the seasonal dynamics and interactions between *Orius* species and pest insects across different plant groups and ecological areas.

3. Results

3.1. Distribution of Orius species and harmful insects on the crop plants

The survey, conducted in the Balcalı region, yielded a total of 134 *Orius* species collected from winter vegetables (Figure 1a). Among these, *O. laevigatus* was dominant, with 105 individuals, making up 78.36% of the total adult population. Regarding pest insects, the survey revealed that, in winter vegetables, 735 individuals of thrips were collected, constituting 50.94% of the total number of adult pest individuals (Figure 1b). The second most common pest taxon was leafhoppers, with a rate of 43.73% and a total of 631 individuals collected (Figure 1b). A total of 77 aphid individuals were recorded, accounting for 5.93% of the total pest population. Thrips was most frequently found in association with *Orius* species, which aligns with previous studies indicating that *Orius* primarily prefer thrips as their prey and some *Orius* species are important predators of thrips. On fruit trees, *O. laevigatus*, *O. niger* and *O. vicinus* individuals were detected, with an incidence of 77.27%, 18.18%, and 4.55%, respectively (Figure 1c). However, *O. albidipennis* species was not found in the survey.

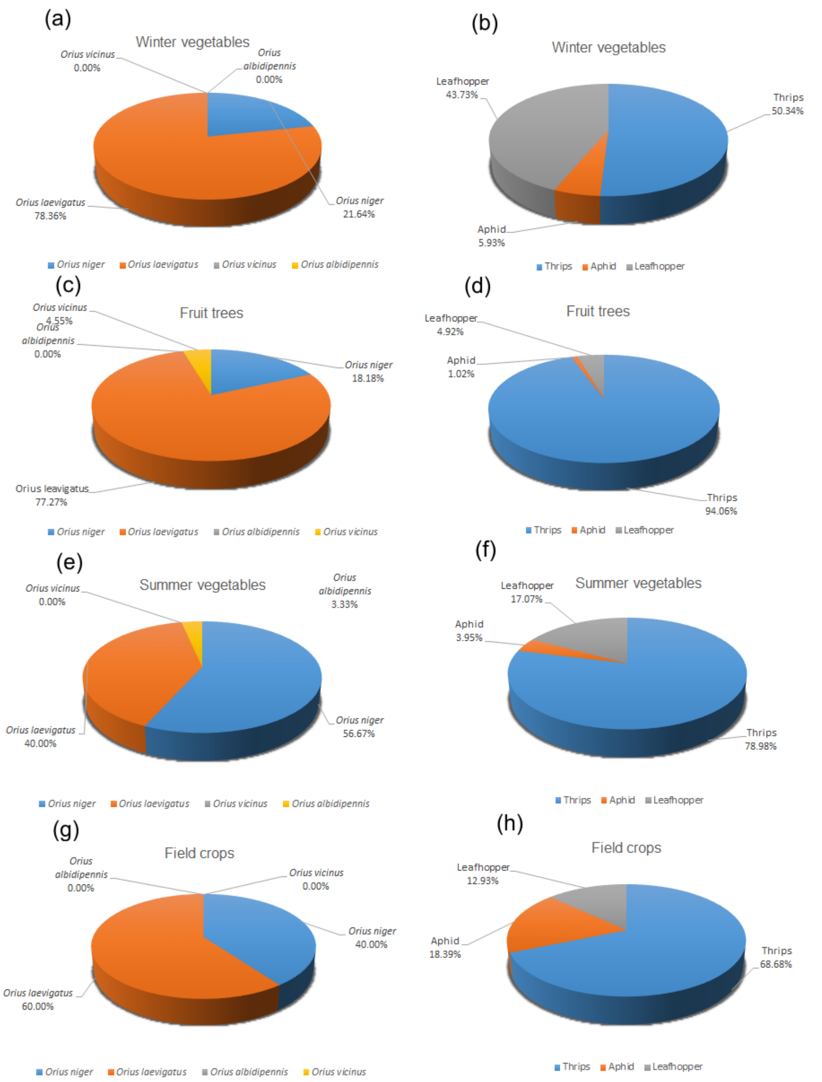

Figure 1- Distributions of *Orius* species and prey groups (aphids, thrips and leafhoppers) on different arable crops, a, b: winter vegetable, c, d: fruit tree, e, f: summer vegetable, g, h: field crop

On fruit trees, thrips were the most common insects, with 620 individuals, accounting for 94.06% of the total pest population (Figure 1d). Aphids and leafhoppers were collected in very low numbers, with three and thirteen individuals, respectively, accounting for 1.02% and 4.92% of the total number of individuals (Figure 1d). In summer vegetables, *O. niger* was the most common species, with 17 adults, representing 56.67% of the total adults (Figure 1e). *Orius laevigatus* was the second most common species, with 20 individuals, comprising 40% of the total individuals (Figure 1e). Individuals of aphids were observed in lower numbers in summer vegetables, constituting 3.95% of the total prey population species (Figure 1f). In field crops, *O. laevigatus* dominated the *Orius* populations (Figure 1g). Individuals of thrips (mainly *F. occidentalis*) were the most common pests, with 239 individuals collected, constituting 68.68% of the total adult herbivorous insect population (Figure 1h). These survey findings provide valuable insights into the population dynamics and interactions between *Orius* species and pest insects in different plant groups within the location Balcalı contributing to our understanding of natural pest control mechanisms.

3.2. Numbers of Orius and harmful insects on the crop plants

Table 2 presents a list of plant species, along with *Orius* species and total prey densities (pooled pest insects), collected in 2019–2020. *Orius* species were not found on plants belonging to the Amaranthaceae, Brassicae and Cucurbitaceae families, or were recorded only rarely (Table 2). Cauliflower had the highest total number of insect pests detected. Broad bean plants from the Fabaceae family had a high number of harmful insects in their flowers, and *Orius* species (especially *O. laevigatus*) were relatively more abundant in these plants. Leafhoppers were found in high numbers on the leaves of broad bean plants (Table 3). Although the number of pests in bean plants was high (301 individuals), the number of *Orius* individuals was lower (21 *Orius* individuals in total). During the flowering period of potato plants grown in Çukurova, the number of thrips was high (252 thrips individuals), and 23 *Orius* individuals were recorded alongside the thrips in the flowers (Table 2). Thrips and *Orius* numbers were relatively higher in loquat and apple blossoms compared to other fruit trees (Tables 2 and 3). The tables (Tables 4, 5, 6 and 7) show the total average numbers of harmful insects and *Orius* during the same sampling periods in crop plants grown in the same ecosystem. In winter vegetables (Table 4), the average number of *Orius* individuals was significantly higher (3.14 ± 0.92 individuals/20 plants) in broad bean plants, while the number of *Orius* was low and similar in other plant species. Thrips were detected in significant and high numbers in potato plants (11.45 ± 1.02 individuals/20 plants). In summer vegetables, *Orius* individuals were sampled in similar numbers in pepper (0.17 ± 0.09 individuals/20 plants) and bean (0.54 ± 0.22 individuals/20 plants) plants but significantly higher when compared to other plant species (Table 5). Similarly, the mean number of thrips was significant and higher in peppers (13.72 ± 0.87/20 plants) and beans (10.22 ± 1.00/20 plants). The total mean numbers of prey groups (thrips, aphids and leafhoppers) in fruit trees were similar and low among other plant species (Table 6). In field crops (Table 7), the total average numbers of *Orius* were generally low, but in sesame (0.71 ± 0.23 individuals/20 plants) and cotton (0.38 ± 0.12 individuals/20 plants) (Table 7), the numbers of *Orius* individuals were statistically significant and higher compared to other plant species. Thrips sampled from sesame flowers had significantly higher numbers (4.09 ± 0.46 individuals/20 plants) than those numbers found in other field crops.

Table 2- Total numbers of *Orius* species with pest insects in some cultivated plants sampled in Balcalı in 2019-2020

Family/English and Latin name	*Orius niger*		*Orius laevigatus*		*Orius albidipennis*		*Orius vicinus*		*Total no of pest insects*
	♀	♂	♀	♂	♀	♂	♀	♂	
Amaranthaceae									
Spinach (*Spinacia oleracea* L.)	0	0	0	0	0	0	0	0	10
Asteraceae									
Lettuce (*Lactuca sativa* L.)	0	0	0	0	0	0	0	0	30
Brassicaceae									
Cauliflower *(Brassica oleracea var. botrytis* L.)	1	0	0	0	0	0	0	0	167
Red cabbage (*Brassica oleracea var,appitate f. Rubra* L.)	0	0	0	0	0	0	0	0	142
Cabbage (*Brassica oleracea* L.)	0	0	0	0	0	0	0	0	106
Rocket (*Eruca vesicaria* (L.))	0	0	0	0	0	0	0	0	2
Peppergrass (*Lepidium sativum* L.)	0	0	0	0	0	0	0	0	15
Radish (*Raphanus sativus* L.)	0	0	0	0	0	0	0	0	44
Cucurbitaceae									
Cucumber (*Cucumis sativus* L.)	1	0	0	0	0	0	0	0	28
Winter Squash (*Cucurbita moschata* Duch.)	0	0	0	0	0	0	0	0	11
Fabaceae									
Broad bean (*Vicia faba* L.)	18	6	77	9	0	0	0	0	642
Bean (*Phaseolus vulgaris* L.)	6	6	4	4	0	1	0	0	301
Soybean (*Glycine max* L.)	0	0	0	1	0	0	0	0	60
Peanut *(Arachis hypogaea* L.)	1	0	0	0	0	0	0	0	27
Lythraceae									
Pomegranate (*Punica granatum* L.)	0	0	2	0	0	0	0	0	9
Malvaceae									
Cotton (*Gossypium hirsitum* L.)	6	0	0	2	0	0	0	0	100
Pedeliaceae									
Sesame (*Sesamum indicum* L.)	3	0	7	5	0	0	0	0	130
Rutaceae									
Lemon (*Citrus lemon* (L.) Osbeck)	0	0	0	0	0	0	0	0	48
Rosaceae									
Pear *(Pyrus communis* L.)	0	0	0	0	0	0	0	0	7
Almond (*Prunus dulcis* Mill. D. A. Webb)	1	0	1	0	0	0	0	0	2
Apple (*Malus domestica* L.)	1	0	6	2	0	0	1	0	76
Plum (*Prunus domestica* L.)	0	0	0	0	0	0	0	0	6
Nectarine (*Prunus persica* L.)	0	0	0	0	0	0	0	0	59
Loquat (*Eriobotrya japonica* (Thunb.) Lindl.)	2	0	4	1	0	0	0	0	81
Solanaceae									
Pepper *(Capsicum annuum* L.)	3	0	1	2	0	0	0	0	356
Tomato (*Solanum lycopersicum* L.)	0	0	0	0	0	0	0	0	23
Potato (*Solanum tuberosum* L.)	4	0	7	12	0	0	0	0	261
Eggplant (*Solanum melongena* L.)	1	0	0	1	0	0	0	0	67
Total	48	12	109	39	0	1	1	0	2809

Table 3- Total numbers of pest insects with *Orius* spp. in some cultivated plants sampled in Balcalı in 2019-2020

Family/English and Latin name	*Thrips*	*Aphid*	*Leafhopper*	*Total no of Orius*
Amaranthaceae				
Spinach (*Spinacia oleracea* L.)	9	1	0	0
Asteraceae				
Lettuce (*Lactuca sativa* L.)	17	11	2	0
Brassicaceae				
Cauliflower *(Brassica oleracea* var. botrytis L.)	152	15	0	1
Red cabbage (*Brassica oleracea* var. capitata f. Rubra L.)	138	4	0	0
Cabbage (*Brassica oleracea* L.)	103	3	0	0
Rocket (*Eruca vesicaria* L.*)*	0	0	2	0
Peppergrass (*Lepidium sativum* L)	1	13	1	0
Radish (*Raphanus sativus* L)	15	14	15	0
Cucurbitaceae				
Cucumis (*Cucumis sativus* L.)	23	4	1	1
Squash (*Cucurbita moschata* L.)	8	0	3	0
Fabaceae				
Broad bean (*Vicia faba* L.)	24	7	611	110
Bean (*Phaseolus vulgaris* L.)	225	8	68	21
Soynbean (*Glycine max* L)	59	0	1	1
Peanut (*Arachis hypogaea* L.)	44	54	2	1
Lythraceae				
Pomegranate (*Punica granatum* L.)	8	0	1	2
Malvaceae				
Cotton (*Gossypium hirsitum* L.)	18	5	4	8
Pedeliaceae				
Sesame (*Sesamum indicum* L.)	86	3	41	15
Rutaceae				
Lemon (*Citrus lemon* L.)	48	0	0	0
Rosaceae				
Pear (*Pyrus communis* L.)	5	1	1	0
Almond *(Prunus dulcis* Mill. D. A. Webb)	2	0	0	2
Apple (*Malus domestica* L.)	72	2	2	10
Plum (*Prunus domestica* L.)	6	0	0	0
Nectarine (*Prunus persica* L.)	58	0	0	0
Loquat (*Eriobotrya japonica* (Thunb.) Lindl)	77	0	4	7
Solanaceae				
Pepper (*Capsicum annuum* L.)	303	5	48	6
Tomato (*Solanum lycopersicum* L.)	12	0	11	0
Potato (*Solanum tuberosum* L.)	252	9	0	23
Eggplant (*Solanum melongena* L.)	50	14	3	2
Total	1815	173	821	210

Table 4- Total average number of *Orius* spp. and pest insects (mean ± SE/20 plants) in winter vegetables in Balcalı in 2019-2020

Winter vegetables	*Orius*	*Thrips*	*Aphid*	*Leafhopper*
Broad bean	3.14 ± 0.92a[a]	1.36 ± 0.29c	0.21 ± 0.10a	19.8 ± 5.87a
	(110)[b]	(24)	(7)	(611)
Spinach[b]	0.00 ± 0.00b	0.40 ± 0.32c	0.04 ± 0.04a	0.00 ± 0.00b
	(0)	(9)	(1)	(0)
Red cabbage[b]	0.00 ± 0.00b	6.27 ± 0.55b	0.18 ± 0.14a	0.00 ± 0.00b
	(0)	(138)	(4)	(0)
Cauliflower	0.02 ± 0.02b	6.59 ± 0.60b	0.68 ± 0.36a	0.00 ± 0.00b
	(1)	(152)	(15)	(0)
Cabbage[b]	0.00 ± 0.00b	4.69 ± 0.58b	0.13 ± 0.13a	0.00 ± 0.00b
	(0)	(103)	(3)	(0)
Lettuce[b]	0.00 ± 0.00b	0.77 ± 0.44c	0.22 ± 0.16a	0.09 ± 0.06b
	(0)	(17)	(11)	(2)
Rocket[b]	0.00 ± 0.00b	0.00± 0.00c	0.00± 0.00a	0.09 ± 0.09b
	(0)	(0)	(0)	(2)
Peppergrass[b]	0.00 ± 0.00b	0.04 ± 0.32c	0.59 ± 0.59a	0.04 ± 0.04b
	(0)	(1)	(13)	(1)
Radish[b]	0.00 ± 0.00b	0.68 ± 0.30c	0.63 ± 0.59a	0.68± 0.44b
	(0)	(15)	(14)	(15)
F	10.227	44.268	0.746	11.227
df	10,374	10,231	10,231	10,231
P	<0.0001	<0.0001	0.681	<0.0001

[a] When the columns are examined, means with the same letter are statistically insignificant according to the Tukey HSD test (P>0.05). [b]Values in parentheses indicate the total number of individuals

Table 5- Total average number of *Orius* spp. and pest insects (mean ± SE/20 plants) in summer vegetables in Balcalı in 2019-2020

Summer vegetables	*Orius*	*Thrips*	*Aphid*	*Leafhopper*
Pepper	0.17 ± 0.09a[a]	13.72 ± 0.87a	0.13 ± 0.09a	10.04 ± 2.53a
	(6)[b]	(303)	(5)	(48)
Tomato**[b]**	0.00 ± 0.00b	0.00 ± 0.00b	0.36 ± 0.23a	1.33 ± 0.28b
	(0)	(12)	(0)	(11)
Potato	0.65 ± 0.33a	11.45 ±1.02a	0.40 ± 0.23a	0.00 ±0.00c
	(23)	(252)	(9)	(0)
Bean	0.54 ± 0.22a	10.22 ±1.00a	0.45 ± 0.21a	3.04 ± 0.85b
	(21)	(225)	(8)	(68)
Cucumis	0.02 ± 0.02b	1,04 ± 0.65b	0.18 ± 0.12a	0.04 ± 0.04c
	(1)	(23)	(4)	(1)
Squash**[b]**	0.00 ± 0.00b	0.36 ± 0.36b	0.00 ± 0.00b	0.13 ± 0.09c
	(0)	(8)	(0)	(3)
Eggplant	0.00 ± 0.00b	1.54 ± 0.60b	0.00± 0.00b	0.13 ± 0.07c
	(2)	(50)	(14)	(3)
F	4.709	78.061	1.635	12.972
df	5,204	5,126	5,126	5,126
P	<0.0001	<0.0001	0.156	< 0.0001

[a] When the columns are examined, means with the same letter are statistically insignificant according to the Tukey HSD test (P>0.05). [b] Values in parentheses indicate the total number of individuals

Table 6- Total average number of *Orius* spp. and pest insects in fruits trees (mean ± SE /20 plants) in Balcalı in 2019-2020

Fruit trees	*Orius*	*Thrips*	*Aphid*	*Leafhopper*
Pear[b]	0.00 ± 0.00b[a] (0)[b]	0.12 ± 0.12a (5)	0.00 ± 0.00a (1)	0.02 ± 0.02a (1)
Plum	0.38 ± 0.19a (2)	0.05 ± 0.05a (2)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
Apple	0.32 ± 0.26a (10)	1.84 ± 0.70 (72)	0.05 ± 0.03a (2)	0.05 ± 0.03a (2)
Plum[b]	0.00 ± 0.00 (0)	0.15 ± 0.15a (6)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
Pomegranate	0.00± 0.00b (2)	0.20 ± 0.10a (8)	0.00 ± 0.00a (0)	0.02 ± 0.02a (1)
Nectarine [b]	0.00 ± 0.00b (58)	1.48 ± 0.79a (0)	0.00 ± 0.00a (0)	0.02 ± 0.02a (0)
Lemon[b]	0.00 ± 0.00b (48)	1.23 ± 0.62a (0)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
Loquat	0.00 ± 0.00b (7)	1.97 ± 1.51a (77)	0.00 ± 0.00a (0)	0.10 ± 0.04a (4)
F	4.852	1.435	1.503	1.705
df	7,304	7,304	7,304	7,304
P	0.008	0.0191	0.166	0.107

[a]: When the columns are examined means with the same letter are statistically insignificant according to the Tukey HSD test (P>0.05). b: Values in parentheses indicate the total number of individuals.

Table 7- Total average number of *Orius* spp. and pest insects ((mean ± SE/20 plants) in field crops in Balcalı in 2019-2020

Field crop	*Orius*	*Thrips*	*Aphid*	*Leafhopper*
Cotton	0.38 ± 0.12a[a] (1)[b]	2.09 ± 0.86b (18)	2.57 ± 0.51a (5)	0.09 ± 0.00b (4)
Soybean	0.00 ± 0.00b (1)	2.66 ± 0.51a (59)	0.00 ± 0.00b (0)	0.04 ± 0.04b (1)
Sesame	0.71 ± 0.23a (15)	4.09 ± 0.46a (86)	0.14 ± 0.14b (3)	1.95 ± 0.52a (41)
Peanut	0.04 ± 0.04b (8)	0.85 ± 0.42c (44)	0.23 ± 0.15b (54)	0.19 ± 0.11b (2)
F	6.141	5.098	19.500	11.443
df	3,80	3,80	3,80	3,80
P	0.001	0.003	<0.0001	<0.0001

[a]When the columns are examined means with the same letter are statistically insignificant according to the Tukey HSD test (P>0.05). [b]Values in parentheses indicate the total number of individuals.

3.3. Population dynamics of Orius and harmful insects on the crop plants

The population dynamics of *Orius* in relation to pest species on various winter and summer vegetables, fruit trees and field crops are illustrated in Figure 2. In winter vegetables (Figure 2a), the number of thrips and aphids increased steadily since the first sampling date and, accordingly, *Orius* species were recorded. However, the densities of *Orius* remained lower than those of the prey insects. From 15th January to 15th March 2020, *Orius* populations showed fluctuations, likely influenced by the densities of both thrips and leafhoppers. In winter vegetable species, considering the average population densities of predatory and herbivorous insects, statistically significant and positive relationships were found only between *Orius* and thrips populations (*Orius*–Thrips: R= 0.55, P= 0.009; *Orius*–aphids: R= 0.20, P= 0.398; *Orius*–leafhoppers: R= 0.39, P= 0.443). On fruit trees (Figure 2b), *Orius* individuals were recorded alongside thrips and leafhoppers during the first sampling week. Relatively high thrips densities were detected in fruit trees on 22nd January and 29th April 2020, and the numbers of.

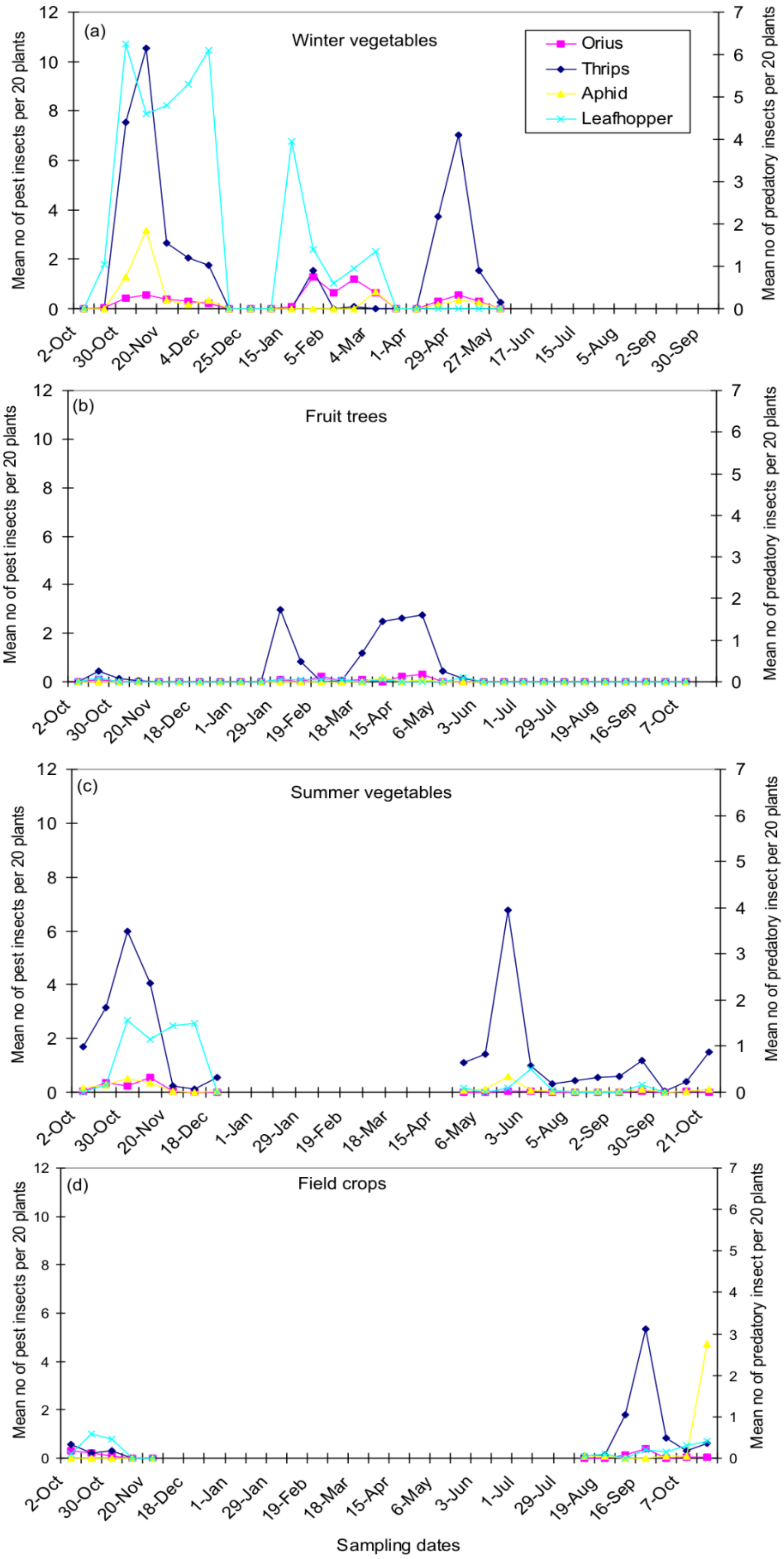

Figure 2- Seasonal densities of *Orius* spp. and different prey groups on different cultured plant groups; a: winter vegetables, b: fruit trees, c: summer vegetables and d: field crops.

Orius fluctuated in response to the thrips populations. In fruit trees, the *Orius* population showed a strong dependence on the thrips populations, and significant positive relationships were detected (*Orius*–thrips: R = 0.65, P = 0.0001; *Orius*–aphids: R = 0.22, P = 0.226; *Orius*–leafhoppers: R = 0.21, 0.316). In summer vegetables (Figure 2c), thrips and leafhoppers increased during the October–December period in 2019 and, correspondingly, the *Orius* population increased as well. In 2020, thrips and *Orius* exhibited similar population patterns until the last sampling date; the densities of *Orius* appeared to be lower than the thrips densities. However, statistically significant positive relationships were observed among the *Orius* and both thrips and aphid populations (*Orius*–thrips: R = 0.58, P = 0.009; *Orius*–aphids: R = 0.54, P = 0.007; *Orius*–leafhoppers: R = 0.43, 0.062). In field crops (Figure 2d), there was a short-term increase in the thrips population on 28th September 2020 and, during this period, the *Orius* density also increased temporarily, although it remained at a lower level than that of the thrips populations. At the last sampling date, although the aphid population increased on the leaves, *Orius* individuals were not found. A significantly strong and positive relationship was found only between the *Orius* and thrips populations (*Orius*–thrips: R = 0.73, P = 0.006; *Orius*–aphids: R = 0.14, P = 0.655; *Orius*–leafhoppers: R = 0.22, 0.488). Overall, the population dynamics of *Orius* seemed to be closely associated with the fluctuations in thrips populations in the different plant environments.

4. Discussion

Although the numbers of thrips were higher in winter vegetables than in other crop plants, except for broad beans, the reason that *Orius* individuals were found in very low numbers may be related to the absence of flowers during this period. It is worth noting that broad bean plants showed lower numbers of thrips than leafhoppers. The high number of *Orius* in broad bean plants could be attributed to the plants' morphological structure and chemical content such as the presence of extrafloral nectars for them to feed on, rather than relying solely on prey such as thrips. In the Çukurova region, broad bean plants remain in bloom from December to early March, providing large covered flowers that may serve as protection, shelter and even sites for mating and egg laying for predatory species like *Orius*. Moreover, broad bean plants are known to be richer in extrafloral nectars, attracting many beneficial insects (Nuessly et al. 2004). Notably, *O. niger* and *O. laevigatus* species were detected in both winter and summer vegetables in the Çukurova region (Zeren & Düzgüneş 1983). To summarise, the presence of flowers and extrafloral nectars in broad bean plants may have contributed to the higher number of *Orius* individuals, while their low presence in other winter vegetables could be attributed to the absence of these essential resources during that season.

Frankliniella occidentalis and *T. tabaci* were widely found in summer vegetables in this region. *Frankliniella occidentalis* was abundant in eggplant and pepper flowers, but its presence was minimal in tomato flowers (Atakan 2010b). According to Bahşi (2011), the most common *Orius* species in Antalya was *O. laevigatus* (66%), followed by *O. niger* (33%). Additionally, *O. limbatus* (Wagner), *O. majusculus* (Reuter), *O. minutus* (L.) and *O. vicinus* were also recorded in this region. Thrips were the most prevalent pest group in various plant samples, often found alongside *Orius* species. While *O. laevigatus* was most common in winter vegetables (mainly broad beans), *O. niger* dominated in summer vegetables (mainly beans; Table 5). *Orius* populations were relatively low in fruit trees, indicating a preference for herbaceous habitats regardless of prey species and their densities. In Adana, *O. niger* played a crucial role in controlling pest thrips like *Frankliniella* flower thrips in unsprayed cotton fields (Atakan and Özgür 2001). A study conducted in a clover field in the same region revealed that *F. occidentalis* was the most harmful thrips species, with *O. niger* and *O. laevigatus* frequently found together with these pest thrips throughout the year (Atakan & Tunç 2004).

Although the number of thrips in pepper plant flowers was higher compared to other summer crops like tomatoes, eggplants and beans, only a few *Orius* specimens were detected (Table 5). In Florida, *O. tristicolor* effectively suppressed *Frankliniella* flower thrips in field-grown peppers (Funderburk et al. 2000). Researchers (Dissevelt et al. 1995) reported that eggplants and, more particularly, peppers due to their abundant pollen were more attractive to *Orius* species. The lack of *Orius* in tomato plants may be attributed to reduced mobility of nymphs or adults due to the plants' secretions negatively affecting *Orius*' searching ability and, thus, their predation on pest insects (Coll & Ridgeway 1995). *Orius* species densities were lower in field crops like cotton when compared to previous studies in the same ecological area (Table 7). Previous research in a pesticide-free cotton field in the region recorded a significant number of *Orius* adults preying on flower thrips in cotton flowers (Atakan 2006). *Orius niger* closely followed the populations of *Frankliniella* flower thrips, indicating that this predatory bug could effectively suppress pest thrips in unsprayed cotton fields (Atakan 2006; Atakan 2010a).

The distribution and population of *Orius* in different plant groups varied primarily due to thrips populations, which appeared to be their main prey. Studies on the field peppers (Funderburk et al. 2000; Hansen et al. 2003; Funderburk et al. 2018) and cotton (Atakan and Özgür 2001; Atakan 2006) confirmed that *Orius* plays an essential role in controlling *Frankliniella* flower thrips, leading to reduced thrips populations in the absence of pesticide application. Thrips and leafhoppers, along with *Orius*, were recorded on summer vegetables and newly planted broad beans in autumn. In spring, the insects found on fruit trees were mainly thrips and limited numbers of *Orius* individuals. After the flowering period of the sampled trees, thrips and *Orius* shifted together to summer vegetables, indicating similar distribution patterns and population dynamics throughout the year, according to the flowering cycle of cultivated plants.

Thrips and *Orius* were collected primarily from the flowers of the sampled cultivated plants, except for broad beans, where few predators were found during the winter months, likely due to the lack of blooming. *Orius* nymphs and adults were observed gathering in the same flowers infested by thrips, depending on thrips density (Ramachandran et al. 2001; Reitz et al. 2003). The scarcity of thrips larvae in the sampled plants may be because the predators prefer thrips larvae, as they are less mobile and easier to prey on than adult thrips (Funderburk et al. 2000; Baez et al. 2004). Despite low *Orius* numbers in the sampled plants compared to previous studies in the area, the prey/predator ratios were low in many plants, suggesting that thrips, in particular, were at risk of being preyed upon. For instance, in field conditions in Florida, 40 thrips per predator were sufficient to keep the thrips population in control (Funderburk et al. 2000; Reitz et al. 2003).

5. Conclusions

The study reveals that *O. laevigatus* has been observed in both cold interior and warm regions of Turkey. The population dynamics of *Orius* and thrips were found to be influenced by the flowering patterns of the sampled crop plants. *Orius* populations showed fluctuations in response to changes in thrips populations. Notably, in regions where pesticide use is limited, *Orius* emerged as a significant biological factor in controlling thrips density, particularly in plants belonging to the Fabaceae family, such as broad beans. This indicates the potential of *Orius* as an important natural predator for managing thrips populations in agricultural settings without heavy reliance on pesticides.

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Content of Organic Acid and Essential Oil in Natural Sweet Chestnuts (*Castanea Sativa* Mill) Growing in Giresun/TURKEY

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ABSTRACT
This study investigated organic acid and essential oil components in sweet chestnut (*Castanea Sativa* Mill) fruits collected from naturally growing chestnut trees within the borders of Giresun province. For this purpose, chestnut samples were collected from trees in 10 different areas in Giresun. The organic acid composition of chestnuts was determined by high-performance liquid chromatography (HPLC). Essential oil components were identified with gas chromatography-mass spectrometry (GC-MS). Five different organic acids, including oxalic, quinic, maleic, citric and succinic acid, were investigated in chestnut samples, and the highest concentrations in chestnut samples were oxalic and citric acid. In the GC-MS analysis of chestnut samples, 18 essential oils were detected and terpinolene (TPO) was the most abundant. Another vital component found in chestnut samples is limonene.

Keywords: Chestnut, organic acid, essential oil, HPLC, GC-MS, monoterpenes.

1. Introduction

Chestnut is a tree from the beech family with upright trunk, reddish bark and hard leaves. Thirteen chestnut species are found in the northern hemisphere. The most common type of chestnut is "*Castanea sativa* Mill", which is delicious and consumable (Burnham et al.1986; Soylu 2004; Serdar et al. 2014). It is the most common chestnut species in Turkey, which is the reason for selecting it for research. Sweet chestnut (*Castanea sativa* Mill.) forests represent an important landscape component in European forest ecosystems, covering more than 2.5 million hectares. Sweet chestnut has a long history in Europe and its distribution area has expanded since the IV century BC (Silva et al. 2002; Silva et al. 2004a; Conedera et al. 2004; Conedera et al. 2016).

Chestnuts play an important role in human nutrition due to containing vitamins, minerals, amino acids, and antioxidant compounds (Alasalvar & Shahidi 2008). Chestnut has important health effects linked to its nutritional composition. It is cholesterol-free, rich in vitamin C, high in fiber and gluten-free. Its importance in human nutrition increases for cases of coronary heart disease, cancer, and celiac disease (Gonçalves et al. 2010). Although chestnuts contain less fat than other types of nuts, they are a rich source of essential oil, especially linoleic acid, which is essential for the human body. The omega-3 content in chestnuts slows down the growth of cancerous cells, increases the effectiveness of chemotherapy and reduces side effects of chemotherapy (Hardman 2002).

There are studies in the literature that determined the organic acid content (Vaughan & Geissler 1997; Silva et al. 2002; Silva et al. 2004a) and essential oil content in foods (Ferreira-Cardoso et al. 1999; Benatti et al. 2004). Organic acids are compounds that have acidic properties, are classified according to the number of carboxylic functions, and have a protective effect against various diseases due to their antioxidant activities. They are involved as intermediate or final products in many important metabolism and catabolism mechanisms in biological processes, animals and plants (Vaughan & Geissler 1997; Silva et al. 2002; Silva et al. 2004a; Silva et al. 2004b; Chahardoli 2020). The nutritional value of chestnuts is affected by the nature and concentration of organic acids. Some organic acids, such as citric, succinic, fumaric and malic acids, have important roles in human metabolism (Delgado et al. 2018). Essential oils have important effects in regulating plasma lipid levels, cardiovascular diseases and many chronic diseases relating to the immune system, insulin levels, neuron development and visual function (Ferreira-Cardoso et al. 1999; Benatti et al. 2004).

The aim of this study was to examine the fruits of natural chestnut trees growing in Giresun province in terms of organic acid and essential oil components. There is no research about organic acids and essential oil, which have significant effects on human health, in chestnuts grown in the Giresun region. In addition, the number of studies about this subject worldwide is quite low. It is thought that the results obtained will contribute to the scientific literature and future research.

2. Material and Methods

2.1. Chestnut samples

Chestnut samples were taken from chestnut trees in 10 different locations close to each other in Giresun province. Approximately 2 kg of chestnut samples were collected from each sampling area. The locations of the sampling areas are given in Figure 1 and their coordinates are given in Table 1. The shells and pellicles of the chestnuts were peeled by hand. The peeled samples were kept in a refrigerator at +4 °C in sterile bags and the analysis was started the next day.

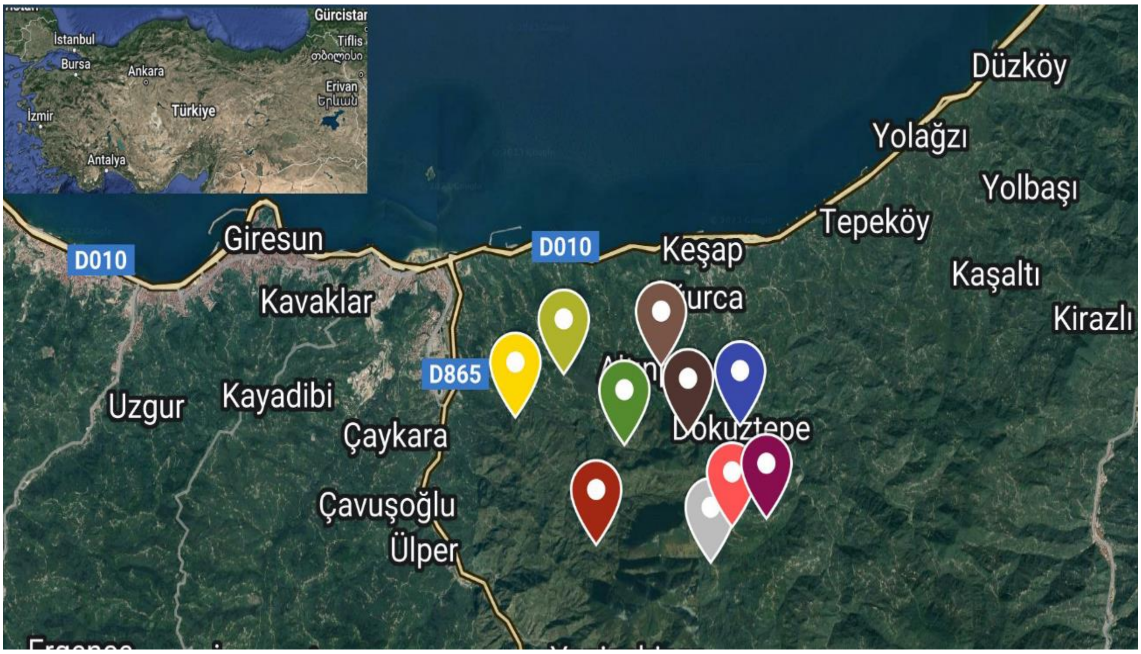

Figure 1-  **Location points represent Güveç, Alataş, Sarvan, Kayabaşı, Dokuztepe, Sayca, Demirci, Altınpınar, Bayrambey, and Güneyköy, respectively**

Table 1- Sampling areas and coordinates

Sample No	Location name	Latitude (°N)	Longitude (°E)
1	Güneyköy	40° 53′ 34″	38° 28′ 29″
2	Kayabaşı	40° 52′ 32″	38° 29′ 11″
3	Güveç	40° 52′ 20″	38° 28′ 53″
4	Sarvan	40° 52′ 41″	38° 27′ 49″
5	Altınpınar	40° 53′ 37″	38° 30′ 45″
6	Sayca	40° 52′ 52″	38° 30′ 32″
7	Dokuztepe	40° 52′ 57″	38° 31′ 24″
8	Alataş	40° 51′ 55″	38° 31′ 46″
9	Demirci	40° 51′ 51″	38° 31′ 16″
10	Bayrambey	40° 51′ 24″	38° 30′ 53″

2.2. Reagent and solutions

Oxalic acid, quinic acid, maleic acid, citric acid, and succinic acid with analytical standard (500 mg; Supelco) were purchased from Sigma-Aldrich Chemie GmbH. Ultrapure deionized water (≈18.2 MΩ.cm) used in sample preparation and analysis was obtained from Sartorius Arium Model Ultra-Pure Water Systems. All other reagents and chemicals used were of analytical grade and solvents were HPLC gradient grade and obtained from a variety of commercial vendors (Merck and Sigma-Aldrich).
An ISOLUTE C18 solid-phase extraction column (SPE; average particle size 50 µm, pore diameter 60 Å, sorbent type non-polar, 10g /70mL) was used for the extraction of the chestnut samples.

2.3. Extraction of organic acids

Extraction of organic acids from chestnut samples was performed according to the method used by Silva et al. (2004b) and modified by Ribeiro et al. (2007) (Silva et al. 2004b; Ribeiro et al. 2007). Five grams of each chestnut sample was weighed and extracted by grinding with 50 mL of methanol at 40 °C with a mixer. The methanolic extract was filtered and the supernatant was re-extracted with methanol. The collected filtrates were passed through an ISOLUTE C18 column (solid phase extraction column) preconditioned with 30 ml of methanol and 70 mL of HCl (pH 2). Non-polar compounds remained in the column, polar ones such as organic acids remained in aqueous solution. The aqueous solution was evaporated, redissolved in 0.01 N sulfuric acid (1 mL) and analyzed by HPLC. The analysis results were calculated on a dry matter basis from the moisture content of chestnut samples determined by drying them in an oven at 105 °C.

2.4. HPLC/UV analysis of organic acids

HPLC/UV analyses of organic acids were carried out with an Agilent 1260 Infinity series with UV detection at 210 nm, using phosphate buffer (KH_2PO_4 - H_3PO_4) with pH 2.70 as the mobile phase, at a flow rate of 0.2 mL/min with an injection volume of 5 µL. The analytical column was Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm, 5 µm) and column temperature was 30 °C.

2.5. Essential oil extraction with Clevenger apparatus and GC-MS analysis

Twenty five grams of chestnut sample was placed in a 500 mL glass bottle belonging to the Clevenger apparatus and boiled with 250 mL distilled water for 2.5 hours to obtain essential oils under reflux. The oil in the capillary, where the essential oil was collected, was sent to the separatory funnel and 5 mL of n-hexane and about 1 g of NaCl were added, and then a sufficient amount of Na_2SO_4 was added to retain the remaining water.

The extracts were transferred to vials and analyzed with GC-MS (Agilent 7694 model with FID detector and 5975C series MS) instrument. Device parameters were HP-5MS column (30 m length, 0.25 mm inner diameter, 0.25 m film thickness), injection temperature 250 °C; carrier gas helium; flow rate: 1 mL/min; electron energy: 70 eV; and detector temperature: 280 °C. The oven temperature program had initial temperature of 50 °C for 4 minutes, increasing at 10 °C/min to 200 °C, left for 1 minute, and finally increasing with 20 °C/min to 230 °C and left for 1 minute. The data were identified using MS Library NIST and Wiley Library (Sevindik et al. 2019).

2.6. Linearity

The calibration curve values, limits of detection (LOD) and limits of quantification (LOQ) of chestnut samples confirming the HPLC-MS/MS analytical method are given in Table 2. The calibration curve method was used to calculate LOD and LOQ (Şengül 2016). Stock organic acid standard solutions of 5.00 mg/mL were prepared with ultrapure water and 5-point calibration curves in the range of 0.100-0.500 mg/mL were plotted for quantitative analysis of organic acids in chestnut samples. Good linearity was observed for all of them. Each sample was studied with three parallel repeats.

3. Results and Discussion

The peaks detected with HPLC analysis for organic acid standards are given in Figure 2 and good separation was observed.

The oxalic acid peak was observed first, followed by quinic acid, maleic acid, citric acid, and succinic acid peaks. In Table 2, the arrival times of the peaks, linear correlation coefficient (R^2) values for the calibration curves and LOD and LOQ values are given. The R^2 values were >0.999.

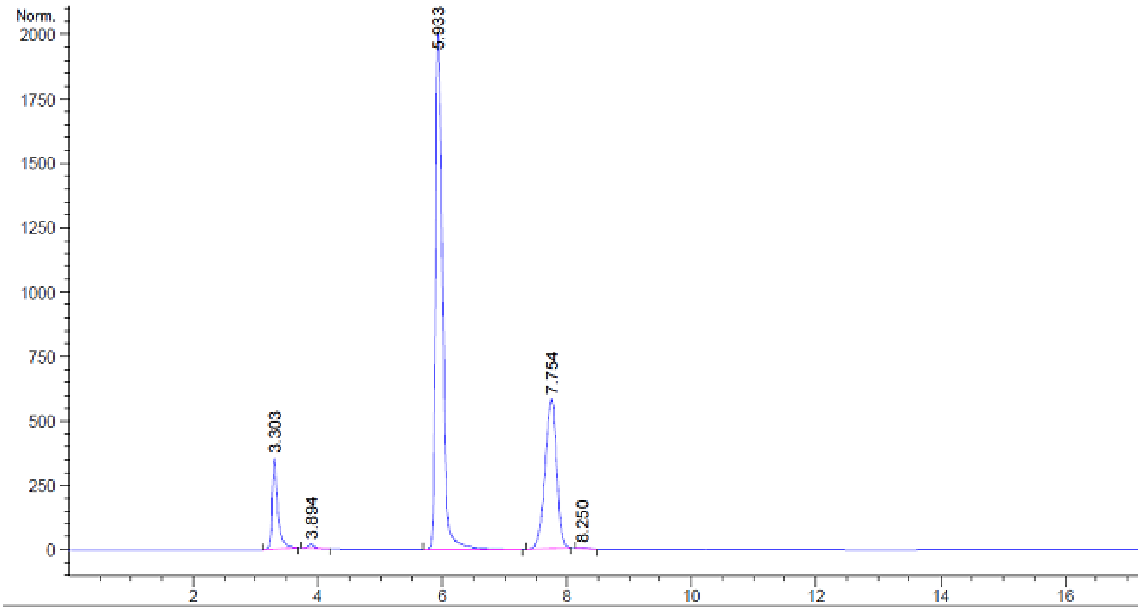

Figure 2- HPLC chromatogram for standard organic acid mix solution (according to retention times 3.303-oxalic acid, 3.894-quinic acid, 5.933-maleic acid, 7.754-sitric acid, 8.250-succinic acid)

Table 2- Calibration curve, LOD and LOQ values for chestnut sample analyses

Organic acids	*R^2**	*Retention time (min)*	*LOD* (mg/mL)*	*LOQ* (mg/mL)*
Oxalic acid	0.9994	3.486	0.0013	0.0156
Quinic acid	0.9995	3.784	0.0009	0.0108
Maleic acid	0.9995	5.895	0.0009	0.0108
Citric acid	0.9998	7.765	0.0007	0.0084
Succinic acid	0.9995	8.241	0.0010	0.0116

*R^2: Linear correlation coefficient; LOD: Detection limit; LOQ: Quantification limit

The structure and amounts of organic acids are important factors affecting the flavor of fruits and vegetables. The presence or absence and amount of each organic acid is important for taxonomic studies. In addition, organic acids may have protective roles against various diseases due to their antioxidant activity; therefore, the qualitative and quantitative availability and analysis of organic acids in chestnuts are very important (Ribeiro et al. 2007). Organic compound concentrations in chestnut samples are given in Table 3 and Table 4.

Table 3- Organic acid content in chestnut samples (n=10, mg/g)

Organic acids	*Mean*	*Median*	*±SD**	*Min.*	*Max.*
Oxalic acid	0.988	1.038	0.593	0.304	1.834
Quinic acid	0.362	0.364	0.237	0.123	0.597
Maleic acid	0.004	0.003	0.004	0.001	0.014
Citric acid	0.498	0.584	0.168	0.199	0.640
Succinic acid	0.254	0.157	0.220	0.059	0.748

* SD: Standard deviation

Among the organic acids, oxalic acid had the highest value followed by citric, quinic, succinic and maleic acid in all samples. Oxalic acid is found in many plants, and in addition to its antioxidant properties, consumption of plants with high oxalic acid content can have a negative effect on metabolism as oxalates bind calcium and other minerals and form calcium oxalate urinary stones (Kayashima & Katayama 2002). The concentration of oxalic acid in chestnut samples was between 0.304-1.834 mg/g, with an average concentration of 0.988 mg/g. The organic acid amounts in two Portuguese chestnut cultivars were studied by Riberio et al. (2007) and the oxalic acid concentrations were found in the range of 0.0013-0.0019 mg/g in Judia cultivar and 0.0014-0.0017 mg/g in Longal cultivar (Ribeiro et al. 2007). These values are quite different from our oxalic acid results. The differences between these results may be due to differences in chestnut variety, soil conditions, cultural practices, climate, and analysis method (Gölükcü & Tokgöz 2018; Santos et al. 2022). In the study conducted by Suárez et al. (2012), oxalic acid was in the range of 0.073-1.667 mg/g in chestnuts from Spain, with an average of 0.525 mg/g (Suárez et al. 2012). In their study, Carocho et al. (2013) determined the oxalic acid concentration in Portuguese chestnuts was 0.7 mg/g (Carocho et al. 2013). The results of these last two studies are consistent with our findings and support our results. If the organic acid values of chestnuts are compared with vegetables containing high concentrations of oxalic acid, values were; spinach (3.64-11.45 mg/g), rhubarb (5.11-9.84 mg/g), beets (0.369-7.94 mg/g) (Attalla & Monga 2014), the tropical vegetables of taro (*Colocasia esculenta*) (2.78-5.74 mg/g FW), sweet potato (*Ipomoea batatas*) (4.70 mg/g FW) and yam (*Dioscorea alata*) (4.86-7.81 mg/g) (Noonan & Savage 1999). The organic acid content of chestnuts is slightly below these values. Oxalic acid was found in significant amounts in chestnuts.

Quinic acid is a natural compound with antioxidant and anti-inflammatory properties found abundantly in plants. It increases the production of tryptophan and nicotinamide in the gut, which in turn increases the concentrations of all lipoproteins that can improve metabolic health and are effective in the treatment of diabetes (Heikkilä et al. 2019; Dong et al. 2022). Quinic acid was detected in only three of the ten chestnut samples and quinic acid concentrations had an average of 0.362 mg/g, ranging between 0.123-0.597 mg/g. In similar studies, Ribeiro et al. (2007) found malic acid+quinic acid concentrations in the range of 0.036-0.051 mg/g (Judia) and 0.055-0.113 mg/g (Longal) in two different chestnut species (Ribeiro et al. 2007). Carocho et al. (2013) found the quinic acid concentration in chestnuts was 13.000 mg/g (Carocho et al. 2013). Our results are close to the study by Ribeiro et al. but quite different from Carocho et al. Quinic acid (1, 3, 4, 5-tetrahydroxy cyclohexane carboxylic acid) is mostly found in coffee beans, cinchona bark, potatoes, apples, and peaches (Ercan & Doğru 2022). Moldoveanu (2012) determined quinic acid concentrations in 23 tobacco samples and found they ranged from 0.750-11.450 mg/g (Moldoveanu 2012). Since there is no study in the literature about the content of quinic acid in foods, it is only possible to state that the amount of quinic acid in chestnuts has lower concentration than in tobacco.

Maleic acid (MA) is a very important chemical intermediate widely used in industrial chemistry. It is used as a raw material in the production of lubricant additives, unsaturated polyester resins, surface coatings, plasticizers, copolymers, and agrochemicals (Wojcieszak et al. 2015). Maleic acid is found at very low amounts in plants and is produced commercially by synthesizing from maleic acid anhydride, 5-hydroxymethylfurfural (HMF), or furfural (Thiyagarajan et al. 2020). Organic acids are known as phytoremediation agents and exogenous preservatives, but this property of MA has not been investigated yet (Mahmud et al. 2017). Among the organic acids in our chestnut samples, the organic acid with lowest concentration was maleic acid. It was found in the concentration range of 0.001-0.014 mg/g in 8 of 10 chestnut samples. Its average concentration was 0.004 mg/g. In studies about the organic acid content of plants, maleic acid has been investigated very little and no study was found examining the maleic acid content in chestnuts. Related to maleic acid in the literature, the organic acid content of honey was investigated, and it was found in the range of 0.0002-0.0005 mg/g in chestnut honey (Suárez-Luque et al. 2002). Maleic acid concentrations are low in both chestnuts and chestnut honey.

Citric acid is an important organic acid for metabolism. The citric acid cycle is the most important metabolic pathway that provides energy to the body, which living cells use to completely oxidize biofuels to carbon dioxide and water. It also provides many intermediate products required for the synthesis of amino acids, glucose, heme, etc. (Akram 2014). Citric acid is found mostly in citrus fruits and the concentration of citric acid in lemon is around 48 mg/g (Penniston et al. 2008). In our sweet chestnut samples, the highest concentration was observed for citric acid after oxalic acid among organic acids. The average citric acid concentration was 0.498 mg/g and the concentration range was 0.199 to 0.640 mg/g. Citric acid concentrations in organic acid research conducted in Portugal on sweet chestnuts: were 5.220 mg/g according to Delgado et al. (2018) for the Longal variety and 9.550 mg/g for the Judia variety (Delgado et al. 2018). Riberio et al. (2007) found values were between 0.040-0.107 mg/g for the Longal variety and 0.050-0.090 mg/g for the Judia variety (Ribeiro et al. 2007). Gonçalves et al. (2010) detected citric acid in the range of 1.460–8.790 mg/g in ten Portuguese cultivars (Gonçalves et al. 2010). Vekiari et al. (2006) found citric acid concentrations between 1.27 and 4.30 mg/g in fifteen European chestnut varieties collected from different European countries (Vekiari et al. 2006). The organic acid contents were identified in 21 chestnuts obtained from Tenerife (Spain) by Suárez et al. (2012 and the average citric acid concentrations were between 0.261 and 1.135 mg/g (Suárez et al. 2012). Differences in the organic acid content of plants are affected by the type of chestnut, soil conditions, climate and even the time of collection (Gölükcü & Tokgöz 2018). Organic acid levels vary between species, cultivars, and even individual tissues of a plant grown under the same conditions (López-Bucio et al. 2001). For these reasons, there are differences in the citric acid values in chestnuts between these studies and our study.

Succinic acid is a good antioxidant and immune system stimulant, stabilizing the structure and functional activity of mitochondria (Lieshchova et al. 2020). In nine of the ten chestnut samples examined, an average of 0.254 mg/g succinic acid was found, and the concentration range was 0.059-0.748 mg/g. There is no study in the literature that determined the succinic acid concentration in chestnuts. Succinic acid is found more in foods such as broccoli, rhubarb and sugar beet. Succinic acid was identified in dry rhubarb stems and its concentration was found to be 2.600 and 8.990 mg/g in two different varieties (Golubkina et al. 2022). The concentration of succinic acid in tomatoes was examined by Marconi and Montanari, and the range was 0.088-0.190 mg/g in 5 of the 7 tomato juice samples (Marconı et al. 2007). Succinic acid concentrations in chestnuts and tomatoes are close to each other.

Table 4- Organic acid content (mg/g) of chestnut samples from each location (n=3)*

Sample No	*Oxalic acid*	*Quinic acid*	*Maleic acid*	*Citric acid*	*Succinic Acid*
1	0.304±0.023	0.364±0.132	0.004±0.003	0.200±0.063	0.441±0.140
2	1.427±0.178	<LOD	0.014±0.002	0.640±0.020	0.332±0.107
3	1.329±0.407	<LOD	<LOD	0.600±0.140	0.146±0.104
4	1.834±0.117	<LOD	0.004±0.003	0.603±0.087	0.113±0.102
5	1.433±0.293	<LOD	0.007±0.003	0.438±0.070	0.059±0.016
6	1.594±0.215	<LOD	0.006±0.003	0.602±0.035	<LOD
7	0.308±0.015	<LOD	0.002±0.001	0.622±0.029	0.160±0.069
8	0.746±0.474	0.597±0.020	0.002±0.001	0.508±0.046	0.748±0.082
9	0.541±0.182	<LOD	<LOD	0.568±0.070	0.157±0.134
10	0.365±0.097	0.123±0.121	0.001±0.001	0.199±0.019	0.128±0.036

*: mean±SD

The oil obtained from chestnut samples with the Clevenger apparatus was analyzed for essential oil components by GC-MS and the chromatogram is shown in Figure 3. When the analysis output was evaluated, a total of thirty-three peaks were found, as seen in Figure 3. These peaks were evaluated using the Wiley7 and NIST05 libraries on the device. Eighteen of these peaks were clearly identified and are shown in Table 5. Essential oils, characterized by their strong odor, are aromatic hydrophobic volatile liquids obtained from different parts of plants, such as flowers, roots, bark, leaves, seeds, bark and fruits. (O'Bryan et al. 2015). Essential oils are an important group of plant secondary metabolites and include a variety of volatile terpenes, aldehydes, alcohols, ketones and simple phenolics (Bunse et al. 2022). Terpenes, and especially monoterpenes, are the most common components in essential oils. Among the eighteen compounds identified with GC-MS analysis of chestnut samples, the most prominent ones are terpinolene (TPO) and limonene. Among the compounds identified, terpinolene appears to be the most abundant compound (retention time: 19.929). Terpinolene and limonene are monoterpenes. Terpinolene is a bioactive compound with important pharmacological activities. This compound is commonly found in the chemical composition of aromatic plants, especially plants of Asian origin such as black currant and saffron (*Curcuma longa*). It has pharmacological activity such as antifungal, antioxidant and insecticide features (Menezes et al. 2021). TPO has a wide range of biological activities including anticancer, antioxidant, antifungal and antiviral effects. Limonene (retention time: 14.764 and 18.668) was another important component among the 18 components in chestnut samples. Limonene is a monoterpene with a citrus odor at room temperature, commonly found in plants of the Rutaceae family. It is abundant in citrus plants such as lemon, grapefruit, and orange and in grapes (Amini et al. 2020; AlSaffar et al. 2022). Monoterpenes are known to have anticancer properties (Sobral et al. 2014). D-limonene, the most common isomer of limonene, was classified as having a wide range of biological activities, including antioxidant, anti-inflammatory, antibacterial, antiviral, anticancer, anti-fibrotic, vasodilator, and anti-hypertensive effects (Sun 2007, AlSaffar et al. 2022). Studies show that the anti-tumorigenic properties of d-limonene were evaluated for various types of cancer including breast, stomach, prostate, bladder and colon cancer (Anandakumar et al. 2021). In addition to its medicinal potential, limonene also has a wide range of uses in cosmetics. It is a commercially important molecule given its use in soft drinks, many sweetening products, and other household uses (Erasto & Viljoen 2008). The fact that chestnuts contain these two important components increases their importance as a nutritional substance.

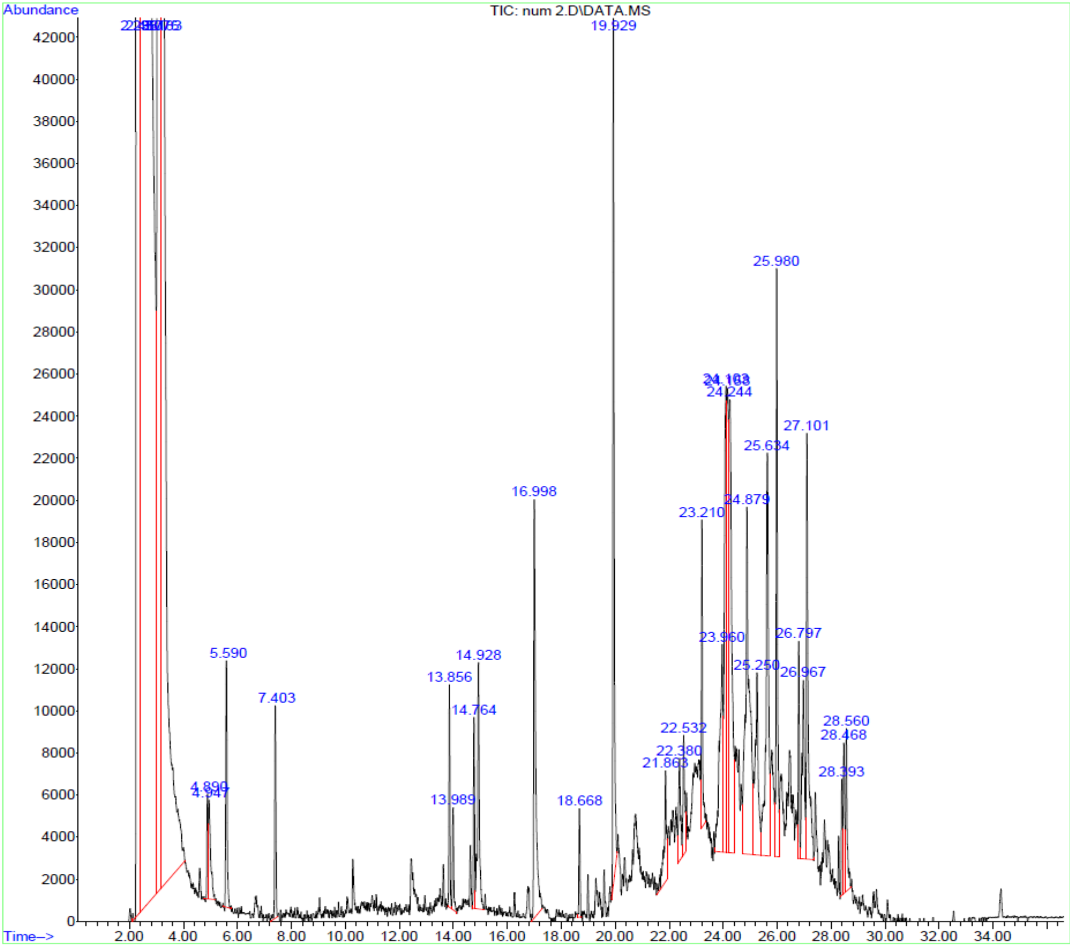

Figure 3- GC-MS chromatogram for chestnut essential oil

Table 5- Chestnut essential oil composition based on Wiley7 and NIST05 libraries

Retention time	*Component name*
4.890	3-methyl-1-butanol (isoamyl alcohol)
5.590	Toluene
7.403	Hexamethylcyclotrisiloxane
13.806	Octamethylcyclotetrasiloxane
14.764	Limonene
14.928	1,2-Dichlorobenzene
16.998	Benzoic acid, methyl ester (methyl benzoate)
18.668	Limonene
19.929	Terpinolene (3-Cyclohexene-1-methanol, α,α 4-trimethyl-)
22.380	5-Eicosyne
23.210	1,1,6-Trimethyl-1,2-dihydronaphthalene
24.104	Longifolene, 1,4-Methanoazulene, decahydro-4,8,8-trimethyl-9-methylene-
25.634	Tritetracontane
26.797	Naphthalene
27.101	Pentatriacontane
28.393	1-Octanol, 2-butyl
28.468	Hexadecane, 2,6,11,15-tetramethyl (crocetane)

4. Conclusions

In this study, organic acid and essential oil contents of chestnut fruits from trees growing spontaneously in forest areas within the borders of Giresun province were determined. There were significant differences in organic acid concentration between samples taken within the same provincial borders. Organic acids and essential oil are natural sources of antioxidants and are therefore very important for metabolism. Due to these properties, studies identifying the content of these components in edible plants need to be increased. According to the research results, chestnut is a useful source of organic acids and essential oils.

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Conflict of Interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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A Novel Approach for Tomato Leaf Disease Classification with Deep Convolutional Neural Networks

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ABSTRACT

Computer-aided automation systems for the detection of plant diseases represent a challenging and highly impactful research domain in the field of agriculture. Tomatoes, a major and globally significant agricultural commodity, are cultivated in large quantities. This study introduces a novel approach for the automated detection of diseases on tomato leaves, leveraging both classical machine learning methods and deep neural networks for image classification. Specifically, classical learning methods employed the local binary pattern (LBP) technique for feature extraction, while classification tasks were carried out using extreme learning machines, k-nearest neighborhood (kNN), and support vector machines (SVM). In contrast, a novel convolutional neural network (CNN) framework, complete with unique parameters and layers, was utilized for deep learning. The results of this study demonstrate that the proposed approach outperforms state-of-the-art studies in terms of accuracy.

The classification process covered various scenarios, including binary classification (healthy vs. unhealthy), 6-class classification, and 10-class classification for distinguishing different types of diseases. The findings indicate that the CNN model consistently outperformed classical learning methods, achieving accuracy rates of 99.5%, 98.50%, and 97.0% for 2-class, 6-class, and 10-class classifications, respectively. Future research may explore the use of computer-aided automated systems to detect diseases in diverse plant species.

Keywords: Automated agriculture, Machine learning in agriculture, Convolutional neural networks in plant pathology, Deep learning in agriculture, Smart farming

1. Introduction

Diseases and pests have a detrimental impact on the production of various agricultural goods, resulting in reduced yields. The timely detection of these diseases and pests is imperative to mitigate the resultant damage. Currently, visual observation by agricultural experts serves as the primary method for detection. The development of computer-aided systems for early disease diagnosis and treatment is proposed to address these challenges.

Computer-aided systems have been widely adopted in agriculture and various other fields. Classical and deep learning methods are frequently employed for the classification of different plant species and disease detection. In recent years, several studies have concentrated on disease detection in various plants, such as tomatoes (Tian et al. 2019; Chen et al. 2020; Karthik et al. 2020; Ouhami et al. 2020; Wspanialy & Moussa 2020; Altuntaş & Kocamaz 2021; Gonzalez-Huitron et al. 2021; Sembiring et al. 2021), rice (Jiang et al. 2020; Sethy et al. 2020; Temniranrat et al. 2021), apple (Park et al. 2018; Kuta et al. 2020; Zhong & Zhao 2020; Rehman et al. 2021), and carrot (Methun et al. 2021) plants. Sustainable agriculture underscores the necessity for efficient, cost-effective, and environmentally friendly techniques. With advancements in computer hardware and software technology, image processing and computer vision have emerged as crucial tools in agriculture, enabling the automated and rapid identification of crop diseases (Xu et al. 2017). The advantages of these technologies include high processing speeds, minimal errors, and improved accuracy. Agricultural research is not limited solely to the automatic detection and classification of crop diseases, it also encompasses other aspects that enhance the overall efficiency of the agricultural sector.

1.1. Related works

In the past 15 years, numerous studies have been undertaken to examine plants and detect diseases by utilizing computer-based systems. Early studies primarily employed classical techniques, including feature extraction, selection, and fundamental image processing methods. The outcomes generated from these techniques were subsequently incorporated into classical learning methods to facilitate learning processes. More recently, there has been a notable surge in the adoption of deep learning methods, which consolidate all these stages into a unified approach. In general, these research endeavors can be broadly classified into two primary categories: (1) classical learning methods and (2) deep learning methods. In the subsequent section, we will delve into the specifics of studies conducted within these two classifications.

1.2. Classical machine learning-based methods

Liming and Yanchao harnessed machine vision technology to enhance the commercial value of strawberries and classify them within the agricultural sector. They employed the k-means clustering method for strawberry classification and adopted multi-specific decision theory to address multifaceted issues. The study provided impressive results, with strawberries being graded in an average of just 3 seconds. Furthermore, the strawberry size detection error remained below 5%, the color grading accuracy reached 88.8%, and the shape classification accuracy exceeded 90% (Liming & Yanchao 2010). In a related study, Burgos-Artizzu and colleagues presented various image processing-based methods for estimating crop, soil, and weed percentages in cultivation area images. They utilized a genetic algorithm to obtain the optimal combination of method and parameter for different image groups, resulting in an average success rate of up to 96% for winter cereal images and 84% for maize images (Burgos-Artizzu et al. 2010). Adebayo and their team reviewed the application of backscatter imaging to monitor food quality in agriculture, specifically discussing laser light backscatter imaging (LLBI), multispectral laser backscatter imaging (MBI), and hyperspectral laser backscatter imaging (HBI). The study examined the effects of moisture, firmness, acidity, and external defects on agricultural and food quality and emphasized the importance of real-time ranking and evaluation for successful quality assessment (Adebayo et al. 2016). Dutta and colleagues proposed an image-processing method to differentiate untreated and pesticide-treated grapes, addressing the high cost, time-consuming nature, and specialized expertise required for chemical methods of pesticide identification. Their study introduced an effective image processing-based non-destructive method for grape classification, achieving 100% accuracy using a Haar filter and support vector machine (SVM) classifier (Dutta et al. 2016). These studies represent promising tools for quality assessment and highlight the potential for future applications to other agricultural products.

Arakeri and Lakshmana discussed the tomato grading process in India, emphasizing the need for careful handling during grading due to the sensitivity of the fruit. To address this, their study proposed an automatic computer-based system. Various experiments were conducted on tomato images, resulting in the proposed method accurately classifying tomatoes as defective or flawless with 100% accuracy. Additionally, an accuracy of 96.47% was achieved for the classification of ripe and unripe tomatoes (Arakeri 2016). Xu and their team developed a system to rapidly and accurately determine wheat leaf rust (BYP) disease to take timely measures to prevent significant decreases in wheat production. BYP is one of the main fungal diseases that cause crop losses. The system integrated embedded Linux and digital image processing techniques and was successfully implemented on the ARM9 microprocessor. Digital image processing and GUI programs were written and compiled with the help of Qt software for crop disease detection and grading. Results were displayed on an LCD screen (Xu et al. 2017).

Compared to expert diagnoses, the system proposed in the study had accuracy rates of 96.2% and 92.3%, which is close to the decision accuracy of the human eye. In addition, the system was more convenient than human judgment. The proposed system could be used as an agricultural robot to examine field areas and detect, identify, diagnose, and grade all wheat diseases (Liming & Yanchao 2010). In their study, Rahman and colleagues aimed to target disease detection and treatment of tomato plants. The study extracted 13 different statistical features using the GLCM method and classified using the SVM method. The study achieved a 100% success rate for the healthy class, while 95% success was achieved for early blight, 90% for Septoria leaf spot, and 85% for late blight (Rahman et al. 2023). Gerdan and colleagues detected tomato diseases in their study. They used convolutional neural networks (CNN), DenseNet201, InceptionResNetV2, MobileNet, and Visual Geometry Group 16 methods. The most successful method in their study was the CNN method.

Table 1 provides an overview of studies conducted using classical machine learning methods, including information about the plant species, methods employed, and success rates achieved.

Table 1- Studies in the Literature Based on Machine Learning Methods

Author(s)	*Plant Type*	*Methods*	*Success*
Tian et al. 2019	Tomato	k-means algorithm	Accuracy: 90.00%.
Xu et al. 2017	Wheat	Gray level histogram change, edge detection, noise removal.	Recognition rate: 96.20%. Accuracy: 92.30%.
Liming & Yanchao 2010	Strawberry	Segmentation, edge detection, feature extraction, K-means clustering, and classification.	Color grading accuracy: 88.80%. Shape classification accuracy: 90.00%
Burgos-Artizzu et al. 2010	Crop	Segmentation, removal of unwanted regions, genetic algorithm.	Up to 96.00% average success on grain images and 84.00% success on corn images.
Adebayo et al. 2016	Apple	Laser light backscatter imaging (LLBI), multispectral laser backscatter imaging (MBI), and hyperspectral laser backscatter imaging (HBI).	Varying results for LLBI, MBI, and HBI at different wavelengths. For example, in a study conducted on bananas with LLBI in 2013, the detection accuracy was 96.10% at 532 nm wavelength.
Dutta et al. 2016	Grape	Haar filter, segmentation, feature extraction, SVM.	100.00% accuracy was achieved with the SVM classifier.
Arakeri 2016	Tomato	Segmentation, feature extraction, and selection, artificial neural networks.	Accuracy for imperfect and perfect classification: 100.00%. Accuracy for mature and immature classification: 96.47%.
Rahman et al. 2023	Tomato	GLCM, SVM	Accuracy for: Healthy: 100.00% Early blight: 95.00% Septoria leaf spot: 90.00% Late blight:85.00%
Gerdan et al. 2023	Tomato	CNN, DenseNet201, InceptionResNetV2, MobileNet, Visual Geometry Group 16	CNN Acc.: 99.82%, DenseNet201 Acc.: 92.12%, InceptionResNetV2 Acc.: 92.75%, MobileNet Acc.: 91.50% Visual Geometry Group 16 Acc.: 84.12%

1.3. Deep learning-based methods

Brahimi and colleagues conducted a classification process on tomato leaves with nine different diseases, utilizing a dataset containing 14,828 images. They employed the CNN method for classification and achieved an impressive accuracy rate of 99.18%. The authors suggested that this method can serve as a practical tool for farmers to protect tomatoes against diseases (Brahimi et al. 2017). In the study by Durmuş et al., the AlexNet and SqueezeNet CNN models were tested using a GPU. The AlexNet model achieved 95.65% accuracy, while the SqueezeNet model achieved 94.3% accuracy, slightly outperforming AlexNet. The SqueezeNet model, which was nearly 80 times smaller than AlexNet, is considered a suitable choice for mobile deep learning classification due to its lightweight nature and low computing requirements. Furthermore, using a smaller network reduces data costs and enhances update rates when mobile applications are updated via mobile communication (Durmuş et al. 2017). Prajwala and their team identified 10 different diseases in tomato crops and utilized LeNet, a convolutional neural network model, in their study. They emphasized that this method, offering an accuracy rate of 94-95%, can assist farmers in accurately identifying leaf diseases with minimal computational effort (Tm et al. 2018). In the study by Karthik et al., deep learning architectures were applied to detect infections in tomato leaves. They first implemented residual learning and then applied the attention mechanism. The study used a dataset featuring three diseases, namely early blight, late blight, and leaf blight on tomato leaves. The proposed approach achieved an overall accuracy of 98%, thanks to the features learned by CNN (Karthik et al. 2020). Ouhami and their team aimed to determine the most suitable machine-learning model for detecting tomato crop diseases in RGB images. They utilized transfer learning models, including DensNet161, DensNet121, and VGG16. The study targeted the automatic detection of six different plant diseases. The results showed that DensNet161 and DensNet121 achieved an accuracy of 94.93%, while VGG16 achieved an accuracy of 90.58% (Ouhami et al. 2020). Sembiring and their team developed a deep-learning model based on the CNN architecture baseline for detecting tomato leaf diseases. The study aimed to classify ten classes of tomato leaves, including one healthy class and nine leaf diseases. They compared their model with VGG Net, ShuffleNet, and SqueezeNet architectures, demonstrating that the proposed architecture outperformed existing architectures with an accuracy of 97.15% (Sembiring et al. 2021).

Bhandari and their colleagues classified tomato leaves into nine different infectious diseases (bacterial spot, early blight, etc.) in their study. They used the EfficientNetB5 transfer learning method in their research and reported an average training accuracy of 99.84% and an average testing accuracy of 99.07% (Bhandari et al. 2023). Tian and their team proposed a model to identify diseases using tomato leaf images. In this study, three different deep learning network architectures (VGG16, Inception_v3, and Resnet50) were used, and an Android application was also developed. The application can identify tomato diseases with a test accuracy of 99% (Tian et al. 2022).

It is worth noting that there are more studies employing deep learning approaches in the literature than those mentioned above. Table 2 presents an overview of the studies reviewed.

Table 2- Studies in the Literature Based on Deep Learning Methods

Study	***Plant Type***	***Methods***	***Success***
Chen et al. 2020	Tomato	ABCK-BWTR and B-ARNet Model	Accuracy: 89.00%.
Wspanialy & Moussa 2020	Tomato	CNN (U-Net)	PlantVillage dataset accuracy: 97.00%. Left-out-disease dataset accuracy: 98.70%.
Gonzalez-Huitron et al. 2021	Tomato	CNN implemented in Raspberry Pi 4	MobileNetV2 accuracy: 75.00%. NasNetMobile accuracy: 84.00%. Xception accuracy: 100.00%.
Karthik et al. 2020	Tomato	Residual CNN	Overall accuracy: 98.00%.
Altuntaş & Kocamaz 2021	Tomato	Concatenated Deep Features	Overall accuracy: 96.99%.
Ouhami et al. 2020	Tomato	DensNet161, DensNet121, VGG16	DensNet161 accuracy: 95.65%. DensNet121 accuracy: 94.93%. VGG16 accuracy: 90.58%.
Sembiring et al. 2021	Tomato	CNN	Accuracy: 97.15%.
Jiang et al. 2020	Rice	CNN + SVM	CNN + SVM accuracy: 96.80%. AlexNet accuracy: 93.79%. VGG accuracy: 91.65%.
Sethy et al. 2020	Rice	CNN (ResNet50) + SVM	Accuracy: 98.38%
Temniranrat et al. 2021	Rice	CNN (YOLOv3)	Average true positive rate: 95.60%.
Park et al. 2018	Apple	CNN	Sensor type: Hyper-spectral Classifier: Local norm. ReLU accuracy: 86.11%.
Kuta et al. 2020	Apple	Manual Harvesting	Optimal height: 1000 mm. The highest surface pressure picks heights: 500 mm and 2000 mm.
Zhong & Zhao 2020	Apple	CNN (DenseNet-121)	Accuracy: 93.71%.
Rehman et al. 2021	Apple	Resnet-50, MASK RCNN	Accuracy: 96.60%.
Methun et al. 2021	Carrot	Inception v3	Accuracy: 97.40%.
Durmuş et al. 2017	Tomato	CNN (AlexNet and SqueezeNet)	AlexNet accuracy: 95.65%. SqueezeNet accuracy: 94.30%.
Tm et al. 2018	Tomato	CNN (LeNet)	Accuracy: 94.00% -95.00%.
Brahimi et al. 2017	Tomato	CNN (Alexnet and GoogleNet)	AlexNet accuracy: 98.66%. GoogleNet accuracy: 99.18%.
Bhandari et al. 2023	Tomato	EfficientNetB5	Accuracy: 99.07%
Tian et al. 2022	Tomato	VGG16, Inception_v3, and Resnet50	Accuracy: 99.00%

This study is primarily concerned with the detection and classification of tomato leaf diseases, employing both classical and CNN deep learning techniques. The study provides several significant contributions, including the proposal of an effective and robust method for detecting tomato leaf diseases, the development of an original CNN model, the utilization of classical learning methods in a novel approach, the demonstration of the proposed method's versatility across various class numbers (2, 6, and 10), and the ability to adapt the proposed method to diverse plant datasets. The study also reports more favorable results compared to the previous literature. The suggested method has the potential to identify tomato diseases at an early stage and reduce harm by enabling timely disease treatment.

The contributions of this study to the detection and classification of tomato leaf diseases, utilizing classical learning methods and CNN deep learning methods, are outlined as follows:

1. An effective, successful, and robust method to detect tomato leaf diseases was proposed by analyzing images of infected tomato leaves.

2. An original CNN model was created and implemented within the scope of the study.
3. Classical learning methods were used with a unique approach.
4. A robust approach for tomato leaf disease is presented for different classes (2, 6, and 10 classes), and more successful results were obtained than other studies in the literature. To the best of our knowledge, this study is the first attempt to detect disease in tomato leaves using different class numbers (2, 6, and 10 classes).
5. The proposed method is independent of the dataset, making it applicable to different plants and datasets.
6. This study will help to detect tomato diseases early and minimize the damage to farmers by ensuring measures can be taken to treat the diseases on time.

Overall, the study provides valuable insights into the detection and classification of tomato leaf diseases and underscores the effectiveness of both classical learning methods and deep learning methods in this domain.

2. Material and Methods

In this section of the study, comprehensive explanations are given regarding the tomato dataset used, as well as the technical intricacies of classical and deep learning methods. The stages of classical methods are elaborated sequentially, providing a step-by-step understanding of their application. Additionally, each layer of the CNN model employed in our research is individually described, including associated parameters and configurations.

2.1. Tomato dataset

The tomato dataset utilized in this study is an open-access repository sourced from Kaggle, featuring a total of 18 345 images categorized into 10 distinct classes. These categories encompass nine disease categories and one category representing healthy tomato leaves [26]. The tomato dataset encompasses all major leaf diseases that can have a substantial impact on tomato production. Each of these leaf diseases has distinct underlying causes, necessitating different strategies, such as fertilization, spraying, and other interventions, to combat them effectively. The tomato dataset employed in this study (Lamrahi 2021) classifies these diseases into nine distinct types, and includes one category for healthy tomato leaves, as detailed in Table 3.

Table 3- Disease types and properties of Tomato Dataset

Disease Type	***Image***	***Properties of the Diseases***
Mosaic Virus		The presence of light green, yellow, and dark green mosaic stains on the leaves is indicative of a disease caused by the tobacco mosaic virus and its various strains or breeds.
Two-Spotted Spider Mite		Red spiders have the potential to reproduce in areas where tomatoes are cultivated, whether in a greenhouse or an open field. Without prompt and effective measures, the infestation of red spiders can lead to significant losses in tomato crops.
Leaf Mold		The infestation of red spiders can sometimes spread to cover entire leaves, resulting in a substantial reduction in crop yield. Conditions that contribute to the prevalence of this disease include sudden temperature changes, excessive humidity, and the presence of shadowless greenhouses.
Septoria Leaf Spot		While this disease primarily affects leaves, it can also manifest on the stems, and flower stalks of plants. It typically begins as small yellowish areas on the leaves and subsequently changes in color, taking on a gray or brown appearance.
Bacterial Spot		Small brown to black spots may develop on the leaves, stems, flowers, and fruit stems. As the disease advances, these small spots have the potential to merge and form larger spots on the leaves. Furthermore, you may observe small, dark brown superficial blisters or lesions on the fruits.
Early Blight		The initial symptoms of the disease are typically observed on older leaves. These symptoms manifest as light green or yellowish-brown spots with a yellow halo. Small spots, each with a diameter of around 0.5 mm, gradually merge to cover the surface of the leaf. As the disease advances, the affected leaves may wither and die, leading to a deterioration in the quality of the fruit due to sun damage.
Leaf Curl Virus		In general, symptoms such as leaf shrinkage, blistering, inward curling, and deformity can be observed. Additionally, the leaves may exhibit yellowing starting from the edges, along with varying degrees of discoloration between the veins, ultimately resulting in an overall yellowed appearance.
Target Spot		The initial symptoms on the leaves present as small, misshapen, and greasy spots. In the later stages, these spots can merge and cause the entire leaf to dry out.
Late Blight		This disease results in the development of pale green to brown spots on the leaves, and occasionally, purplish spots may also appear. The edges of the leaf spots might exhibit a pale green color or show signs of waterlogging.
Healthy		Healthy leaves typically exhibit a proper, undistorted shape and maintain a vibrant green color.

2.2. Preprocessing for classical learning methods

To improve classification accuracy, our study initially applied preprocessing steps to the images used. As illustrated in Figure 1, the first step involved converting the images into grayscale. The images in the tomato dataset are originally in color. The subsequent preprocessing step was noise removal since it is highly unlikely that real-world images are entirely noise-free. Therefore, noise removal is commonly carried out as a preliminary step in many studies. In our research, we utilized the Wiener method for noise removal following grayscale transformation (Lim & Oppenheim 1979). The Wiener method is proficient in reducing image blur and is defined by the following formula:

$$W(u, v) = \frac{H(u,v)}{|H(u,v)|^2 + S_{nx}(u,v)} \quad (1)$$

$S_{nx}(u, v)$ is the signal-to-noise ratio and H (u, v) is the sinc function of the target pixel.

During the preprocessing stage, image sharpening was performed on the images, followed by the application of contrast enhancement. In Figure 1, the preprocessing steps are applied to an image from the bacterial spot class in the dataset. Upon closer inspection, it becomes evident that the preprocessing steps significantly enhance the quality of the original image.

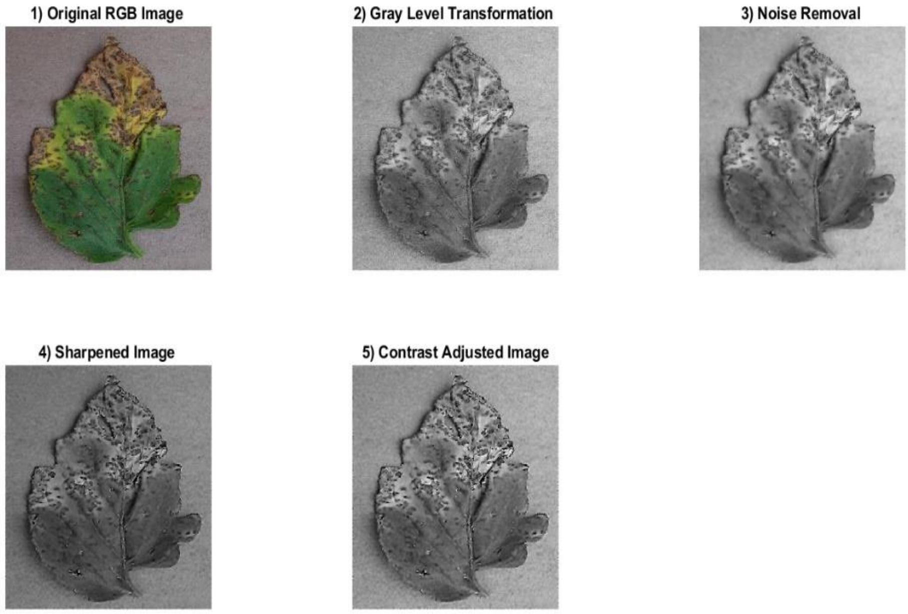

Figure 1- Demonstration of the preprocessing steps in a sample leaf image

2.3. *Feature extraction for classical learning methods*

In this study, the local binary pattern (LBP) method was employed as a nonparametric feature extraction technique (Ojala et al. 2000). The core principle of this method involves assessing the relationships between pixels by analyzing their neighborhood associations. The computation of LBP is carried out using Equation 2:

$$\mathrm{LBP}_{P,R}(x_c) = \sum_{p=0}^{P-1} u(x_p - x_c)2^p, \; u(y) = \begin{cases} 0, & \text{if } y < 0 \\ 1, & \text{if } y \geq 0 \end{cases} \quad (2)$$

Where: x_c is the pixel center, x_p is the central pixel neighbors, R is the distance to neighbors, and P is the number of neighbors.

2.4. *Classification with classical learning methods*

In this stage, the extreme learning machine (ELM), SVM, and k-nearest neighbor (kNN) methods were used. These methods were shown to be effective in various studies in the literature and yield successful results for different class numbers. SVM, developed by Vladimir Vapnik and Alexey Chervonenkis, is a method based on the principle of constructing a hyperplane that separates two classes (Schölkopf & Smola 2002). Here, $(x_i, y_i)_{1 \leq i \leq N}$ indicates training examples, for each example, $x_i \in R^d$, d feature space, y_i class label. The main purpose of SVM is to obtain a hyperplane where samples of the same class will coexist. This hyperplane is expressed with a line equation as in Equation 3.

$$y_i(w.x_i + b) > 0, \qquad i=1, \ldots, N \quad (3)$$

The kNN algorithm belongs to the category of non-parametric classification methods (Arya et al. 1998). ELM is a model of a single hidden layer feed-forward neural network (SLFN) (Huang et al. 2004, 2006, 2011). The output function of ELM for generalized SLFNs can be expressed as follows:

$$f_L(x) = \sum_{i=1}^{L} \beta_i h_i(x) = h(x)\beta \quad (4)$$

Where: $\beta = [\beta_1, \ldots, \beta_L]^T$ is the output weight vector between the output layer and the hidden layers, h(x) is the output vector of the hidden layer, $h(x) = [G(a_1, b_1, x), \ldots, G(a_L, b_L, x)]$ then G(a, b, x) nonlinear piecewise continuous function, and $\{(a_i, b_i)\}_{i=1}^{L}$ activation functions are randomly generated input values.

Parameter values for all methods used in the study are presented in Table 4.

Table 4- Purpose and Parameters of the Classical Methods

Operation	*Operation Type*	*Parameters*
Sharpening	Pre-Processing	Radius:1 Strength of the sharpening effect: 0.7
RGB to Gray Conversion	Pre-Processing	Default
LBP	Feature Extraction	Window Size: 3 x 3 Cell Size: 32
kNN	Classification	k:1 Distance: Minkowski
SVM	Classification	Default
ELM	Classification	Kernel: RBF # of Hidden Neurons: 4096 C: 1e-1

In Figure 2, a structure comprising three fundamental components is observed: preprocessing, feature extraction, and classification, each of which encompasses sub-steps. This figure illustrates the flowchart for the classical learning model employed in our study.

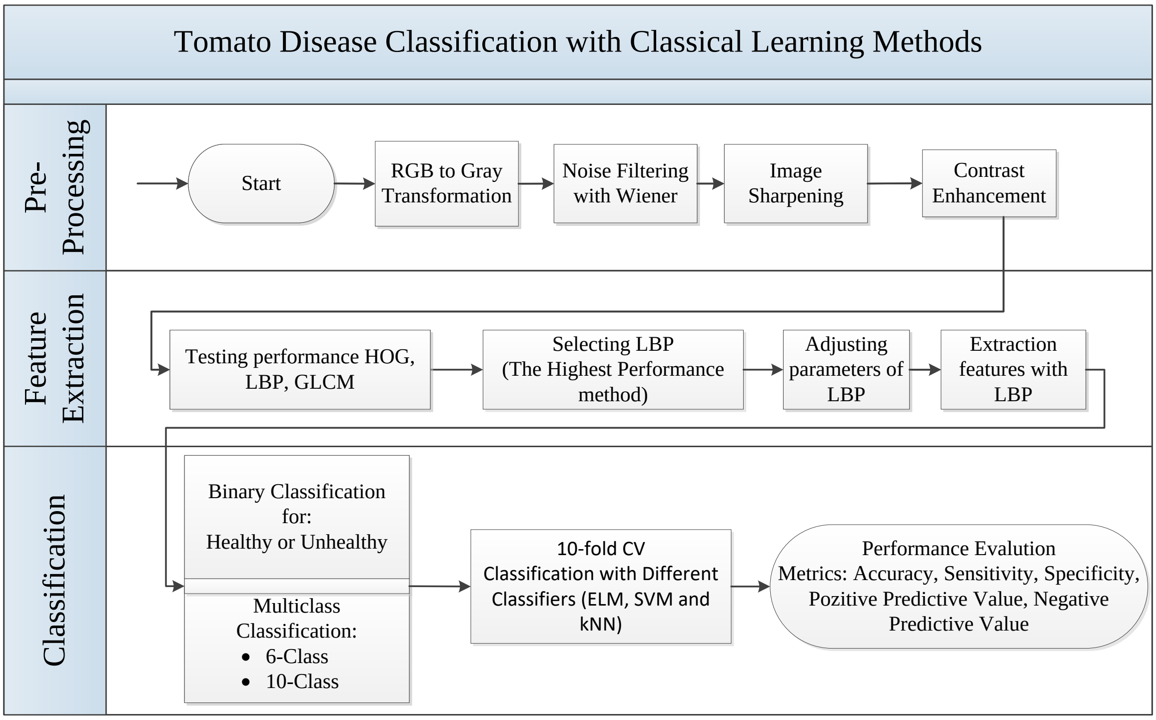

Figure 2- Flowchart for the Process using Classical Learning Methods

As depicted in Figure 2, both preprocessing and feature extraction methods were applied before the utilization of classical learning methods. A novel approach was adopted in terms of the methods used and their application. Various methods with different parameter values were experimentally tested both in feature extraction and preprocessing stages, and the most successful ones were selected. As shown in Figure 2, it is not feasible to perform studies solely on raw data when applying classical methods. Direct classification with raw data can result in very low success rates. Therefore, in classical methods, raw data are pre-processed and subjected to feature extraction, as shown in Figure 2. The success achieved by the system is directly related to the suitability of the feature extraction method.

Feature extraction methods can assess the effectiveness of images based on their structural characteristics. However, it is important to note that the same feature extraction method may not yield similar success across all image datasets. Consequently, the selection of the most successful feature extraction method was determined by comparing the performance of various methods, as evident in the feature selection section of the flowchart above. This choice has a substantial impact on the model's success. Furthermore, during the classification stage, the system was configured for various class numbers using the 10-fold cross-validation (CV) method. This approach contributes to the effectiveness and reliability of the obtained results.

2.5. *Classification with deep learning methods*

Classical learning methods face limitations when dealing with high-dimensional data, especially when inputs and outputs are extensive. As the complexity of the problem increases, processing such data becomes more challenging in terms of both performance and accuracy. In such scenarios, deep learning provides solutions to intricate real-world problems by constructing more complex network structures of neurons for information transmission.

Deep learning can directly learn features from the data provided. Neural networks can adeptly capture attributes and relationships between data points that may be challenging for other algorithms to discern. By employing layers of neurons that mathematically manipulate data, neural networks can develop complex models. Typically, a neural network model comprises an input layer, an output layer, and one or more hidden layers that facilitate the flow of information between the input and output layers. The term "deep learning" is used to describe models with numerous hidden layers. In Figure 3, each circle represents a neuron, which is a mathematical function with weight, bias, and activation function values. Neurons receive one or more inputs, and information is relayed from the input layer to the hidden layers for processing and, ultimately, to the output layer.

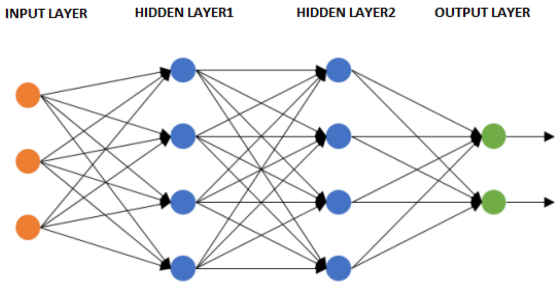

Figure 3- General View of Neural Network Architecture

The activation function plays a critical role in determining whether a neuron in an artificial neural network will be active or not. There are several activation functions to choose from, including "sigmoid", "tanh,", "relu" (rectified linear unit), and "SoftMax". The selection of the appropriate activation function depends on the specific problem being addressed. An artificial neuron model is seen in Figure 4. "X_1, X_2, ..., X_n" are the input values and "W_1, W_2, ..., W_n" are the corresponding weight values and "b" is a bias value. "f" is the activation function and is the function applied to the value by adding the bias value to the sum of the products of the inputs and the weights.

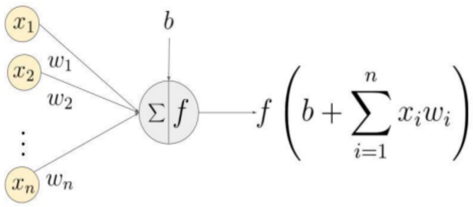

Figure 4- An Artificial Neuron Model

The loss function calculates the disparity between the predicted output and the actual target variable, indicating the level of error. Various error functions, such as "binary cross-entropy" and "negative log-likelihood" can be employed for classification tasks. Throughout the learning process, the goal of the model is to minimize this error to reduce the rate of false predictions. This is achieved by adjusting the weight and bias values, with the ultimate aim of learning the weight and bias values that yield the minimal error rate. Optimization algorithms like "stochastic gradient descent", "Adagrad", "RMSProp" and "Adam" can be used to facilitate this process.

Convolutional neural networks (CNNs) are a subset of deep learning often used for the analysis of visual information, particularly in image and video recognition tasks. They fall under the category of multi-layer neural network models. Compared to classical learning methods, CNNs tend to be more successful and typically do not require additional techniques for feature extraction or preprocessing (Tm et al. 2018). The structure of a CNN, as depicted in Figure 5, includes convolutional, non-linearity, pooling, flattening, and fully connected layers. The convolutional layer is used for feature detection, the non-linearity layer introduces non-linearity to the system, the pooling layer reduces the number of weights and assesses their suitability, the flattening layer prepares the data for the network, and data classification occurs in the fully connected layer.

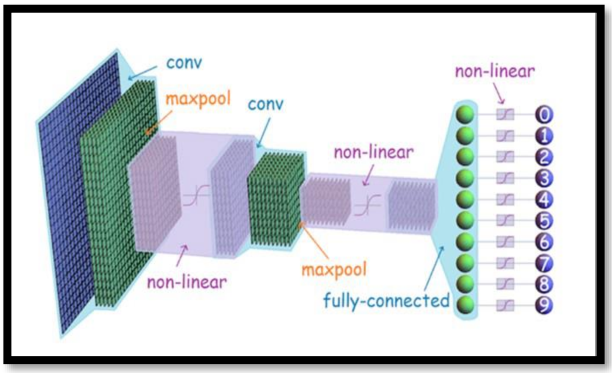

Figure 5- CNN Structure (Anonymous 2021a)

The convolutional layer is the initial layer in CNN algorithms that operates on images. This layer consists of a series of operations between an input 'I' and a set of 'n' convolutional filters 'F^E' followed by a non-linear activation function. As a result of these operations, the output volume of 0 is expressed in the formula (5).

$$O_m(i,j) = a\left(\left(\sum_{d=1}^{D}\sum_{u=-2k-1}^{2k+1}\sum_{v=-2k-1}^{2k+1}F_{md}^{E}(u,v)I_d(i-u,j-v)\right)+b_m\right) \quad (5)$$

Where: '2k+1' is the side of a square with an odd convolutional filter, 'a' refers to the activation function, 'b_m' refers to the bias for the m^{th} feature map

Each convolutional layer in this architecture is responsible for learning patterns to detect the type of disease in the tomato leaf (Karthik et al. 2020).

The non-linearity layer, also known as the "activation layer," employs one of the activation functions. In the past, nonlinear functions such as "sigmoid" and "tanh" were used; however, the rectifier linear unit (ReLU) function is currently preferred because it provides the best performance in terms of neural network training speed. In this study, the ReLU function was utilized, which is expressed in formula (6) and has a value range of [0, +infinity].

$$f(x) = \begin{cases} 0 \; for \; x \le 0 \\ x \; for \; x > 0 \end{cases} \quad (6)$$

The pooling layer, similar to the convolutional layer, aims to reduce dimensionality. This reduction not only saves processing power but also filters out irrelevant features, emphasizing the more important ones. CNN models often use two primary pooling techniques: maximum (Max) pooling and average pooling. In this study, the maximum pooling technique was used. The image size after the pooling process is calculated as follows:

Size of the generated image = $W_2 \; x \; H_2 \; x \; D_2$ (7)

$$W_2 = (W_1 - F) / N + 1 \quad (8)$$

$$H_2 = (H_1 - F) / N + 1 \quad (9)$$

$$D_2 = D_1 \quad (10)$$

Where: W_1 = Width value of input image size, H_1 = Height value of input image size, D_1 = Depth value of input image size F = Filter size, N = Number of steps, In the pooling process, F = 2 and N = 2 were chosen.

The flattening layer is responsible for preprocessing data for the subsequent "fully connected layer." Neural networks typically accept input data in the form of one-dimensional arrays. The data used in this layer is in the form of a one-dimensional matrix obtained from the convolution and pooling layers.

The fully connected layer transforms the matrix image, which has passed through the convolutional and pooling layers multiple times, into a flattened vector.

In the classification layer, the Softmax activation function was chosen for the output layer of our CNN model. This choice was made because more than two classifications (10 classes) were required for tomato leaf diseases.

2.6 Our application of CNN

In our study, the proposed approach consists of three significant stages: data acquisition, data pre-processing, and classification, as depicted in Figure 6.

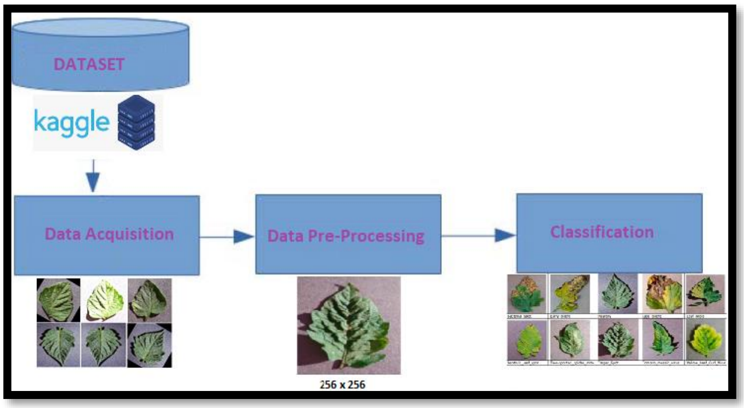

Figure 6- Flowchart for the Process with the Deep Learning Methods

In our study, a tomato leaf dataset sourced from the publicly accessible Kaggle platform was utilized, containing 18 345 images categorized into 10 distinct classes, including 9 categories representing diseased leaves and 1 category for healthy leaves. These categories cover all leaf diseases that can impact tomato production. To improve modeling efficiency and reduce processing time, we resized the images to 256 x 256 resolution. The classification process was carried out using a deep-learning CNN model. The study was conducted in the Python programming language within the Google Colaboratory (Colab) Notebook environment and employed Python libraries such as OpenCV, Keras, Numpy, Os, Sklearn, and Matplotlib.

The dataset included a total of 18 345 images, with approximately 2 000 images per class. For binary classification, 500 samples were randomly selected for both the healthy and unhealthy classes. For the 6-class classification, we chose around 167 samples from each class. In the 10-class classification, 100 samples were selected from each class, resulting in a total of 1 000 sample images. The dataset was split, reserving 20% for testing and using the remaining portion for training.

Before modeling, the input images were resized to 256 x 256 pixels. The CNN model was constructed using a sequential model with sequential layers. To prevent overfitting and encourage generalization, we incorporated a dropout layer during training. The CNN model was applied after resizing and defining the number of classes, which included nine different disease types and one healthy class. The CNN architecture in our study was tailored to our specific requirements, involving multiple layers. The CNN model was configured with the following parameters:

- The training phase included 25 epochs.
- Each iteration involved the use of 32 images.
- Input images were resized to dimensions of 256 x 256 pixels.
- The images were labeled for classification into 2, 6, and 10 different classes.

Experiments to detect tomato leaf diseases were conducted using our model, and the steps applied in our CNN model can be observed in Figure 7.

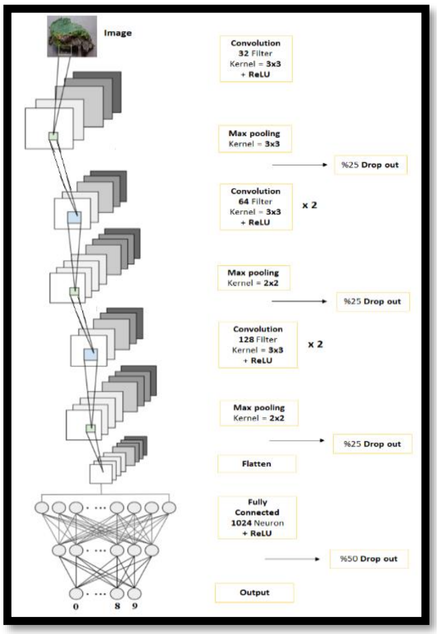

Figure 7- Our CNN Model

In our CNN model, the specified parameters were employed to conduct the following operations:

i. ReLU-activated convolution layer with 32 filters of size 3x3.
ii. A Maxpool layer with a 3x3 dimensional frame.
iii. Dropout of 25% of neurons after the Maxpool layer.
iv. ReLU-activated convolution layer with 64 filters of size 3x3.
v. ReLU-activated convolution layer with 64 filters of size 3x3.
vi. A Maxpool layer with a 2x2 frame.
vii. Dropout of 25% of neurons after the Maxpool layer.
viii. ReLU-activated convolution layer with 128 filters of size 3x3.
ix. ReLU-activated convolution layer with 128 filters of size 3x3.
x. A Maxpool layer with a 2x2 frame.
xi. Dropout of 25% of neurons after the Maxpool layer.
xii. Flattening of the data in preparation for the fully connected layer.
xiii. ReLU-activated fully connected layer with 1024 neurons.
xiv. Dropout of 50% of neurons after the fully connected layer.
xv. Softmax-activated neurons in the output layer, with the number of neurons equal to the number of classes.

The CNN model employed in our study exhibited strong performance for the tomato dataset. This success can be attributed to its shorter average epoch round and higher accuracy compared to other CNN models. The key to this success lies in the experimental determination of the optimal number of layers and parameters for the CNN model, resulting in an effective and efficient model. A detailed comparison of model performances can be found in the discussion section of our study. Furthermore, the approach we used to determine the number and parameters of layers is not limited to the tomato dataset; it can be applied to other datasets as well. This eliminates dependency on a specific dataset and allows for successful application to datasets with varying numbers of classes, as demonstrated in our experimental studies.

To assess the performance of our model, the tenfold cross-validation method was used in the training and testing phase. This method involves a thorough examination of all sections of the dataset, ensuring that the model has been exposed to every sample, which contributes to a more effective learning process. Cross-validation, in particular, randomly divides the dataset into "k" groups, designating one as a test set and using the remaining groups for training. This procedure is repeated for each group, enabling the model to be trained and tested with all parts of the data, ultimately enhancing its accuracy. The cross-validation process is visualized in Figure 8.

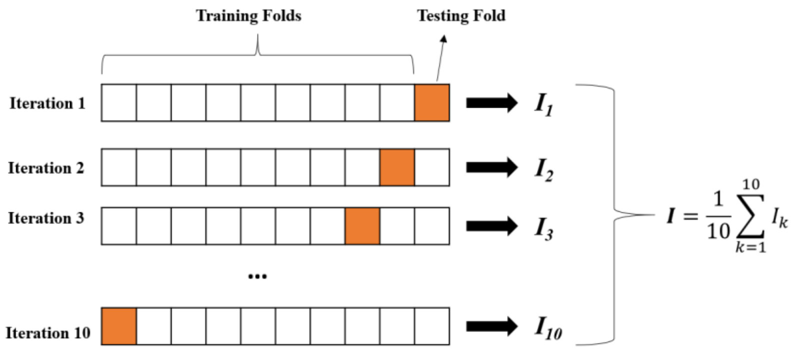

Figure 8- 10-fold cross-validation

2.7 Visualizing feature maps in our CNN model

Feature maps are the output structures generated by applying filters within a CNN. Essentially, they represent the evaluation of a particular layer. Normalizing these features aims to enhance the understanding of the detected features. In deep learning techniques, initial layers primarily identify low-level features (e.g., colors and edges), while subsequent layers identify high-level features (e.g., shapes and objects). Therefore, we incorporated feature visualization into our model. Figure 9 displays the visualization of the features of tomato leaves, while Figure 10 showcases symptom visualization. These images reveal numerous activations related to edges and textures, with a particular focus on outlining the leaf.

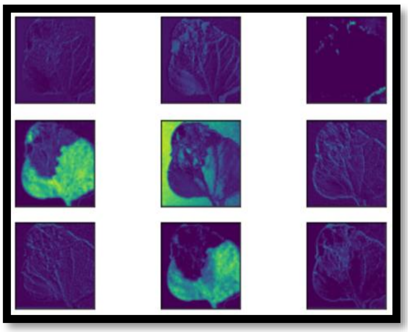

Figure 9- Feature visualization for tomato leaves

In Figure 10, the differentiation of the pixel values is seen in areas with symptoms.

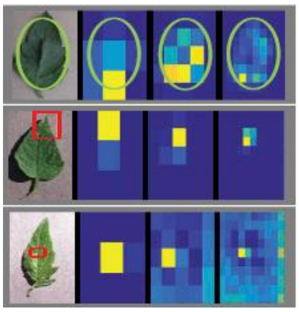

Figure 10- Symptom visualization for tomato leaves

2. Results

A comparative analysis of experimental results using various metrics is a crucial aspect to consider. To assess the effectiveness of our models, five distinct metrics were employed. The performance metrics used in our study, along with their corresponding formulas, are presented in Equations (11-15). In these equations, TP, TN, FP, and FN represent true positive, true negative, false positive, and false negative, respectively.

$$\text{Accuracy} = \frac{\text{TP+TN}}{(\text{TP+TN+FP+FN})} * 100 \quad (11)$$

$$\text{Sensitivity} = \frac{\text{TP}}{(\text{TP+FN})} * 100 \quad (12)$$

$$\text{Specificity} = \frac{\text{TN}}{(\text{TN+FP})} * 100 \quad (13)$$

$$\text{PPV} = \frac{\text{TP}}{(\text{TP+FP})} * 100 \quad (14)$$

$$\text{NPV} = \frac{\text{TN}}{(\text{TN+FN})} * 100 \quad (15)$$

PPV and NPV serve as indicators of a test's clinical significance. Sensitivity signifies the percentage of true positives (e.g., 95% sensitivity means that 95% of individuals with the targeted disease will test positive), while specificity indicates the percentage of true negatives (e.g., 95% specificity suggests that 95% of individuals without the targeted disease will test negative). Accuracy is a measure of correctly identifying both diseased and healthy datasets. Tables 5, 6, and 7 present the classification results for conventional learning models in our study. Table 5 showcases our model's ability to distinguish between diseased and healthy tomato leaf images. A closer examination of the table reveals that the SVM method attained the highest accuracy rate, reaching 92.5%. Furthermore, the kNN method, a simple yet effective approach, achieved the highest sensitivity rate of 98%, as depicted in the same table.

Table 5- Binary class classification performance metrics with classical learning methods (the most successful method for each metric is shown in bold)

Methods	*Accuracy (%)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
ELM	91.80	95.80	87.80	88.70	95.40
SVM	**92.50**	96.00	**89.00**	**89.70**	95.70
kNN	89.00	**98.00**	80.00	83.10	**97.60**

Table 6 illustrates the performance metrics when six classes are created by merging the five most frequent diseases with the healthy class. Similar to binary classification, SVM stands out as the most effective method. However, after the six-class classification, it appears that the outcomes of all methods are quite similar.

Table 6- 6-Class classification performance metrics with classical learning methods (the most successful method for each metric is shown in bold)

Methods	*Accuracy (%)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
ELM	81.30	95.90	96.90	84.50	99.30
SVM	**83.00**	94.00	**98.60**	**92.10**	98.90
kNN	82.30	**97.30**	96.40	82.90	**99.50**

Table 7 presents the results obtained by incorporating all classes in the dataset. In the table, success decreases as the number of classes increases. The results of the model with 10 different classes indicate that the SVM method achieved a higher accuracy rate compared to other methods.

Table 7- 10-Class classification performance metrics with classical learning methods (the most successful method for each metric is shown in bold)

Methods	*Accuracy (%)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
ELM	78.70	94.10	97.40	78.90	99.40
SVM	**81.40**	92.70	**98.90**	**89.40**	99.20
kNN	79.60	**95.03**	97.80	81.40	**99.50**

When the results obtained from Tables 5, 6, and 7 are analyzed, it becomes evident that the kNN method excels in terms of successfully detecting diseased samples, particularly for sensitivity. On the other hand, the SVM method proved to be the most effective in distinguishing healthy images, emphasizing its specificity.

Table 8 presents the implementation times of the methods utilized in our study. The table illustrates how the number of classes impacts the implementation time of these methods. Binary classification emerges as the quickest method, while the ELM method had the fastest performance when classifying all 10 classes. Notably, the SVM method, despite providing the highest accuracy rates, exhibited slower performance when classifying 6 and 10 classes.

Table 8- Average time of classification for methods

Methods	*Binary*	*6-Class*	*10-Class*
ELM	12.90	39.80	115.90
SVM	5.60	235.20	1938.60
kNN	2.30	45.90	470.90
CNN	265.00	432.00	505.00

As depicted in Table 9, CNN exhibited the highest accuracy rates for binary, 6, and 10 classes, but it required a longer training duration compared to classical learning methods. Interestingly, binary classification also proved to be the quickest method within the CNN framework. What's particularly intriguing is that our proposed CNN architecture displayed less sensitivity to the number of classes when contrasted with ELM, SVM, and kNN methods, where an increase in the number of classes substantially augmented execution time. For instance, in SVM, the execution time difference between binary classification and 10-class classification was approximately 345 times, while in the CNN method, this ratio was only about 2 times. This observation implies that our proposed CNN method is more versatile and original.

The classification results obtained with our deep learning models are presented in Table 9, revealing that all accuracy results are quite similar. Furthermore, as the number of classes increases, accuracy tends to decrease.

Table 9- Classification performance metrics according to different class numbers with deep learning methods (the most successful method for each metric is shown in bold)

Number of classes	*Accuracy (%)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
Binary Class	**99.50**	**100.00**	98.97	**99.04**	**100.00**
6-Class	98.50	98.50	**99.70**	98.50	99.70
10-Class	97.00	97.00	99.67	97.00	99.67

In our study, modeling was conducted for three different class numbers: binary, 6-class, and 10-class. Table 10 compares the accuracy values of the CNN architecture with those of classical learning methods. Upon reviewing the table, it is clear that the original CNN model yielded considerably more successful results than classical learning methods. This can be attributed to the fact that the parameters and layers employed in the CNN model were determined through extensive experimental research, a fact reflected in Table 10.

Table 10- Accuracy values according to different class numbers with deep and classical learning methods (the most successful method for different numbers of classes is shown in bold)

Number of classes	*CNN (%)*	*ELM (%)*	*SVM (%)*	*kNN (%)*
Binary Class	**99.50**	91.80	92.50	89.00
6-Class	**98.50**	81.30	83.00	82.30
10-Class	**97.00**	78.70	81.40	79.60

Figures 11, 12, and 13 illustrate the current state of the dataset and the number of correct and incorrect classifications made by the model. The vertical axis represents the actual values, while the horizontal axis represents the predicted values. The confusion matrix is utilized to determine the number of TP, TN, FP, and FN. TP values are displayed as numerical values on the diagonal, signifying highly accurate disease classification.

Upon analyzing the confusion matrix, it can be concluded that each class exhibits the highest rate of correct estimations. More specifically, according to Figure 11, the highest correct estimation is for the healthy state, while the lowest correct estimation is for leaf mold disease (Figure 12) and yellow leaf curl virus disease (Figure 13). Furthermore, when inspecting the incorrect predictions, it is apparent that bacterial spot, target spot, and yellow leaf curl virus are diseases that are most frequently confused with each other. To mitigate this confusion between these diseases, it may be beneficial to analyze mixed features of the images and implement certain preprocessing steps to enhance the accuracy of classification.

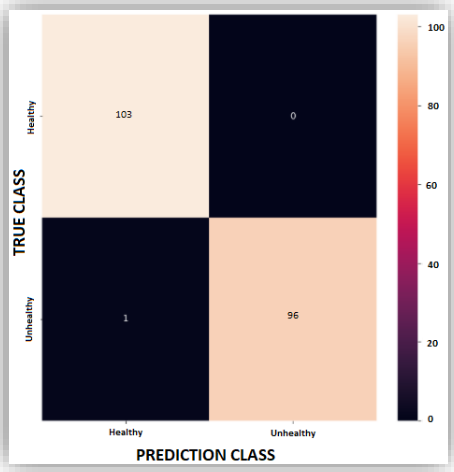

Figure 11- Confusion Matrix for Binary Class

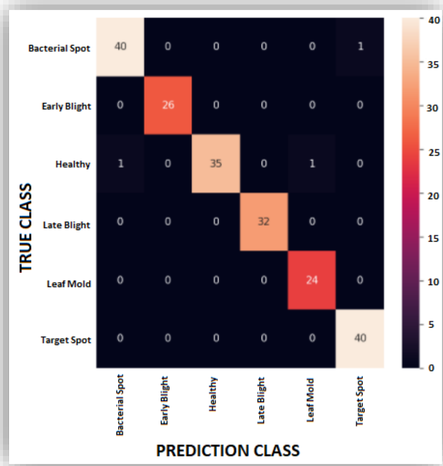

Figure 12- Confusion Matrix for 6-Class

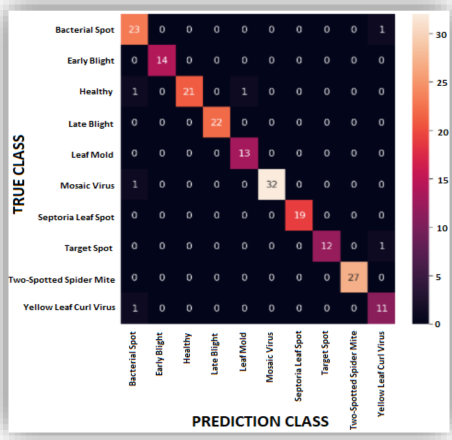

Figure 13- Confusion Matrix for 10-Class

3. Discussion

Our study aimed to develop a novel CNN framework for the automatic classification of tomato leaf images (Lamrahi 2021). We conducted a comparative analysis of the CNN method with models created using different classical learning methods, as demonstrated in Tables 5, 6, 7, and 9, which present the performance metrics of these models. The results consistently indicate that the CNN method outperformed the classical models across all class numbers. This comparison emphasizes the advantages of deep learning models over classical machine learning methods, particularly their capacity to extract features from raw data without the need for expert knowledge.

The findings of our study hold significant implications for the field of agriculture. Traditionally, disease detection in crops relies on manual observations by farmers, which can be time-consuming and costly, especially when diseases are detected late. Our method can be applied to process images captured from vast agricultural areas using drone-like devices, enabling the early detection of plant diseases and facilitating timely interventions to prevent yield loss. Table 11 provides a comparison of our study with other studies related to the tomato dataset. It is worth noting that the studies (Kapucuoglu 2011; Anonymous 2021b; c) listed in the table are not academic studies, as the dataset was not used in an academic context; they were sourced from Kaggle for comparison purposes.

Table 11- Comparison table of current studies of the tomato (Lamrahi 2021) dataset

Study	*Model*	*Number of Classes*	*Loss (%)*	*Accuracy (%)*	*Average Epoch Tour*
(Anonymous 2021b)	Sequential	10	14.20	95.35	308
(Anonymous 2021c)	VGG-16	10	1.84	97.96	314
(Kapucuoglu 2011)	AlexNet	10	8.92	97.20	233
Our Study	Sequential	2	0.04	99.50	11
Our Study	Sequential	6	2.13	98.50	17
Our Study	Sequential	10	9.33	97.00	20

As indicated in Table 11, our study achieved the lowest loss rate, the shortest average epoch count, and the highest accuracy for 2-class classification. In comparison to the study (Kapucuoglu 2011) which utilized AlexNet for 10-class classification and achieved an accuracy rate of 97.20%, our model attained an accuracy of 97% with a shorter average epoch count when subjected to 10-fold cross-validation. Furthermore, when our method is applied to classify the 5 most common diseases and one health status (6-class), we obtained favorable results. The proposed approach and CNN model consistently demonstrated high success across all classification methods while requiring fewer epochs than other studies, underscoring the effectiveness and robustness of our method.

4. Conclusions

The results demonstrate that the deep learning model, particularly the CNN architecture, outperforms classical methods in terms of both accuracy and efficiency. The CNN model consistently achieved high accuracy rates for all the different classification methods employed in the study, including the identification of multiple diseases and health status. Furthermore, the CNN model's ability to automatically extract features from raw data without requiring expert input is a notable advantage over classical feature extraction methods, which often rely on domain-specific knowledge for dataset-specific feature selection.

This study has significant implications for the agricultural sector, as early disease detection in plants is crucial for improving productivity and reducing costs. Digital detection tools developed through smart agriculture studies can facilitate early diagnosis and treatment of plant diseases, ultimately leading to higher crop yields and higher-quality agricultural products. Overall, this study underscores the potential of deep learning methods, particularly CNN models, in the realm of plant disease detection, and emphasizes the importance of ongoing research in this field.

Tomato plants are susceptible to various types of diseases. As a result, our study initially focused on distinguishing between diseased and healthy leaves and subsequently categorizing the diseased leaves into 6 or 10 different types. The results reveal that our original CNN model achieved the highest success rates for 2, 6, and 10-class classifications, with accuracy rates of 99.5%, 98.50%, and 97.0%, respectively. In comparison to classical methods, the CNN network we designed consistently delivered significantly superior results. Classical methods typically involve data preprocessing, while CNN methods can directly extract features from raw data, eliminating the need for additional feature extraction techniques. This attribute significantly contributes to the success of our model. Our findings underscore the importance of computer-aided recognition and detection systems for enhancing agricultural productivity. Additionally, the independence of our model from the dataset enables its application to different plant species and various disease types, making it a valuable contribution to the field of agriculture.

In future studies, we plan to develop a system capable of detecting various plant species and disease types, while also assessing disease severity based on images of afflicted plants. Our goal is to make this system compatible with mobile devices, further supporting the agricultural industry by enhancing production and efficiency.

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Data Availability

The present work utilizes one dataset which is tomato data from Kaggle developed by Nouaman Lamrahi. This dataset is cited in the manuscript in Lamrahi (2021).

Conflict of interest

The authors declare that there is no conflict of interest.

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Rapid synthesis of Silver Nanoparticles with *Rheum ribes* L Fruit Peels: Anticancer and Antimicrobial Effects with Biocompatible Structures

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ABSTRACT

Silver nanoparticles (AgNPs) are substances with a wide range of uses. Utilizing extracts obtained from the peels of *Rheum ribes* L. (Rr) fruit growing in Erzurum region, silver nanoparticles were rapidly created in this study with a quick, easy, and environmentally friendly technique without harmful processes. In order to evaluate the attributes of the synthesized Rr-AgNPs, FE-SEM or TEM micrographs were utilized to characterize their morphology. A UV-visible spectrophotometer was used to assess the highest absorbance bands of RR-AgNPs. These data were used to define RR-AgNPs, which were characterized as having exclusively negative surface charges of -25 mV, spherical shape, maximum absorbance at 428 nm wavelength, and 96 nm size distribution. The effectiveness of the produced AgNPs for use in medical applications was assessed using the MTT technique with microdilution. Minimum inhibition concentrations of Rr-AgNPs for pathogen strains ranged from 0.03 to 0.50 mg/L. Additionally, it was discovered that AgNPs effectively suppressed malignant cells, with rates of 86.27%, 74.67%, and 73.49%, in the investigation of the anticancer effects of AgNPs. Healthy cells were not subject to any inhibitory effects at the same concentrations.

Keywords: Rr-AgNPs, Fruit peels, FTIR, TGA, UV-Vis

1. Introduction

Among the nanosciences, green nanotechnology is one of the most interesting areas with widespread areas of use producing and controlling elements at the molecular level (SI et al. 2020). Nanoparticles (NPs) are used in both scientific study and industrial applications, and they are crucial to both (J. Singh et al. 2018). The range of use of nanoparticles is quite wide. They are products with superior physical properties such as surface area, as well as chemically important properties (Kumar et al. 2017; Baran 2019a). Metallic nanoparticles such as zinc (Zn), palladium (Pd), gold (Au), and silver (Ag) are commonly used (Ismail et al. 2017; Baran 2019; Pandiyan et al. 2019; Baran et al. 2021).

AgNPs have uses in medical applications as antimicrobial, antioxidant, anticancer and anti-inflammatory agents. In addition, AgNPs are valuable particles in many areas such as bioremediation applications, the food industry, catalysis studies, and cosmetics (Velmurugan et al. 2014; Francis et al. 2017; V. Kumar et al. 2017; 2019; Thomas et al. 2018 Mohammadi et al. 2019; Abu-Dief et al. 2020; Arroyo et al. 2020). It is possible to obtain these valuable products using various methods. Among these methods, green synthesis methods, which are among biologically-sourced synthesis methods, have various advantages over physical, chemical, and other biologically-sourced environmentally friendly methods. Some of these advantages include being economical with low cost, no toxic chemicals used in synthesis, easy process, not requiring special conditions, and high yield of product obtained as a result of the synthesis (Al-ogaidi et al. 2017; Patil et al. 2018b; Rolim et al. 2019).

AgNPs are used in medical applications as antimicrobial and anticancer agents. Their use as an antimicrobial agent can contribute to the search for a solution to antibiotic resistance, which is a very important global problem in today's world (Kumar et al. 2017; A. Singh et al. 2018; Oliveira et al. 2019; Azmi et al. 2021). A terrible disease that has been studied extensively in the search for a cure is cancer. In addition to traditional approaches, studies about different treatment processes have attracted a great deal of attention. As a result, numerous studies have been conducted about the use of Rr-AgNPs as anticancer agents (Remya et al. 2015; Chung et al. 2016; Sarkar et al. 2018; Satpathy et al. 2018; Abu-Dief et al. 2020; Zein et al. 2020).

Rheum ribes L. (Rr), known as "Işgın" or "Kurdish banana", is a species belonging to the Polygonaceae family. This perennial herbaceous plant grows in the Iran-Turan floristic region. It is naturally found in rocky areas in regions such as Erzurum, Hakkari, Bingöl and Elazığ in Eastern Anatolia in Turkey. The length of its shoots can reach approximately 40 cm. The plant is beneficial against ulcers, diabetes, obesity, hypertension and digestive problems. It is frequently consumed as food for these reasons. In addition, the plant forms the raw material for many medicines in the Middle East. The plant contains significant amounts of phytochemical components such as phenolic acid and flavonoids. These phytochemicals have antimicrobial, anticancer and antioxidant bioactivities (Munzuroğlu 2000; Tosun & Kizilay 2003; Naqishbandi et al. 2009; Çınar Ayan et al. 2020).

This study used an extract from the fruit peel of Rr cultivated in Erzurum to create Rr-AgNPs by bioreduction and evaluated their antibacterial and anticancer activities in medical applications.

2. Material and Methods

2.1. Materials

Rr fruit was collected in Erzurum Çat district on May 2, 2022. The species was identified by Dr. Kenan Akbaş at Muğla Sıtkı Koçman University, Department of Biology Herbarium and was given Herbarium number K.A 1658-A.

2.1.1. Preparation of extract from fruit peels and silver nitrate solution

Fruit peels were removed and rinsed with distilled water before being dried. Dried fruit peels were weighed to 20 g and placed in a 500 mL beaker. Then 250 mL of distilled water was added and the mixture was heated until boiling. After being filtered with filter paper and brought to room temperature, the extract was ready for synthesis. A solution with a concentration of 5 mM was prepared from Sigma-Aldrich silver nitrate ($AgNO_3$) salt to be used to obtain Rh-AgNPs by bioreduction.

2.2. Methods

2.2.1. Bioreduction synthesis of Rr-AgNPs with fruit peel extract

For this, 200 mL of fruit peel extract and 200 mL of 5 mM $AgNO_3$ solution were combined 1:1 in a 1000 mL glass Erlenmeyer and left to sit at room temperature. The color shift was observed. By obtaining samples from the reaction medium at different times and performing UV-vis wavelength scans, along with color change, the presence of AgNPs at maximum absorbance was identified (Baran et al. et al. 2021; Atalar et al. 2022).

2.2.2. Characteristics of the produced Rr-AgNPs

To exhibit the formation of AgNPs synthesized from *R. ribes* fruit peel extract (Rh-AgNPs), samples were taken from this medium, with reaction-related color change after mixing the extract with $AgNO_3$ solution, and a Perkin Elmer One UV visible spectrophotometer was used (UV-vis). Maximum absorbances were measured in the 300-800 nm wavelength range. Using Fourier transformation infrared spectroscopy (FTIR-Perkin Elmer), frequency changes in the range of 4000-800 cm^{-1} for both the extract and the liquid obtained at the end of the reaction were examined to evaluate bioactive functional groups in the extract responsible for bioreduction. The morphological appearance of the synthesized Rh-AgNPs was evaluated in micrographs taken using Jeol Jem FE-SEM and TEM devices. In addition, topographic structures, shape, and size distributions of Rh-AgNPs were determined by means of AFM. Also, the size distribution was determined using Marven DLS. Graphs obtained using a RadB-DMAX II computer-controlled electron dispersed X-ray (EDX) were examined, and the elemental contents of the synthesized particles were determined. Plane-reflected crystal patterns were identified for Rh-AgNPs in data acquired at 2θ using a Rigaku Miniflex 600 model X-ray diffraction (XRD). With this data, crystal nanosizes were calculated using the Debye-Scherrer equation via XRD data (Baran & Acay 2019; Umaz et al. 2019). The charge distributions of the surface structures of the synthesized Rh-AgNPs were evaluated with Marven Zeta potential. The resistance of Rh-AgNPs to temperature changes was measured between 25 and 900 ℃ using Shimadzu 50 thermogravimetric and differential thermal analysis (TGA-DTA).

2.2.3. Antimicrobial Effects of Rh-AgNPs

Minimum inhibition concentrations (MIC) of Rh-AgNPs synthesized with fruit peel extract were calculated using the microdilution method (Atalar et al. 2022) for suppression of the growth of microorganisms in pathogenic groups. Pathogenic gram-negative and positive bacteria and yeast were used in the experimental study. Each microorganism was grown in suitable media for 24-48 hours of incubation at 37 °C. Then, microorganisms were prepared according to McFarland standard 0.5 (Emmanuel et al. 2015; Patil et al. 2021; Raghavendra et al. 2022) turbidity criteria for each strain. Bacteria and fungi were transferred to 96-well microplates containing the appropriate medium. Some wells on the microplate were selected and defined for the control groups. Then, Rh-AgNPs prepared at different concentrations were transferred to the microplate wells and microdilution was applied. The microorganisms were then transferred to the wells. The prepared microplates allowed interaction between microorganisms and Rh-AgNPs for 24-48 hours in an oven at 37 °C. At the end of the period, growth control was performed and MIC values were determined. İnönü University Medical Faculty Hospital Microbiology Laboratory provided the strains of *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Escherichia coli* (*E. coli*) ATCC 25922, and *Candida albicans* (*C. albicans*) ATCC 10231. Artuklu University Microbiology Research Laboratory provided the strains of *Bacillus subtilis* (*B. subtilis*) ATCC 11774 and *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853.

2.2.4. Anticancer effects of Rh-AgNPs

Cytotoxic effects of the synthesized Rh-AgNPs on cancer cells were evaluated by determining the % survival rate using the MTT method (Baran et al. 2021). Three different cancer cells and one healthy cell were used in experimental studies. The cells were incubated in suitable nutrient media and under optimum conditions. Cells were then suspended at different concentrations using a hemocytometer, transferred to 96-well microplates, and incubated for 24 hours. Then, solutions containing Rh-AgNPs at different concentrations were added to the wells cultured with cell lines and incubated for 48 hours. The absorbance of the cells was then read using a Multi Scan Go (Thermo) instrument at 540 nm. The viability suppressing concentrations of Rh-AgNPs in the cell lines were calculated with the following formula (Remya et al. 2015; Mohmed et al. 2017; Sunderam et al. 2019; Chen et al. 2021).

Viability % = (sample absorbance / control absorbance) x 100 (2)

3. Results and Discussion

3.1. Maximum absorbance bands obtained via UV-vis data of Rh-AgNPs

A color change from yellow to brown was observed 35 minutes after combining Rr fruit peel extract with $AgNO_3$ solution, which is a macroscopic finding indicating that Rh-AgNPs had formed (Acay and Baran, 2019; Jebril et al. 2020; Mamdooh & Naeem, 2021; Mani et al. 2021; Sattari et al. 2021). In addition to this finding, the maximum absorbance bands at 428 nm taken with UV-vis were characteristic, showing the vibrations (SPR) occurring on the plasma surface and the formation and presence of Rh-AgNPs (Figure 1) (Luna et al. 2015; Patra et al. 2016; Francis et al. 2017; Eren & Baran 2019; Anandalakshmi 2021).

In a study in which AgNPs were synthesized using the fruit of the Rh plant and 1 mM $AgNO_3$ solution in a ratio of 10:1, respectively, the maximum wavelength absorbance of the obtained AgNPs was 425 nm (Mustafa, Mahdi Auda, Hajir, Ali Shareef & Bari 2021). In this study, the maximum absorbance of AgNPs obtained by using the fruit peels of the Rh plant and 5 mM $AgNO_3$ solution in a 1:1 ratio was 428 nm. UV-vis findings of the studies show that the maximum wavelengths were almost the same, despite the use of different parts of the plant, different extracts and metal concentrations. In another study using Rh fruit, the maximum wavelength absorbance of AgNPs obtained using the ethanol extract in an medium with 10 mM $AgNO_3$ concentration at 200 rpm at 75 ℃ for two days was 410 nm (Aygün et al. 2020). This study is more advantageous in terms of application conditions such as the method of obtaining the extract, synthesis temperature, shaking and metal concentration.

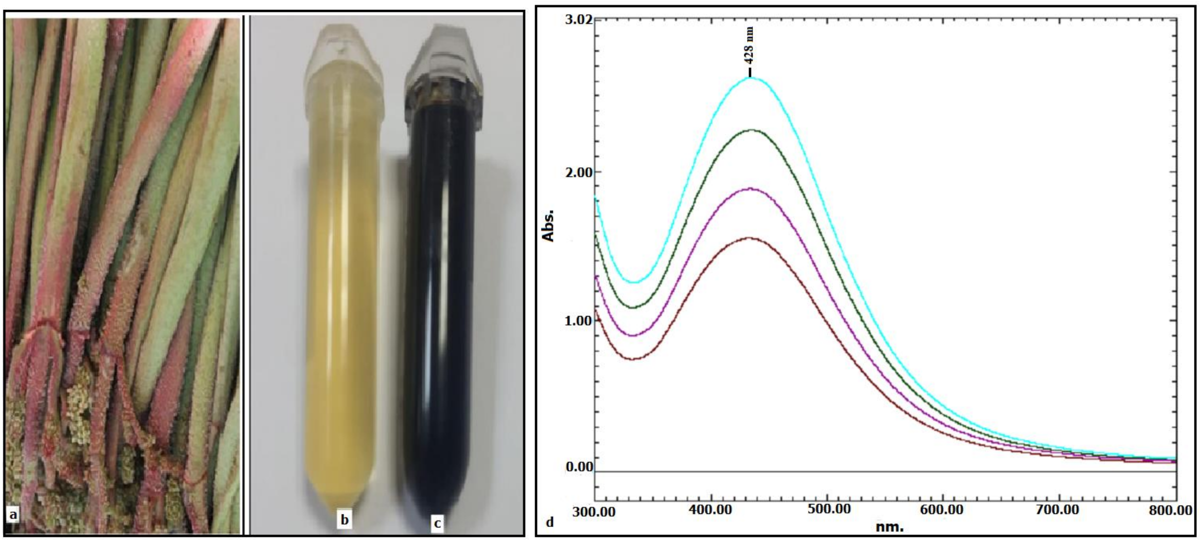

Figure 1- a. Rr fruit b. Rr fruit extract, c. The appearance of the end-reaction fluid indicates the formation of Rr-AgNPs as a result of synthesis, and d. Maximum absorbance spectra dependent on SPR according to UV-vis for the presence of Rr-AgNPs

3.2. XRD Analysis of Rr-AgNPs

In Figure 2, data obtained at 2θ with XRD was evaluated to calculate the crystal patterns and nanosizes of Rr-AgNPs. Widening of the Bragg angle peaks showed that the Rh-AgNPs have a central faceted cubic pattern (fcc) (A. Singh et al. 2018; Wongpreecha et al. 2018a; 2018b; Jebril et al. 2020; Anandalakshmi 2021). The FWHM values of 111°, 200°, 220°, and 311° Bragg angles in the data were determined as 38.72, 44.62, 64.90, and 77.48, respectively. By using Debye-Sherrer's high peak FWHM value for the crystal nano dimensions of Rr-AgNPs, the cubic pattern of Rr-AgNPs was calculated to have crystal size of 58.06 nm. In two green synthesis studies using Rh fruits, the crystal patterns of the obtained AgNPs had similar characteristics with broadening peaks occurring at 111°, 200°, 220°, and 311° Bragg angles (Aygün et al. 2020; Mustafa et al. 2021). In the biosynthesis study of AgNPs using *Cucurbita maxima* L. extracts, the crystal nanosizes of AgNPs were calculated as 67 and 56 nm (Ali 2020). In studies using herbal extracts for the synthesis of AgNPs, the crystal nanosizes were calculated as 24.36 nm (Khan et al. 2022), 40 nm, and 21.17 nm (Aktepe & Baran 2021).

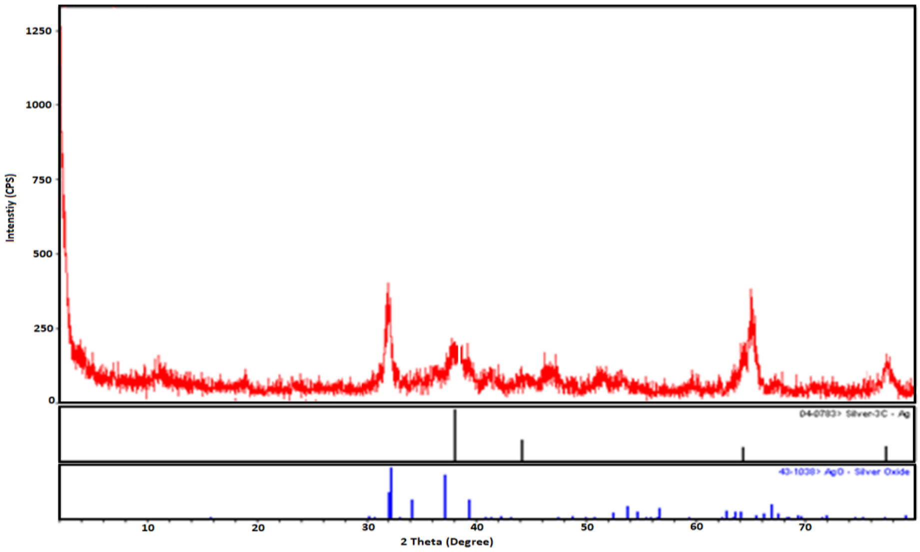

Figure 2- Data from X-ray diffraction showing the crystal structures of Rr-AgNPs.

3.3. FTIR Spectroscopy Data

The FTIR spectra of the liquid media as a result of extract synthesis were analyzed in order to assess the groups of bioactive substances that may control bioreduction and stability. Frequency changes in the data occur at 3337.81-3330 cm^{-1}. In order to assess the groups of bioactive components ensuring bioreduction, stability of Ag^{+} form to Ag^{o} form, and the stability of produced nanoparticles were investigated from 2122.95-2122.80 cm^{-1}, and 1635.48-1635.33 cm^{-1}. These observations demonstrated the potential for aromatic chains with C=C stretching, flavonoid-phenolic groups with C=O stretching, and hydroxyl groups resulting from O-H stretching to affect bioreduction and durability (Figure 3) (B. Kumar et al. 2015; Ahmad et al. 2018; A. U. Khan et al. 2018; Hemmati et al. 2019; Jogaiah et al. 2019; Jebril et al. 2020;Auda et al. 2021).

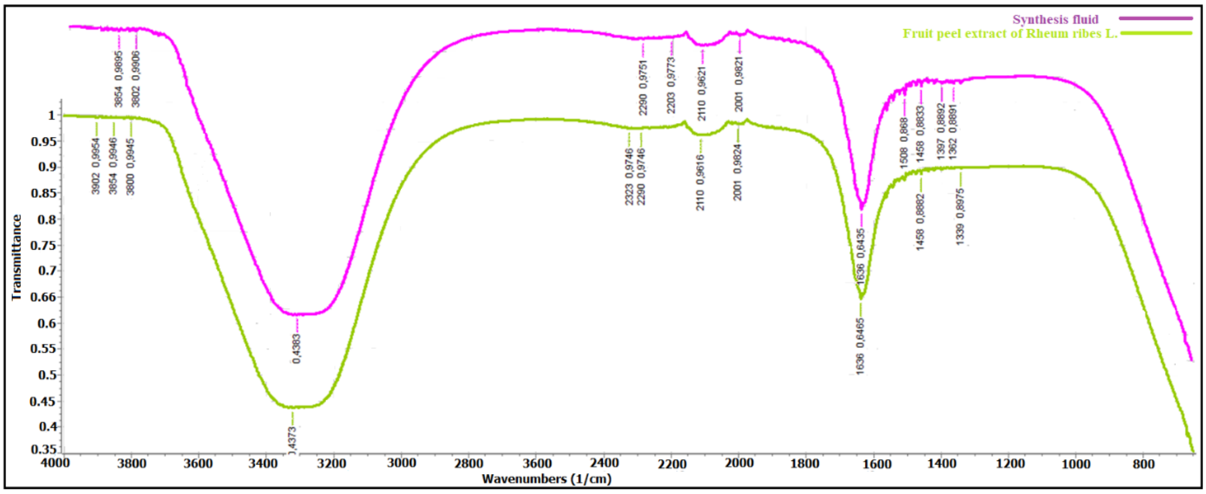

Figure 3- FTIR spectra of Rr fruit peel extract and reaction liquid obtained after synthesis

3.4. SEM and EDX Images of Rr-AgNPs

In SEM micrographs, the synthesized Rr-AgNPs had spherical morphology (Figure 4). According to reports, Rr-AgNPs had spherical morphology in SEM images taken during green synthesis investigations (Satpathy et al. 2018; Jebril et al. 2020; Aktepe & Baran 2021).

The profile of the EDX graph was used to determine the elemental contents of the particles synthesized with fruit peel extract (Figure 5). Strong peaks in the areas for elemental silver indicated that the synthesized particles were Rr-AgNPs (Pallela et al. 2018; Butola et al. 2019; M. R. Khan et al. 2022; Suriyakala et al. 2022). The existence of phytochemicals that actively contribute to the stability of the medium around Rr-AgNPs was suggested by weak peaks for elements in the graph, such as carbon and oxygen (Vastrad 2016; Kumar et al. 2019; Das et al. 2021). FTIR and zeta potential results in Figures 3 and 7, respectively, further confirm this data.

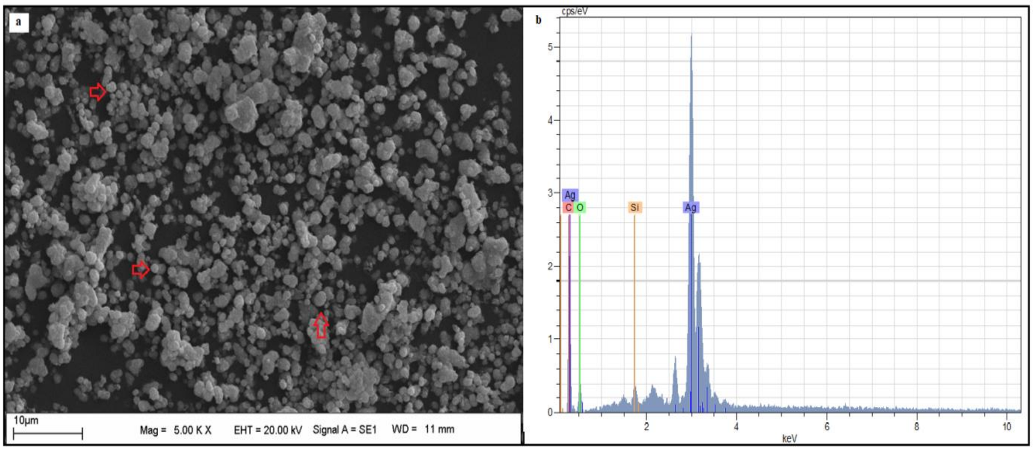

Figure 4- Rr-AgNPs after synthesis with Rr fruit peel extract; a. SEM micrograph of morphology and b. EDX profile

3.5. Appearance of Rr-AgNPs in FESEM and TEM Micrographs

Figure 5 shows images taken with a FESEM and TEM microscope of Rr-AgNPs produced with Rr fruit peel extract. The figure shows that the synthesized Rh-AgNPs exhibited spherical morphological appearance. AgNPs had the same morphological appearance in both studies using Rh fruit (Aygün et al. 2020; Auda et al. 2021). AgNPs synthesized in other green synthesis studies using different plant sources had the same morphological appearance (Velmurugan et al. 2014; Remya et al. 2015; Mamdooh & Naeem 2021; Wang et al. 2021).

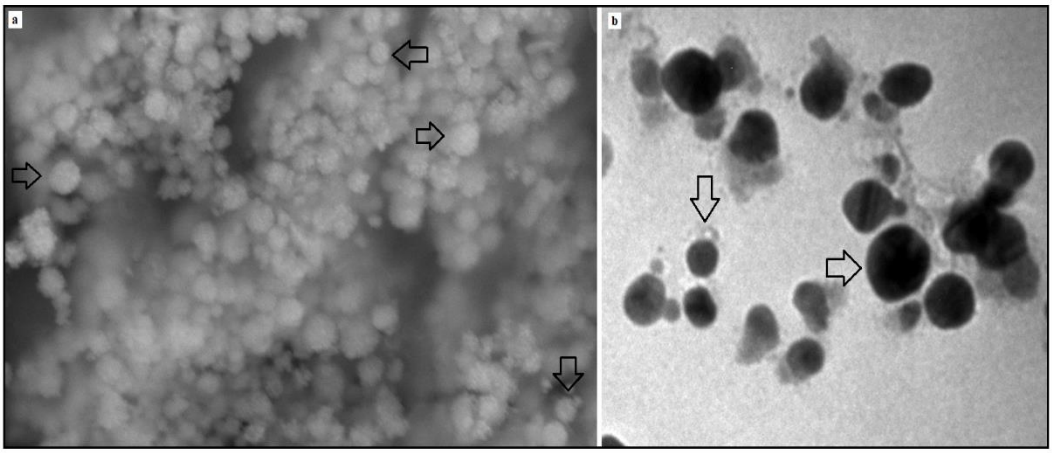

Figure 5- Rr-AgNPs after synthesis with Rr fruit peel extract; a. FE-SEM ve b. TEM micrograph of morphological views

3.6. TGA-DTA analysis of synthesized Rr-AgNPs

The thermal resistance of the synthesized Rr-AgNPs was determined by TGA-DTA analysis data at 10-800 °C. In the data, there were 5.98%, 8.24%, 23.73%, and 15.17% mass losses, respectively, occurring at 27.35-158.88 °C, 159.99-289.13 °C, 291.71-458.03 °C, and 458.03-801.53 °C (Figure 5). The loss of water absorbed was the cause of the initial mass loss (7.07%). The presence of flavonoids surrounding the surface of Rr-AgNPs caused mass losses at the other three temperatures (Baran 2019b; Rolim et al. 2019; Baran et al. 2021). The weak C, O peaks and -25 mV negative surface charge in the EDX and Zeta potential data in Figures 5 and 7, respectively, also support this finding.

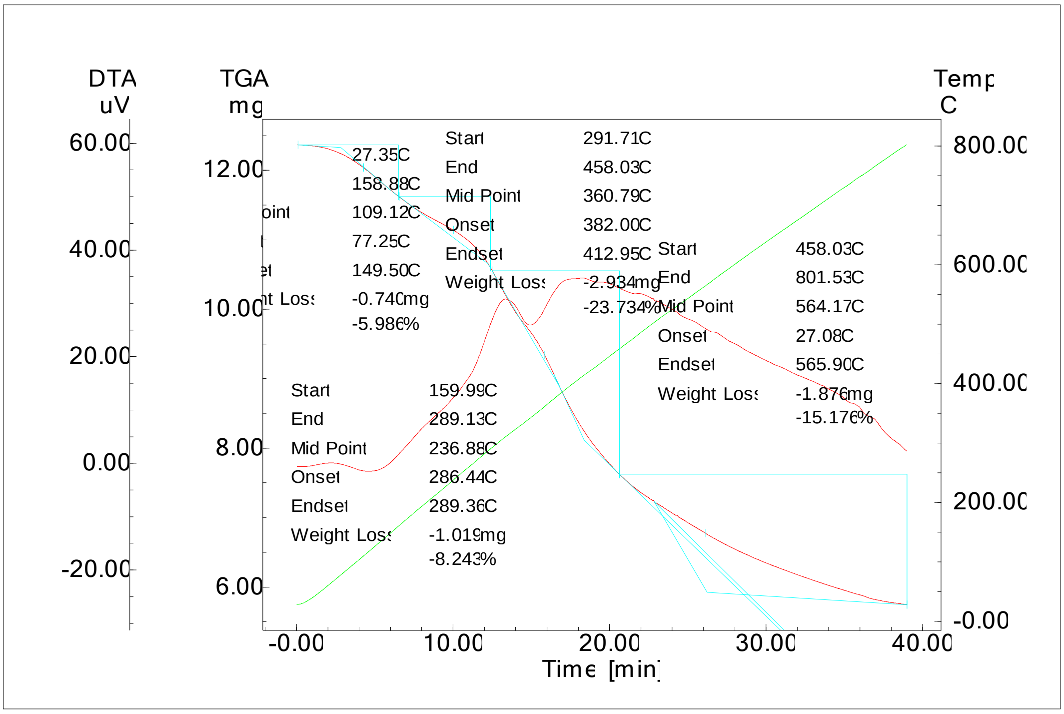

Figure 6- TGA-DTA data for Rr-AgNPs with mass loss points against heat treatment

3.7. Zeta potential analyses of synthesized Rr-AgNPs

The surface charges of Rr-AgNPs synthesized using fruit peel extract were identified as -25 mV by zeta potential analysis (Figure 7). The negative charge distribution of Rr-AgNPs is an important indicator of their stability. Green synthesized Rr-AgNPs exhibit more efficient negative charge distribution compared to those obtained by other synthesis approaches. The existence of flavonoids in the surface structure of Rr-AgNPs influences the distribution of negative charges. A negative charge arrangement stabilizes free electrons in colloidal form by oxidizing the hydroxyl groups. (Pugazhendhi et al. 2018; Wongpreecha et al. 2018b; Oliveira et al. 2019; Jebril et al. 2020; Aktepe et al. 2022). The presence of phytochemicals around Rr-AgNPs in the findings obtained in Figure 3, Figure 5, and Figure 6 also contribute to the explanation for the surface structure. The fact that the surface charges of the synthesized Rr-AgNPs have different charges (negative and positive) triggers the formation of negative results that affect stability, such as fluctuation and aggregation, as the nanoparticles attract with each other by electrostatic attraction (Al-ogaidi et al. 2017; Patil et al. 2018a; Satpathy et al. 2018). The -25 mV surface charge data showed that the synthesized Rr-AgNPs were stable. It was reported that Rr-AgNPs synthesized from different plant sources had surface charge distribution of 25.01 mV (Aktepe et al. 2022), −22 ± 5 mV (Ferreyra Maillard et al. 2018), and -19 mV (Oliveira et al. 2019).

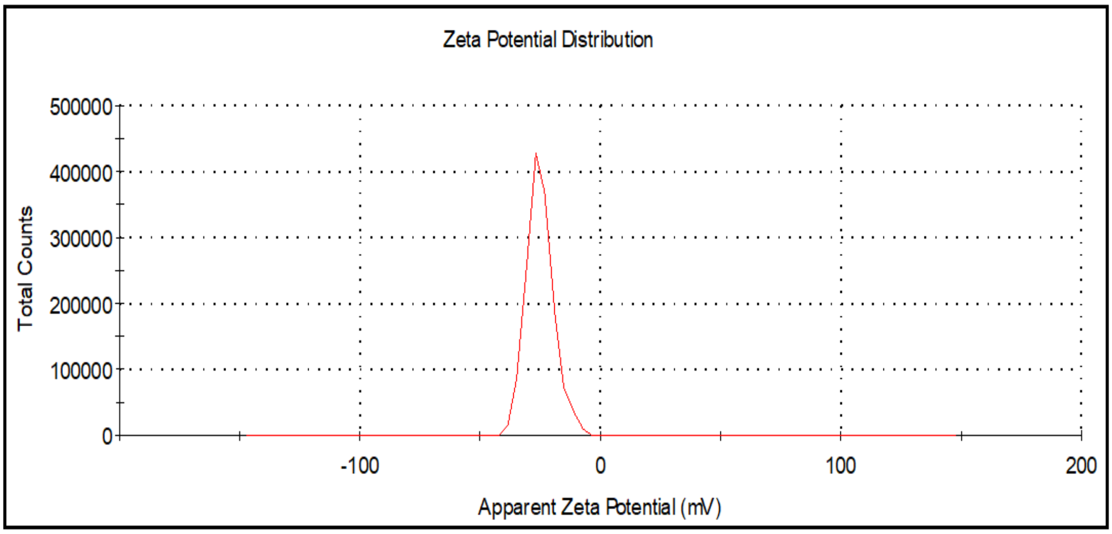

Figure 7- Graphs of zeta potential charge distribution of Rr-AgNPs after synthesis

3.8. Density-dependent Rr-AgNPs size distribution

The mean size distribution of the produced Rh-AgNPs, as seen in Figure 8, is 96 nm. They had an average size distribution of 54 nm in a study about the manufacture of AgNPs utilizing extracts from *Crinum asiaticum* leaves (Shukla et al. 2022). According to Kumar et al. (2017), AgNPs made with *Prunus persica* extract had size distribution of 2-130 nm. According to reports from earlier studies about environmentally friendly synthesis, the produced AgNPs had size distribution between 59.74 nm and 268 nm (Alkhulaifi et al. 2020; Mamdooh & Naeem 2021).

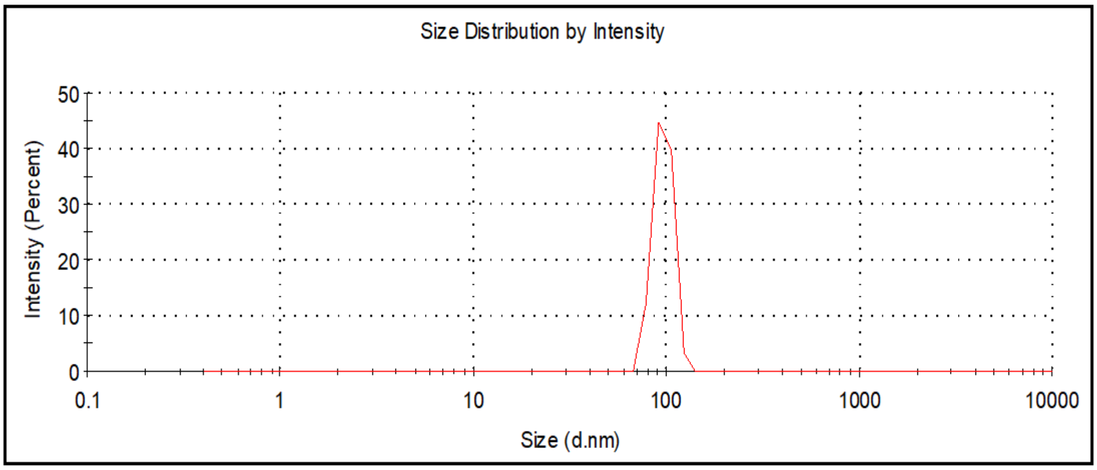

Figure 8- Density-dependent size distribution of synthesized Rr-AgNPs

3.9. AFM Micrograph of the distributions of synthesized Rr-AgNPs

As shown in Figure 9, the topographical features and morphologies of the produced Rr-AgNPs were determined by analyzing AFM data. The data showed that Rr-AgNPs were less than 100 nm, exhibited spherical appearance, and were distributed in a single structure (Swamy et al. 2015; Kumar et al. 2017; Rauf et al. 2021).

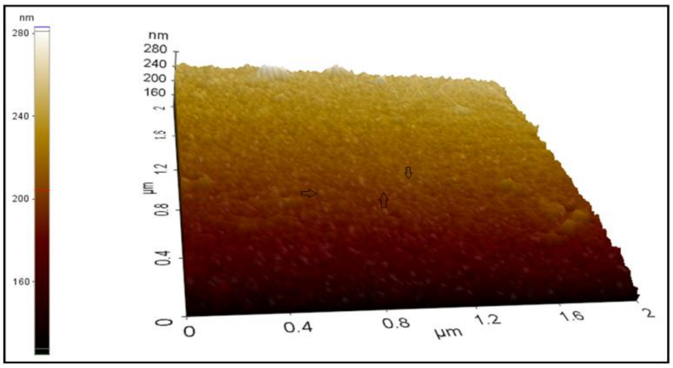

Figure 9- AFM micrograph of Rr-AgNPs synthesized using fruit peel extract

3.10. Antimicrobial effects of synthesized Rr-AgNPs

By using the microdilution method, the MIC values of the produced Rr-AgNPs for the development of pathogenic microbes were examined. In Table 1 and Figure 9, the MIC values affecting the growth of the strains are given. As seen in the data, 0.03-0.50 µmg/L concentrations had a suppressive effect on growth of microorganisms. The effective MIC values of the synthesized Rr-AgNPs were 0.03-0.13 and 0.25-0.50 mg/L for gram positive and negative bacteria, respectively. These values were considerably lower than the effective concentrations of silver nitrate solution and antibiotics. The effective MIC value for the fungus *C. albicans* was found to be 0.03 µmg/L, and this value is also many times lower than the value at which silver nitrate solution and antibiotics were effective. Table 2 gives the MIC values of Rr-AgNPs obtained in some applications using green synthesis.

Rr-AgNPs ionize in liquid media and show high reactivity. They approach one other with the electrical force of attraction, interacting with organisms in the exact same environment (Narayan & Dipak 2015; Ahmed et al. 2016; Chung et al. 2016; Zhang, et al. 2010; Aina et al. 2018). They cause defects in the membrane structure of microorganisms and adversely affect the functions of DNA, RNA, and vital enzymes, which have high affinity for these species due to the increase in reactive oxygen species (ROS). The structure of the biomolecules is damaged by these species and as a result, their functions deteriorate and the microorganism dies (Emmanuel et al. 2015; Shao et al. 2018; Huq et al. 2022).

Table 1- MIC values for Rh-AgNPs, antibiotics and silver nitrate solution with antimicrobial effect on the growth of microorganisms

Microorganisms		***AgNPs µg/mL***	***Silver Nitrate µg/mL***	***Antibiotic µg/mL***
Gram Positive	*B. subtilis* ATCC 11774	0.13	2.65	2.00
	S. aureus ATCC 29213	0.03	1.32	1.00
Gram Negative	*E. coli* ATCC25922	0.25	0.66	2.00
	P. aeruginosa ATCC27833	0.50	1.32	4.00
Fungus (Yeast)	*C. albicans* ATCC 10231	0.03	0.66	2.00

Commercial antibiotics used: **vancomycin** for gram (+) bacteria, **colistin** for gram (-) bacteria, and **fluconazole** for *C. albicans*.

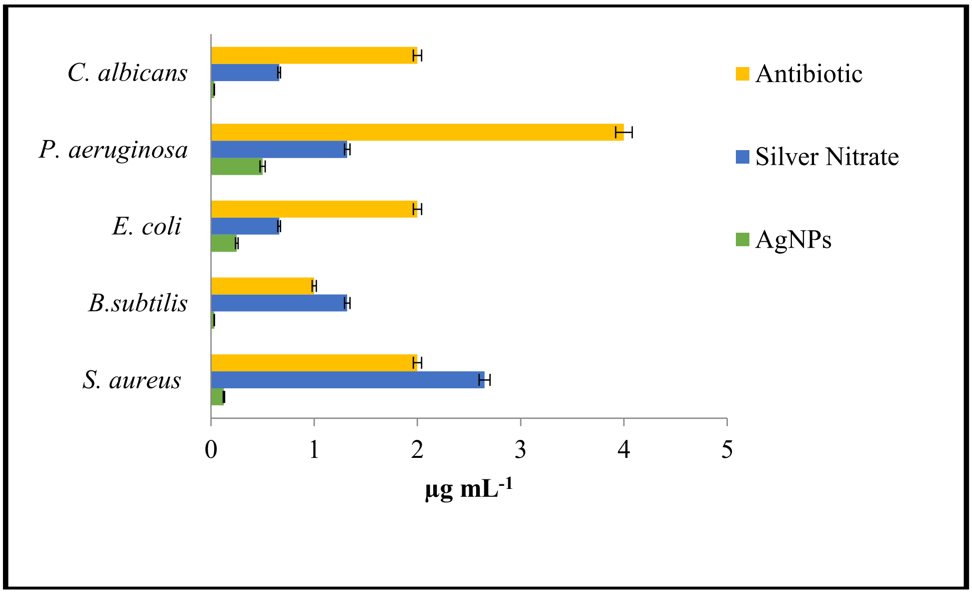

Figure 9- Graph of MIC values of Rh-AgNPs, $AgNO_3$ solution, and antibiotics Commercial antibiotics used: vancomycin for gram (+) bacteria, colistin for gram (-) bacteria, and fluconazole for *C. albicans*.

Table 2- Antimicrobial effects of AgNPs obtained in green synthesis studies

Biological Source	***Size (nm)***	***Gram Negative µg/mL***	***Gram Positive µg/mL***	***References***
Agastache foeniculum	9-19	12.50	6.25	(Polivanova et al. 2021)
Vitis vinifera	18.53	0.31	0.07	(Acay et al. 2019)
Fritillaria sp.	10	1-2	1-4	(Hemmati et al. 2019)
Zataria multiflora	25	1.25	0.5-4	(Barabadi et al. 2021)
Euphorbia hirta	15.5	0.67	0.82	(V. Kumar et al. 2016)
Madhuca longifolia	30-50	80-90	40-60	(Patil et al. 2018b)

3.11. Anti-cancer effects of Rr-AgNPs

The MTT technique was used to investigate the viability-suppressing concentrations of Rr-AgNPs made using Rr fruit peel for healthy cell lines and the suppressive effects for cancer cell lines. Up to a concentration of 25-200 mg/L, the Rr-AgNPs were not hazardous for the normal cell line HDF. However, it had a very strong suppressive impact on lines of cancer cells, indicating a very positive outcome for use as an anticancer drug. Even at a 25 mg/L dosage, a good suppressive effect was seen. With an inhibitory rate of 86.27%, 74.67%, and 73.49% for U118, CaCo-2, and Skov-3, 100 mg/L concentration was discovered to have excellent anticancer effect (Table 3 and Figure 10). Due to the existence of extensive vascular holes in the target cancer cells, the tiny size of NPs impacts the build-up and penetration of NPs into these areas. Additionally, Rr-AgNPs have a propensity to accumulate in crucial components such as membranes of cells, proteins, and nuclei. After localization, Rr-AgNPs with strong oxidative characteristics cause the cell to die by inducing cell death mechanisms such apoptosis and an increase in ROS As a result of these impacts, it is crucial to assess the harmful effects of Ag+ ions in living organisms (Wongpreecha et al. 2018b). Some NP characteristics contribute to harmful impacts.

Table 3- Viability percentages after Rr-AgNP interactions with various cell lines using the MTT technique for 48 hours.

Cell Line	***25 µmg/L***	***50 µmg/L***	***100 µmg/L***	***200 µmg/L***
HDF	79.45	67.55	67.55	41.00
U118	64.82	58.71	13.73	8.68
CaCo-2	23.74	23.08	26.51	34.11
Skov-3	86.18	73.77	25.33	32.22

HDF; human skin fibroblast cells: **U118**; glioblastoma cells: **CaCo-2**; colon cancer cells: **Skov-3**; ovarian sarcoma cells

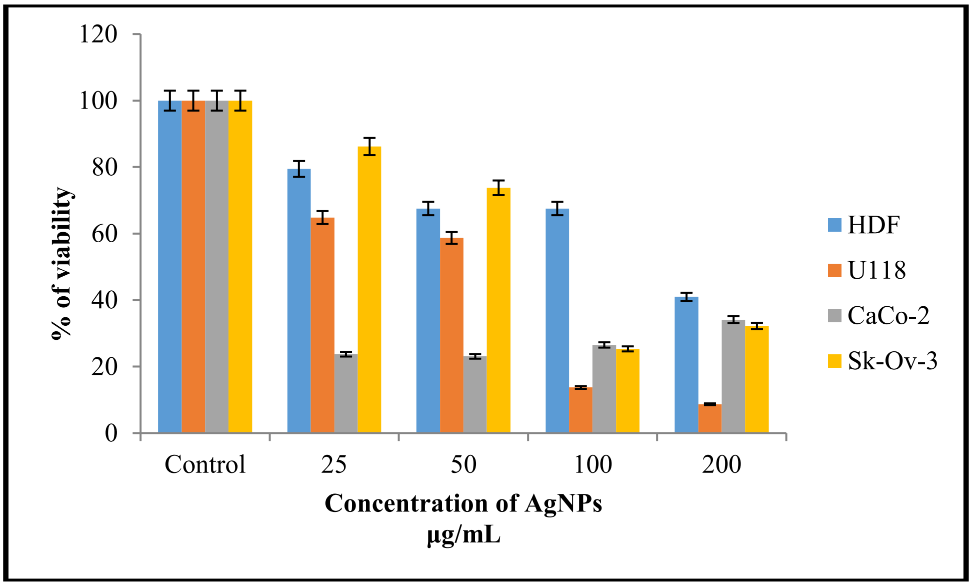

Figure 10- Viability percentages after Rr-AgNP interactions with various cell lines using the MTT technique for 48 hours.In Table 4, the cytotoxic effects on cancer cells of some nanoparticles obtained with green synthesis are compared.

Table 4- Amounts of Rr-AgNPs found in research employing environmentally friendly synthesis methods that contribute to the cytotoxic impact

Biological Source	***Cancer Cell Line***	***Size (nm)***	***Shape***	***Effective Concentration µmg/L***	***Refereces***
Aloe Vera	HDF	20	Spherical	100	(Zhang, et al. 2010)
Aspergillus sp.	CaCo-2	5-30	Spherical	3.75-5	(Mohmed et al. 2017)
Pueraria tuberosa	Skov-3	162.72	Spherical	29.36	(Satpathy et al. 2018)

4. Conclusions

Rr-AgNPs are widely used, which makes them very valuable materials. The manufacture of these items receives a lot of attention daily for green synthesis techniques. The extract from the peel of the Rr fruit, also known as "Işgin" or "Kurdish banana," which grows in the Erzurum region, was used in this study to produce Rr-AgNPs for the first time in a quick, straightforward, ecologically benign method without harmful processes. Data from TEM, AFM, UV-vis, FE-SEM, XRD, EDX, TGA-DTA, Zetasizer, and Zeta potential analyses were used to identify the characteristics of the synthesized Rr-AgNPs. In order to evaluate the functional components of flavonoids that contribute to bioreduction during the manufacturing process, FTIR data were examined. Maximum absorbance, spherical shape, median size range of 96 nm, and charge on the surface of -25 mV at wavelength of 428 nm were all characteristics of the produced Rr-AgNPs. The efficacy of produced Rr-AgNPs in medicinal applications was studied using MTT and microdilution techniques. The minimum inhibitory concentrations of Rr-AgNPs needed to inhibit hospital pathogens were between 0.03-0.50 mg/L. These concentrations were extremely low compared to antibiotics and $AgNO_3$ solution. With inhibitory rates of 86.27%, 74.67%, and 73.49%, Rr-AgNPs were discovered to have a very good anticancer impact on U118, CaCo-2, and Skov-3 cancer cells. The healthy cell HDF was not damaged by the same concentration. In particular for biopharmacology, enhancing application processes will considerably advance the search for antibacterial and anticancer drugs.

Declarations

Conflict of interest

All authors declare there are no conflicts of interest in this research work.

Acknowledgements

We would like to thank Dr Kenan Akbaş who identified the *Rheum ribes* L. plant.

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Halotolerant *Bacillus* Species as Plant Growth Promoting Rhizobacteria from Hyper – Arid Area of Algeria

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ABSTRACT
The aim of this study was to determine the diversity of aerobic halotolerant *Bacillus* plant growth promoting rhizobacteria (PGPR), their production of hydrolytic exo-enzymes and their inoculation effect on two cowpea plants. The soil dilution plate technique was performed on tryptic soy agar complemented with thermal pretreatment to select *Bacillus* strains associated with the *Phoenix dactylifera* rhizosphere growing in hypersaline and arid soil in Algeria (In Salah, Tamanrasset). The inoculation effect of these strains on cowpea plant growth was assessed based on biometric and physiological parameters. As a result, thirteen halophilic, halotolerant and non-halophilic *Bacillus* strains were isolated. Upon screening, all strains were capable of producing at least two hydrolytic enzymes under saline conditions and most of the strains (n=10/13) showed at least two plant growth promoting (PGP) traits. Strains were identified as members of *Bacillus* genera based on their phenotypic and biochemical characteristics. The inoculation of these strains in cowpeas significantly improved biometrics and physiological growth parameters of the inoculated plants. Based on their PGP effects, five strains were identified: RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), RP 12 (*B. circulans*) and RP 13 (*B. cereus*). The isolation and characterization of halophilic and halotolerant *Bacillus* strains increased knowledge about the microflora in the rhizosphere associated with date palms in saline and arid soils. *Bacillus*-PGPR strains proved to be highly effective to improve cowpea plant growth and development.

Keywords: *Bacillus*, Biofertilizers, Rhizosphere, Sustainable agriculture

1. Introduction

Salinity is a natural feature of ecosystems in arid and semi-arid regions due to very low rainfall but it can also be induced by irrigation with saline water, which can be a real hindrance to plant growth. Worldwide, it is estimated that 800 million hectares of agricultural land are affected by salinity (Yasin et al. 2018). A biological approach to improving plant productivity, particularly in saline conditions, can be adopted through the use of halophilic or salt-tolerant bacteria. From this perspective, different ecological niches have been explored to isolate and characterize halophilic and halotolerant bacteria, which include the rhizospheric soil of different cultivated plants. Halophilic bacteria are a heterogeneous physiological group of microorganisms belonging to different genera and capable of developing optimally in media with a wide range of NaCl content (3-15%) (Ventosa et al. 1998). In addition, saline environments support various bacterial populations that have modified and adapted their physiological and structural characteristics under the prevailing saline conditions. These characteristics are very important in taxonomic classification.

The salinity effect is always more pronounced in the rhizosphere, where different halophilic and halotolerant bacterial species can be hosted, due to increased water absorption by plants as a result of transpiration (Ibekwe et al. 2010). Moreover, the rhizosphere is a narrow ground area adjacent to the plants (inside and around the roots) where high microbial activity is characterized by the potential to promote plant growth (Kloepper & Beauchamp 1992). The selection of non-phytopathogenic rhizospheric bacteria with halophilic or salt-tolerant character may increase plant growth in cases of stress generated by aridity, including salinity. Consequently, inoculation with these plant growth promoting rhizobacteria (PGPR) is a biological method to replace chemical fertilizers and pesticides in agricultural practice. Therefore, different mechanisms of action can be used by PGPR to improve the growth and health of the plant such as biofertilization (nitrogen fixation, phosphate and potassium solubilization, siderophore production) and phytostimulation (phytohormone production) (Benaissa 2019). *Bacillus* species with various PGPR properties can be introduced into various root zones. Many researchers around the world use *Bacillus* PGPR to improve the growth of various plants (Kayshep et al. 2019).

Many studies have isolated PGPR from soil, but little attention has been paid to PGPR isolated from saline soil. In fact, soil salinity is a major factor in microbial selection because salt may be considered a limiting factor that reduces bacterial diversity. The adaptation of micro-organisms to salinity conditions due to salt-selective pressure makes them candidates to be salt-tolerant PGPR isolates (Jiang et al. 2018). Kushner (1978) defined the most common categories as: (i) extreme halophiles (optimal growth in salt concentrations of 2.5 to 5.2 M or 14.63-30.45% NaCl), (ii) extreme limit halophiles (optimal growth in salt concentrations of 1.5 to 4.0 M or 8.79-23.44%), (iii) moderate halophiles (optimal growth in salt concentrations of 0.5 to 2.5 M or 2.93-14.63% NaCl), and (iv) halotolerant microorganisms that do not have an absolute need for salt for growth, but tolerate many salt concentrations (1.17-30.45% NaCl) that are often very high (considered extremely halotolerant if the growth interval extends above 2.5 M of salt, and weak halophilic with optimal growth in salt concentrations of 1.17-2.93% NaCl). Non-halophilic organisms require less than 1% NaCl.

The date palm (*Phoenix dactylifera*) is considered a xerophytic and halophyte species where difficult aridity conditions do not pose a problem for cultivation. The study of the rhizobacterial community associated with plants naturally adapted to cope with extreme saline conditions could lead to several knowledge outputs: (i) understanding the plant-microbe interaction under saline conditions, (ii) defining the mechanisms underlying plant growth with promotion under saline stress, and (iii) identifying bacterial strains to design organic fertilizers for agriculture practice in arid and saline lands (Zahran 1997).

To the best of our knowledge, there are no studies conducted about the halophilic bacteria isolated from arid and saline soil in Algeria associated with the *Phoenix dactylofiera* rhizosphere. We hypothesize that the *Bacillus* group of halophilic and halotolerant rhizobacteria may have the potential to enhance plant growth and to cope with arid conditions. Therefore, the main objectives of this study were to characterize halophilic and halotolerant *Bacillus* group rhizobacteria with hydrolase activities, plant growth promoting (PGP) traits such as nitrogen fixation, hydrogen cyanide (HCN) production and phosphate solubilization and to examine their effects by inoculating two cowpea plants. The cowpea plant is particularly cultivated by peasants in small villages in southern Algeria for their own consumption.

2. Material and Methods

2.1. Sample site and collection

Soil samples were randomly collected from three palm groves located in an arid area of Algeria (In Salah) (27° 11′ 55.69″ North, 2° 26′ 45.29″ East) during December 2020 (Figure 1). The sampling technique involved inserting a sterile spatula into the soil to a depth of 15 cm glued to the roots. The sample was placed in a sterile container and transported to the laboratory in an ice box set to 4 °C. The soil texture and physicochemical analysis (Table 1) were performed as described previously (Mathieu & Pieltain 1998; Mathieu & Pieltain 2003). Soil classification (World Reference Base: WRB) was based on the Group of Arid Hapylic Solonchaks (FAO WRB 2006).

Figure 1- Geographic location of the palm grove in the In Salah region, Algeria (Google Earth, 2020) [*rhizosphere soil]

Table 1- Texture and physicochemical parameters of soil

Parameters	*Results*
pH	7.13
Electric Conductivity 1 :5 (ds/m)	2.6
Organic Matter %	0.1
Calcium Carbonate $CaCO3$	0%
Texture (Particle-Size)	Sandy loam

2.2. Isolation and characterization of halotolerant Bacillus strains from rhizospheric soil

Soil dilution plate technique was performed on tryptic soy agar complemented with cycloheximide (40 mg/mL) and nalidixic acid (10 mg/L) (to eliminate gram-negative bacteria and fungi in the soil, respectively) and supplemented with NaCl (0%, 15%) to allow isolation of halotolerant or/and halophilic bacteria (Kushner 1978). Surface seeding of soil dilutions was carried out on Petri dishes to select aerobic strains.

To increase the selectivity of the *Bacillus* isolation medium, thermal pretreatment (10 min at 80° C) of the soil dilution was carried out in order to select bacterial spores and eliminate all vegetative forms.

To determine whether the strains that were grown on the initial 15% NaCl medium are halophilic or halotolerant, the nutrient broth medium (NB) was used, adjusted to NaCl concentrations (0%, 3%, 20%, 25% and 30%). Halotolerant strains were classified if they grew on both media (0 and 15% NaCl), moderate halophilic strains were those grown only on the medium at 3% - 15% NaCl, while extreme halophilic strains grew on media at 15% - 30% NaCl (Kushner 1978). The Petri dishes were incubated at 27 °C for 24 to 48 hours.

The strains were phenotypically characterized using standard procedures of Gram staining and spore position, catalase and oxidase tests complemented with several biochemical tests, such as respiratory type (medium was meat-liver agar, packaged in Prévot tube, and only bacteria with surface growth were considered aerobic), Voges-Prauskeur (VP) and Methyl red (RM) (Clark and Lubs broth was seeded with a drop of bacterial suspension, after incubation for 18 h at 37 °C, the broth was divided into 2 sterile tubes. Each tube was used to reveal one of the 2 ways: RM or VP), arginine di-hydrolase (ADH), ornithine decarboxylase (ODC) (Moeller medium contains only one amino acid, glucose and purple bromocresol as an indicator of pH. Alkalization indicates the presence of these enzymes) and sugars assimilation on inclined nutrient agar containing 1% mannitol, 1% glucose, 1% fructose and 1% saccharose, respectively, with phenol red added as pH indicator. All the strains were preserved in nutrient broth with 20% glycerol added at -80 °C.

2.3. Screening for hydrolytic activity

The activity of six extracellular enzymes was investigated under non-saline (0.9% NaCl) and saline (15% NaCl) conditions. The hydrolysis test for gelatin was performed as described by Egamberdiyeva & Höflich (2004) with modifications, using a nutrient broth supplemented with 50 g/L gelatin powder as solidifying agent. Ureolytic activity was revealed on Christensen urea agar medium (peptone 1 g/L, dextrose 1 g/L, sodium chloride 5 g/L, potassium phosphate monobasic 2 g/L, urea 20 g/L, phenol red 0.012 g/L, agar 15 g/L) according to the protocol of Atlas (2010). Casein hydrolysis was tested on Mueller Hinton agar (MH) supplemented with 10% skimmed milk (Castro-Escarpulli et al. 2003) and amylolytic activity was detected on tryptic soy agar (TSA 1/10) with 1% starch added (Delarras 2014). Cellulose hydrolysis was demonstrated on agar medium with crushed pulp as a source of cellulose (Lesel et al. 1986). Lecithinase was determined on ordinary nutrient agar supplemented by an emulsion of egg yolk and distilled water (2 mL/20 mL) (Delarras 2007).

2.4. Screening for plant growth promoting activities

Molecular nitrogen fixation was tested on the Jensen medium, a free nitrogen medium. Growth on this medium after being transferred ten times in the same medium reflects the ability of bacteria to fix nitrogen. The ability of strains to produce hydrogen cyanide (HCN) was examined using the method of Lorck (1948). The qualitative solubilization activity of phosphate was tested on the National Botanical Research Institutes Phosphate (NBRIP) medium (Nautiyal 1999).

2.5. Assay of Bacillus isolates on cowpea plants growth

The seeds of two cowpea ecotypes (*Vigna unguiculata subsp. unguiculata (L.). Walp*.) from the Tamanrasset region (Bassedik et al. 2021): (accession#1 (NEA10) from Igléne (Abalessa) [4°89'E, 22°88'N], accession#2 (NEA13) from Tit (Tamanrasset) [5°14'E, 22°58'N]), were surface sterilized by brief rinsing in 95% ethanol for 30 s, then rinsed with distilled water for 5 minutes three times. The seeds were evenly distributed over the surface of sterile absorbent paper in petri dishes. The boxes were sealed with film paper and incubated in an oven set at 28 °C. The seeds with a fully widened cotyledon were selected for inoculation. Then, 24-hour cultures of *Bacillus* inoculants were first prepared by growing a colony in 10 mL of nutritious broth. All the isolated strains were tested without taking into account halophilic character so as not to neglect interesting strains that were not halotolerant.

The germinated seeds were then placed in bacterial cultures for about 30 minutes. Three inoculated seedlings were directly planted into local soil from the university of Tamanrasset, previously sterilized at 160 °C/4 h. The pots were then kept under light with 16-hour photoperiod under laboratory conditions at room temperature with regular sprinkling of 10 mL of distilled water. Plant growth was monitored and compared to controls grown without cultured bacteria. For this purpose, biometric parameters were measured and photosynthetic pigment concentrations were determined according to Lichtenthaler (1987), and the relative water content of the leaf was determined using the method described by Barrs (1968).

2.6. Statistical analysis

The data was subjected to statistical analysis using the Microsoft Excel 2010 program. All values are given as mean ± SE (standard error) of three replicates of a single sample for each experiment. The obtained data underwent unidirectional analysis of variance (ANOVA) using the Statistical Analysis System (XLSTAT) version 2016.02. The differences between individual means were considered significant at $P<0.05$. The averages were compared with the LCD test. Principal component analysis (PCA) was performed. This is a multivariate statistical method, which involves transforming interrelated variables (called "correlated" in statistics) into new variables that are not correlated with each other. These new variables are called "principal components" or main axes.

3. Results and discussion

In this study, the microflora of aerobic halotolerant-halophilic *Bacillus* strains from the rhizosphere of *Phoenix dactylifera* growing in arid and saline soil in the Algerian Sahara were studied. Thirteen strains phenotypically close to *Bacillus* and able to grow on salt-based media were selected for further analysis. They were characterized using phenotypical tests, for hydrolytic and PGP activities and inoculation potential.

3.1. Bacteria characterization

Morphological and chemotaxonomic analyses indicate that all isolates are rod-shaped, gram-positive, aerobic, sporulating, catalase positive and oxidase variable (Table 2). All isolates were characterized according to the above-mentioned phenotypic tests as members of the genera *Bacillus*. Phenotypical identification showed that 23.07% of bacterial isolates were identified as *B. circulans,* 23.07% as *B. coagulans,* 15.38% as *B. amylo-liquifaciens* and 7.69% each for *B. megaterium, B. subtilis, B. cereus, B. licheniformis* and *Paenibacillus polymyxa*. Halophilic, non-halophilic or halotolerant strains of *Bacillus* (n=13) isolated from the date palm rhizosphere represent an infinite fraction of the soil microbial community. However, the sporulation capacity of this bacterial genus promotes ubiquity on the one hand and survival in very diverse environments on the other.

Table 2- Morphological and biochemical characterization of *Bacillus* isolates associated with the *Phoenix dactylifera* rhizosphere growing in hyper-arid area of Algeria (DT: deformant terminal, TND: terminal non-deformant, CD: central deformant, CND: central non-deformant, ANAF: aero-anaerobic facultative, AS: aerobic strict)

Code / *Tests*	*RP1*	*RP2*	*RP3*	*RP4*	*RP5*	*RP6*	*RP7*
Form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Grouping mode	chain	chain	chain	chain	chain	chain	chain
Gram	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Spore position	TD	CD	CD	CND	CND	TND	CD
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	-ve	+ve	+ve	+ve
Respiratory type	ANAF	ANAF	AS	ANAF	ANAF	ANAF	ANAF
Voges-Prauskeur	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl red	-ve	+ve	+ve	+ve	+ve	-ve	+ve
Arginine Di-hydrolase	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Ornithine Decarboxylase	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sugar assimilation:							
-Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Fructose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Saccharose	-ve	-ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 0.9% NaCl:							
-Urease	+ve	+ve	+ve	+ve	+ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Cellulase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Lecithinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 15% NaCl :							
-Urease	-ve	-ve	-ve	-ve	-ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	-ve	-ve	-ve	-ve	+ve	+ve	-ve
-Cellulase	-ve	-ve	-ve	-ve	-ve	-ve	-ve
-Lecithinase	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 2(Continue)- Morphological and biochemical characterization of *Bacillus* isolates associated with the *Phoenix dactylifera* rhizosphere growing in hyper-arid area of Algeria (DT: deformant terminal, TND: terminal non-deformant, CD: central deformant, CND: central non-deformant, ANAF: aero-anaerobic facultative, AS: aerobic strict)

Code / *Tests*	*RP8*	*RP9*	*RP10*	*RP11*	*RP12*	*RP13*
Form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Grouping mode	chain	chain	chain	chain	chain	chain
Gram	+ve	+ve	+ve	+ve	+ve	+ve
Spore position	TD	CND	CD	TND	TD	CND
Catalase	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	+ve	-ve	+ve
Respiratory type	ANAF	ANAF	ANAF	AS	ANAF	ANAF
Voges-Prauskeur	+ve	+ve	+ve	+ve	+ve	-ve
Methyl red	+ve	+ve	+ve	+ve	+ve	+ve
Arginine Di-hydrolase	-ve	+ve	-ve	-ve	-ve	-ve
Ornithine Decarboxylase	+ve	-ve	-ve	-ve	-ve	-ve
Sugar assimilation:						
-Mannitol	+ve	+ve	+ve	+ve	+ve	+ve
-Glucose	+ve	+ve	+ve	+ve	+ve	+ve
-Fructose	+ve	+ve	+ve	+ve	+ve	+ve
-Saccharose	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 0.9% NaCl:						
-Urease	+ve	+ve	+ve	+ve	+ve	+ve
-Gelatinase	+ve	-ve	-ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	+ve	+ve	+ve	+ve	+ve	+ve
-Cellulase	+ve	+ve	+ve	+ve	+ve	+ve
-Lecithinase	+ve	+ve	+ve	+ve	+ve	+ve
Hydrolytic activities in presence of 15% NaCl :						
-Urease	-ve	+ve	+ve	-ve	+ve	-ve
-Gelatinase	+ve	+ve	+ve	+ve	+ve	+ve
-Caseinase	+ve	+ve	+ve	+ve	+ve	+ve
-Amylase	-ve	+ve	+ve	+ve	-ve	-ve
-Cellulase	-ve	-ve	-ve	-ve	-ve	-ve
-Lecithinase	-ve	-ve	-ve	-ve	-ve	-ve

The nature of the soil impacts the diversity of the bacterial community. The rhizosphere soil of the date palm in our study is hyper-arid and saline, which are two unfavorable conditions for the growth of microorganisms. In the same way, our microbial ecology study in arid and hypersaline environments reveals that rhizobacteria isolated from saline soils can grow at different salinity levels. From the date palm's rhizosphere, 8 halotolerant *Bacillus* strains (n= 8/13) and one moderately halophilic (n=1/13) strain were isolated (Table 3).

Table 3- Types of *Bacillus* strains and their Plant Growth Promoting activities isolated from *Phoenix dactylifera* rhizosphere

Code	*Phosphate solubilization*	*HCN production*	*Nitrogen fixation*	*Type*	*Species*
RP1	+	+	+	**Halotolerant**	*B. circulans*
RP2	+	+	+	Non-halophilic	*B. coagulans*
RP3	+	-	+	**Halotolerant**	*B. coagulans*
RP4	+	+	+	Non-halophilic	*B. megaterium*
RP5	-	-	+	**Halotolerant**	*B. subtilis*
RP6	+	-	+	**Halotolerant**	*B. amylo-liquifaciens*
RP7	-	-	+	**Halotolerant**	*B. coagulans*
RP8	+	+	-	**Halotolerant**	*B. circulans*
RP9	+	+	+	**Moderately Halophilic**	*B. licheniformis*
RP10	+	-	+	Non-halophilic	*Paenibacillus polymyxa*
RP11	+	-	-	Non-halophilic	*B. amylo-liquifaciens*
RP12	-	+	+	**Halotolerant**	*B. circulans*
RP13	-	+	+	**Halotolerant**	*B. cereus*

3.2. Production of hydrolytic enzymes

Enzymes from halophilic sources are expected to have optimal activity under extreme conditions (de Lourdes Moreno et al. 2013). Our study showed that the 8 isolated halotolerant strains could produce more than three different hydrolytic enzymes, which is highly interesting. All isolates had a combination of hydrolytic activities under normal growing conditions (0.9% NaCl). It appears that almost all strains had the six hydrolytic activities tested (urease, amylase, cellulase, lipase, caseinase and lecithinase). At the same time, in the presence of salt (15% NaCl), the enzymatic activities decreased, in particular the production of urease, amylase and cellulase (Table 3). However, all strains are capable of producing at least two hydrolytic enzymes under saline conditions.

For this reason, the possibility of having a wide variety of halotolerant species producing extremozymes will provide great assistance for biotechnological applications, particularly in the agricultural field. Thus, it is very important to select enzyme-producing halotolerant-halophilic bacteria with optimal activity at different salt concentrations. However, our study showed that the enzymatic activity of our isolates decreased in the presence of 15% NaCl, which implies that salinity can pose an ecological challenge for production of certain extracellular enzymes. Indeed, some of the bacterial isolates were able to grow at 5% NaCl but failed to express hydrolytic activity at the same level. Moreover, it was recently shown that halotolerant bacteria of the genus *Bacillus* produce industrially important hydrolases and their enzymatic activities are more diverse. Enzyme-active bacilli were already isolated in saline soils (Zahran 1997) or salt marshes (Weisser & Truper 1985) with bacteria displaying significant enzymatic activity under saline stress (10% NaCl).

In this study, most of the *Bacillu*s isolates were able to produce hydrolysis enzymes. The genus *Bacillus* is well known as an enzyme producer. Many industrial processes use species belonging to this genus for the commercial production of enzymes. It is interesting to note that the combined hydrolytic activities detected in some strains could have applications for biotechnological purposes. The production of hydrolytic enzymes reflects a good adaptation of halophilic rhizobacteria to harsh environmental conditions and establish themselves in competition for the colonization of the plant's rhizosphere. For this reason, it is very important for these rhizobacteria to exhibit hydrolytic activity under saline conditions. Indeed, halophilic bacteria are a potential source of extracellular hydrolases like proteases, with a wide array of industrial applications (Shivanand & Mugeraya 2011).

3.3. Plant growth promoting traits

In terms of functional diversity, *Bacillus* strains were screened for various plant growth-promoting traits such as nitrogen fixation, HCN production and phosphate solubilization. The halotolerant *Bacillus* strains isolated in this study could for the most part (n=6/8) produce at least two PGP effects and the moderately halophilic one produced all three PGP effects studied, which are of interest in a saline environment. Most of the strains (n=10/13) had at least two plant growth-promoting activities (Table 3). Also, 11 strains (n=11/13) were able to fix nitrogen, 9 strains (n=9/13) showed phosphate solubilization activity and 7 strains (n=7/13) produced HCN.

Based on our results, halotolerant species of *Bacillus*-PGPR were identified as *B. circulans* (RP 1, RP 8 and RP 12), *B. coagulans* (RP 3), *B. amylo-liquifaciens* (RP 6), *B. licheniformis* (RP 9) and *B. cereus* (RP 13). Indeed, a large number of PGPR belonging to the genus *Bacillus* were isolated from the rhizospheres of various plants (Kashyap et al. 2019; Zafar-ul-Hye et al. 2019; Benaissa et al. 2018), including halophytes (Arora et al., 2020). Consequently, PGPRs are able to directly affect plants by promoting their development or indirectly affect them by impacting their responses to environmental constraints such as edaphic salinity (Han & Lee 2005).

3.4. Bacillus strains improves cowpea growth

A variety of biometric, physiological, and biochemical parameters were assessed for inoculated and non-inoculated plants (RP 0: control) cultivated in soil in order to explore the in vitro effects of isolates on two accessions of cowpea. Cowpea seedlings were observed on the 15th day following laboratory inoculation to determine which plants had the best growth.

Following inoculation, the isolates promoted plant development in both of the cowpea varieties studied. For instance, when compared to control plants, the two cowpea plants inoculated with RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), and RP 12 (*B. circulans*) grew more effectively. Therefore, all of the aforementioned strains, aside from RP10 which is non-halophilic, were determined to be halotolerant. However, all strains studied demonstrated favorable impact on one or more physiological and biometric metrics (Table 4). As an illustration, neither strain had a favorable impact on the relative water content of the leaf in either accession.

Analysis of variance also indicated that each of the biometric and physiological parameters were highly significantly different from one strain to another for the two accessions studied (Figure 2).

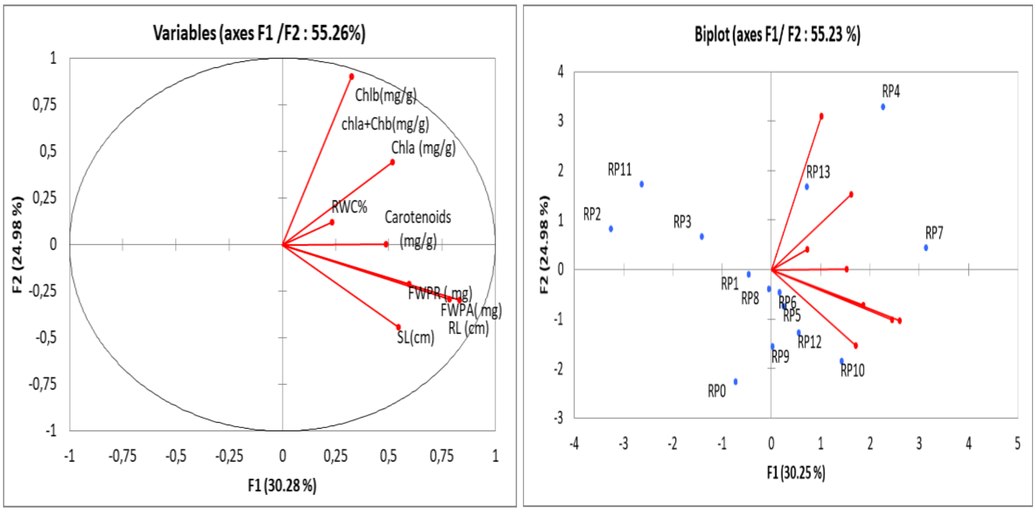
Table 4- Effect of *Bacillus* PGPRs inoculation on stem and root length, fresh weight of shoot and root, photosynthetic pigments and relative water content of cowpea plants. Standard Error (SE) shows very high significant differences (P<0.05)

Accessions	*Strains code*	*FWSP (mg)*	*FWRP (mg)*	*SL (cm)*	*RL (cm)*	*RWC%*
NEA10	RP0	209± 91cd	112.33± 28.5de	8.03±1.51abc	5.73±1.02ab	52.07±13.71ab
	RP1	327± 39b	**327± 15.94a**	8.13±1.24abc	5.23±0.64abc	53.58±18.80ab
	RP2	385.33± 29ab	257.66±15.13 b	9.36±2.53ab	**6.06±1.40abc**	50.81±15.65ab
	RP3	298± 0.57bc	124.33± 13.65cde	7.56±1.09abc	4.33±0.63abcd	48.38±10.32ab
	RP4	311± 79.26b	168.33±19.62 c	6.96±1.47bc	4.46±0.89bcd	46.98±8.21ab
	RP5	387± 1ab	174.66±28.29 c	8.1±1.47abc	4.46±0.89bcd	48.01±5.71ab
	RP6	**470± 36.59a**	131.66± 37.2cd	**10±1.04a**	3.46±0.45de	56.84±1.70ab
	RP7	**433± 3.77a**	122± 19.07cde	8.13±4.10abc	4.16±1.15cde	41.26±10.5b
	RP8	76.33± 80.99e	76.33± 17.32e	9.23±1.87ab	3.40±0.17de	51.56±12.39ab
	RP9	104.66± 67.03e	104.33± 5.85de	**10.03±0.90a**	3.2±0.26e	59.96±17.61ab
	RP10	84.33± 88.96E	84.33±4.61 E	7.63±0.41abc	3.33±0.37e	45.76±9.66ab
	RP11	107.66± 62.44e	107.66± 19.85de	8.30±1.58abc	3.96+1cde	41.35±5.27b
	RP12	123.33± 29.77de	123.33±29.36cde	5.81±0.07c	3.63±0,8de	41.35±5.27b
	RP13	84.33E± 20.55	84.33±16.25 E	7.43±1.56abc	3.5±0.6de	**67.84±27.86a**
NEA13	RP0	255±61ef	237.66±12.01bcd	10.60±3.5a	5.6±0.9bcd	84.41±5.40abc
	RP1	255±42.67ef	193.33±20.55cde	8.33±0.95abcd	4.6±0.79cde	80.76±6.61bcd
	RP2	122.33±63.7g	80±18.02f	4.3±1.47f	2.7±0.43ef	57.14±12.36d
	RP3	259.33±43	80±18.02F	4.8±0.15ef	4.83±0.15cd	87.60±3.97abc
	RP4	304±70 cdef	324.66±15.58ab	7.73±2abcde	5.66±1.4bcd	80.42±6.09bcd
	RP5	280±90.5def	**396±1a**	6.5±0.7cdef	6±0.5bc	89.04±2.72ab
	RP6	204±34.1fg	325±27.9ab	5.4±0.52def	6.96±1.61ab	66.11±7.37bcd
	RP7	**461.66±32.18a**	271.33±14.73bc	8.33±0.76abcd	**8.33±0.55a**	88.45±6.79ab
	RP8	306.66±40.41cdef	246±7.76bcd	7.33±1.15bcdef	5.4±±0.36bcd	**110.65±10.16a**
	RP9	394.33±5.85abc	241±12.50bcd	9.9±3.51ab	4.9±0.96cd	57.62±11.23d
	RP10	**423.66±4.61ab**	174.33±15.50de	8.8±2.60abc	5.9±2.68bc	62.31±8.79cd
	RP11	139.33±19.8g	137±9.29ef	4.8±2.08ef	2.53±0.45f	78.25±17.31bcd
	RP12	380.66±29.3abcd	**380±16.52a**	7.2±1.32bcdef	4.8±2.42cd	90±3.25ab
	RP13	344.33± 16bcde	181±29.53cde	8.43±2.25abcd	3.76±0.25def	89.17±1.92ab

Table 4(Continue)- Effect of *Bacillus* PGPRs inoculation on stem and root length, fresh weight of shoot and root, photosynthetic pigments and relative water content of cowpea plants. Standard Error (SE) shows very high significant differences (P <0.05)

Accessions	*Strains code*	*Chla (mg/g FPM)*	*Chlb (mg/g FPM)*	*Total Chl (mg/g FPM)*	*Carotenoids (mg/g FPM)*
NEA10	RP0	0.206±0.69b	0.244±0.28de	0.795±0.03e	0.200±0.06cde
	RP1	0.456±0.19ab	0.24±0.1de	0.687±0.18e	**0.456±0.15a**
	RP2	0.477±0.22ab	0.498±0.42cde	0.777±0.12e	0.150±0.11de
	RP3	0.456±0.13ab	0.095±0.13e	0.655±0.25e	0.184±0.01cde
	RP4	0.654±0.45a	**1.11±0.26a**	1.474±0.52abc	0.107±0.09de
	RP5	0.368±0.05ab	0.225±0.07de	0.775±0.04e	0.240±0.04bcd
	RP6	**0.7±0.18a**	0.430±0.55de	0.796±0.14e	0.328±0.09abc
	RP7	0.616±0.39ab	0.165±0,13de	0.868±0.26de	0.371±0.20ab
	RP8	0.301±0.07ab	0.381±0.10cde	1.224±0.13bcd	0.240±0.02bcd
	RP9	0.309±0.1ab	0.526±0.07cde	1.309±0.18bc	0.192±0.04cde
	RP10	0.529±0.19ab	0.668±0.21bc	1.599±0.3ab	0.192±0.01cde
	RP11	0.323±0.21ab	0.983±0.22ab	**1.758±0.05a**	0.061±0.04e
	RP12	0.498±0.14ab	0.423+0.01cde	1.262±0.09bc	0.253±0.02bcd
	RP13	0.563±0.05ab	0.550±0.10cd	1.200±0.34cd	0.121±0.13de
NEA13	RP0	0.206±0.05ab	0.264±0.07bc	0.264±0.07e	0.049±0.04b
	RP1	0.456±0.15ab	0.667±0.18bc	0.667±0.29cde	0.203±0.04b
	RP2	0.456±0.25ab	0.565±0.64bc	0.565±0.30cde	0.180±0.04b
	RP3	0.477±0.01ab	0.663bc	0.663±0.02cde	0.134±0.06b
	RP4	0.654±0.12a	**1.862±0.36a**	**1.862a**	0.330±0.04b
	RP5	0.368±0.03ab	0.570±0.07bc	0.570±0.15cde	0.143±0.34b
	RP6	**0.700±0.03a**	0.351±0.54bc	0.352±0.73e	0.228±0.04b
	RP7	0.616±0.01ab	1.094±0.03abc	1.094±0.61b	0.325±0.11&b
	RP8	0.301±0.25ab	0.66±0.03bc	0.666±0.14cde	0.157±0.09b
	RP9	0.309ab	0.528±1.01bc	0.528±0.12de	0,.133b
	RP10	0.529±0.56ab	0.213±1.01c	0.213±0.57e	**0.782±0.96b**
	RP11	0.323±0.06ab	1.014±0.03abc	1.014±0.21bcd	0.166±0.01b
	RP12	0.498±0.62ab	0.326±0.03bc	0.326±0.01e	0.156±0.23b
	RP13	0.563±0.18ab	**1.232±0.69ab**	**1.232±0.03b**	0.307± 0.12b

SL: stem length; **RL:** root length; **FWSP:** fresh weight of the shoots part; **FWRP:** fresh weight of the root part; **RWC:** relative water content; **Chl:** chlorophyll; **FPM**: fresh plant material



(a) **(b)**

Figure 2 - Principal Component Analysis (PCA) (a): Correlation circle between variables, (b): projection of PCA results on a factorial plane (F1 -F2) for the inoculation effect of PGPR-*Bacillus* strains on cowpea ecotype NEA10 growth. The first factor axis appears to oppose the strains with greatest positive effect on chlorophyll a, chlorophyll b, and chlorophyll a+b, compared to RP 4 strain. Strain RP 7 has the highest carotenoid content and appears to characterize the second factor axis. The individual projection on the factorial plane defined by axes 1 and 2 showed a fairly large distribution along the plane (Axis 1 accounted for 30.28% of the variation and was associated the characteristics on the positive side: RWC, chlorophyll a, chlorophyll b, Chlorophyll a+b and carotenoids and FWAP, FWRP, RL, SL which indicates positive correlation between the parameters of the plant, that is to say that they evolve together, with a more or less common effect).

Bacillus-PGPR strains had a positive effect on cowpea growth, which is reflected by a significant increase in biometric parameters of plant growth (Table 4). The fresh weights of shoots part (FWSP) and root part (FWRP) appear to be greater than the control, concerning strains RP 1 to RP 7 for ecotype NEA13 and strains RP 4 to RP 13 for ecotype NEA10. In the inoculated NEA10 accession (Appendix 1), fresh root weight almost tripled with the RP1 strain (327 µg) compared to the RP0 control (112 µg) and fresh weight of the aerial portion was more than double the RP0 control (209 µg) with the RP6 strain (470 µg). On the other hand, in the inoculated NEA13 accession (Appendix 2), the RP10 and RP7 strains had most positive effect on the fresh weight of the aerial part, which varied between 423 µg and 461 µg respectively, compared to the RP0 control (255 µg). Furthermore, in this same accession, the RP5 and RP12 strains were able to improve the root weight to 396 µg and 380 µg, respectively, compared to the RP0 control (237 µg).

Measurements of the lengths of the roots and stems were made both with and without inoculation. According to Table 4, the maximum stem size for inoculated seedlings was 10 cm, while the control stem sizes for NEA13 and NEA10 were 8 cm and 10 cm, respectively. Concerning the results obtained for the root length t, it is noted that the plants inoculated with most of the strains were smaller compared to control.

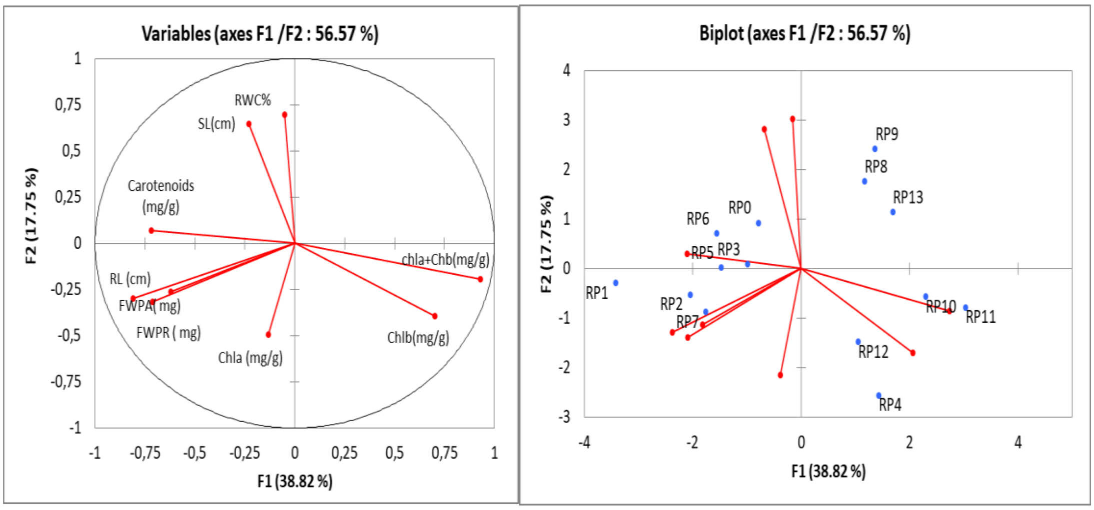
In most instances, it does not appear that inoculated plants had higher relative water content (Table 4), while the RP13 (67.84 %) and RP 8 (110.65%) strains in NEA10 and NEA13, respectively, are more interesting. The carotenoid content was higher in inoculated plants in most cases, more so with RP 5, 6, 7, 8 and RP 12 strains in NEA13 and all strains in NEA10. The total chlorophyll content was higher in plants that were inoculated with all strains, while higher values were found with strains RP11 (1.758 mg/g fresh plant material) and RP4 (1.862 mg/g fresh plant material) in accessions NEA10 and NEA13, respectively.

PCA was used to present most of the variance with a small number of principal components, represented in our case by the biometric and physiological parameters of the plant and by the strains of *Bacillus*-PGPR tested. Also, the correlation matrix (Appendix 3) shows the correlation coefficients between several variables related to plant parameters.

PCA analysis for NEA10 accession (Fig. 2) showed that Axis 1 had the following strains on the negative side: RP 6 and RP 1, RP 5, RP 2, RP 12, RP 9 and RP 10, which indicates that their effect is different on the plant. On the positive side of axis 1, the strains RP 4and RP 13 were present, this indicates that they have approximatively the same effect on the plant. Axis 2 was defined on the positive side by the strains RP 7 and RP 10. On the other hand, the negative part of the axis was defined by the strains RP 11, RP 3, and RP 2.

Concerning the effect of inoculation on NE14 accession, the correlation circle for the plane formed by the first two factorial axes is shown in Fig. 3. All the variables are well represented in this factorial plane since their correlations with the axes are relatively significant (the projections are close to the correlation circle). The first two axes alone of the PCA explain nearly 56.7%

of the variability; they were retained. This means that there is less common effect of inoculation on the plant parameters studied.



(a) **(b)**

Figure 3- Principal Component Analysis (PCA): a) Correlation circle between variables, b) projection of PCA results on a factorial plane (F1 -F2) for the inoculation effect of PGPR-*Bacillus* strains on cowpea accession NEA13 growth. The first factor axis appears to oppose strains with high stem length and water content and very low chlorophyll content, compared to RP 11 strains. The second factor axis is characterized by strains RP 11 and RP 1 with a high content of chlorophyll a, chlorophyll a +b, carotenoides and fresh weight (FWAP, FWRR), respectively. The projection of individuals on the factorial plane defined by axes 1 and 2 showed a fairly large distribution along the plane (Axis 1 isolated the following strains on the negative side: RP 12 and RP 4. On the positive side, the strains RP 13, RP 8, RP 9 and the control were isolated. Axis 2 is defined on the positive side by the RP 11 and RP 10 strains. On the other hand, the strains RP 7, RP 3, RP 1, RP 2, RP 5 and RP 6 defined the negative part of the axis).

Cowpea (*Vigna unguiculata*) is one of the most important food crops of the Fabaceae family in arid regions. The importance of this plant is linked to its seeds, which are edible and rich in protein (Aida et al. 2021). Therefore, the selection of microbial strains with beneficial activities to promote cowpea production is a great need, especially in arid regions. In our study, cowpea crop yield improvement by inoculation of *Bacillus*-PGPR strains was observed in pot experiments, which is in agreement with many studies of the species *B. megaterium*, *B. circulans, B. cereus*, *B. subtilis*, *B. amyloliquefaciens*, and *Paenibacillus polymyxa*. Thus, the use of *Bacillus* strains capable of maintaining and developing the root system has significant beneficial effects on inoculated plants, especially halophytes. In fact, PCA clearly demonstrated that the effect of inoculation was significant for all test parameters of the plant, with similar actions recorded between certain strains and specific parameters. The promotion of cowpea growth by *Bacillus*-PGPR led to a significant increase in morphological parameters such as stem size and weight and root, which were likely due to improved plant nutrition. The highest chlorophyll content was recorded with RP 4 (*B. megaterium*) and RP 11 (*B. amylo-liquifaciens)* treatment, which may be due to increased photosynthesis and nutrient intake. Some studies reported the use of PGPR-*Bacillus* as promoters of water use efficiency in *Vicia faba* (Li et al. 2016), maize and bean (Lima et al. 2019). The genus *Bacillus* is very widespread in the rhizosphere, many of them were listed as PGPR. Indeed, *Bacillus* species used as biofertilizers probably have a direct effect on plant nutrition, growth and health. In recent years, research about halophilic and halotolerant bacteria of the genus *Bacillus* has exploded in different ecological niches and several new species have been discovered, especially those with PGP effects on *Zea mays* (Mukhtar et al. 2020), *Chenopodium quinoa (*Mahdi et al. 2020) and *Triticum aestivum* (Hussain et al. 2020).

4. Conclusions

The isolation and characterization of halotolerant *Bacillus* strains has increased knowledge of rhizobacteria associated with the date palm in saline and arid soils. In general, 8 halotolerant and 1 moderately halophilic strain of the genus *Bacillus* were isolated from arid soil in the rhizosphere of the date palm. It would be interesting to study the biological properties of these microorganisms to understand how they adapt to salinity in the first place, as well as to exploit their potential applications in the second place. The results of PGP and enzymatic abilities are very interesting and show that most strains have at least two PGP effects and three hydrolytic activities even under saline conditions. Furthermore, the resistance to the physico-chemical parameters of the hyper-arid Algerian ecosystem of In Salah among the isolates is the first step to select effective PGPR capable of improving plant growth under extreme climatic conditions.

Therefore, our second investigation focused on the inoculation effects of the isolated halotolerant bacteria on cowpea plant growth. Based on the results of our study, strains RP 7 (*B. coagulans*), RP 8 (*B. circulans*), RP 10 (*Paenibacillus polymyxa*), RP 12 (*B. circulans*) and RP 13 (*B. cereus*) were identified to be highly effective strains in terms of improving cowpea plant growth and development. Moreover, the isolated bacteria are likely to offer new opportunities for biotechnological applications in agro-ecological systems, especially in arid areas known for their saline soils. However, further research should be conducted in field trials in several arid and semi-arid locations and on several crops to provide clear evidence of their usefulness. In future studies, it would be interesting to perform molecular analysis and study the effect of our *Bacillus*-PGPR strains as bioinoculants for plants grown in saline soil.

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