MEDITERRANEAN AGRICULTURAL SCIENCES

Volume: 37
Number: 3
Year: December 2024

E-ISSN: 2528-9675

e-ISSN 2528-9675

MEDITERRANEAN AGRICULTURAL SCIENCES

Previous Name: Akdeniz University Journal of the Faculty of Agriculture

The peer reviewed scientific journal of Akdeniz University Faculty of Agriculture

Three issues are published per year in April, August and December

Abbreviation of the journal: Mediterr Agric Sci

Owner on behalf of Akdeniz University, Faculty of Agriculture

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Web site: www.dergipark.org.tr/en/pub/mediterranean

Publisher

Akdeniz University, Faculty of Agriculture
07058, Antalya, Türkiye

Phone: +90 242 310 2412
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Subscription

Open access journal

Online access free of charge
www.dergipark.org.tr/en/pub/mediterranean

Cover design
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AIM AND SCOPE

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ABSTRACTS AND INDEXING

MEDITERRANEAN AGRICULTURAL SCIENCES is indexed and abstracted in **CABI** data bases (**CAB Direct**), **TUBITAK-ULAKBIM** (National Data Bases-Data Base of Life Sciences), **CLARIVATE, WEB OF SCIENCE, MASTER JOURNAL LIST** (Zoological Records) and **DRJI** (Directory of Research Journals Indexing).

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e-ISSN 2528-9675

www.dergipark.org.tr/en/pub/mediterranean

MEDITERRANEAN AGRICULTURAL SCIENCES

The journal was published under the name "Akdeniz University Journal of the Faculty of Agriculture" and with the number ISSN 1301-2215 until 2016.

Volume: **37** Number: **3** Year: December **2024**

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e-ISSN 2528-9675

MEDITERRANEAN AGRICULTURAL SCIENCES

Volume: **37** Number: **3** Year: December **2024**

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 111-114
DOI: 10.29136/mediterranean.1547996

Investigation effect of pulsed magnetic fields on pepper (*Capsicum annuum* L.) plant growth

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ARTICLE INFO

Received: September 10, 2024
Received in revised form: October 2, 2024
Accepted: October 10, 2024

***Keywords*:**

Pepper (*Capsicum annuum* L.)
Magnetic field
Plant growth

ABSTRACT

Pepper (*Capsicum annuum* L.) plants are extensively utilized in culinary and spice industries, rendering their cultivation pivotal in agricultural production. Enhancing their growth and yield is a critical research area for producers and horticulturists. Recent investigations have delved into the use of pulsed magnetic fields (PMFs) as a potential growth stimulant. Unlike static magnetic fields, PMFs are characterized by transient, high-intensity magnetic bursts, potentially eliciting varied responses in plants. To assess PMFs' impact on pepper plants, several experiments were setup comprising two solenoids, each wound around an 18-cm-diameter rigid plastic pipe but with differing coil turns, one with 40 and the other with 80 turns. These solenoids were utilized to generate PMFs at a frequency of 1 kHz with two intensities: 17 micro-Tesla (µT) and 34 µT. The pepper plants were situated within the PMF zone under controlled conditions, ensuring consistency in light, temperature, and moisture levels. The experimental design included three plant groups: a control group with no PMFs exposure except that of the Earth's magnetic field, and two groups subjected to 17 µT and 34 µT PMFs intensities with Earth's magnetic field, ranging between 25-65 µT. The treatment spanned 15 days, involving 6 hours of daily continuous exposure. Key growth indicators such as plant height, stem diameter, leaf area, and fresh and dry weights of both shoot and root systems were measured and analyzed. This analysis revealed significant increases in plant height, leaf area, and fresh and dry weights of the shoot, but not in root systems. Further research is warranted to deepen the understanding of PMFs' effects on pepper plants.

1. Introduction

Magnetic fields (MFs), a ubiquitous environmental factor on the Earth, exert a profound influence on the biological processes of plants. Contemporary research has increasingly focused on how plants perceive and respond to these fields. Plants exhibit rapid responses to variations in MFs, modulating their metabolic pathways, altering gene expression profiles, and consequently affecting phenotypic outcomes (Maffei 2014). The interaction of MFs with plant cells, especially their membrane structures, has garnered significant interest. This interaction is thought to facilitate enhanced absorption of water and nutrients, a hypothesis supported by studies on plant development and magnetic field interplay. Moreover, the role of paramagnetic biological molecules, such as hemoglobin, cytochrome, and ferritin, containing metal ions, is critical in understanding the influence of MFs on plant physiology (Hozayn and Qados 2010). These molecules may act as mediators in the MF-induced responses observed in plants. MFs also have far-reaching implications for molecular and cellular processes, including mRNA regulation, protein biosynthesis, and enzyme activities (Atak et al. 2003). Such changes cascade through various organ and tissue functions, implying a comprehensive impact on plant development. Despite these insights, the research field is fraught with inconsistencies and lacks a unified theoretical framework (Harris et al. 2009), leading to a spectrum of often contradictory findings regarding the effects of MFs on plant organisms. For example, improved membrane integrity and enhanced germination rates of wheat seeds have been found under static magnetic fields (SMF) (Payez et al. 2013). Conversely, rice (Oryza sativa) seeds exposed to various MF inductions exhibit significantly varying germination times due to a complex and species-specific response to MF exposure (Florez et al. 2004). Further studies indicate that the application of MFs influences seed membrane properties, leading to differential germination outcomes (Hussain et al. 2020). The variability in responses underscores the species-specific nature of MF effects and the importance of considering the type of magnetic field and its interaction with specific metabolic or molecular structures in plants. Recent scientific inquiries, notably those by Himoud et al. (2022) and Tirono and Hananto (2023), have highlighted the advantageous impact of pulsed magnetic fields (PMFs) in agriculture. These studies, extending across cellular and organismal levels, reveal that PMF significantly influences

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various cellular biochemical processes in plants, including alterations in membrane electro-potential, modulation of protein and enzyme activities, and enhancement in photosynthesis and pigment content. The effect of PMFs is also seen in accelerating cell division rates (Radhakrishnan 2019), moving charged particles across cell membranes (Nyakane et al. 2019), and facilitating an expedited uptake of water and nutrients (Nyakane et al. 2019). In general, recent findings show that PMF is an environmentally sustainable agricultural technique, with broader scientific consensus on its importance for sanitary applications in plants (Nair et al. 2018; Radhakrishnan 2019; Himoud et al. 2022; Tirono and Hananto 2023). For the production of resilient crops in varying climatic conditions, magnetic field applications are gaining attention for sanitizing crops from biotic pathogens. Maffei (2014) emphasizes the necessity of incorporating data on the Earth's static magnetic fields, a dimension currently underrepresented in the literature. Additionally, Đukić et al. (2017) and Bajagić et al. (2021) advocate for experimental research conducted in open fields under diverse climatic conditions to understand the effects of MFs more comprehensively. Considering these findings, the current research is directed towards investigating the effects of low frequency pulsed magnetic field stimulation on pepper plant growth and development. Although the research mentioned previously do not fully reveal that the Earth's magnetic field ranges between 25-65 µT, it is important to understand better how the Earth's MFs works on plant development. This study aims to assess the influence of two different PMFs in addition to the Earth's MF on pepper plants' height, leaf area, fresh and dry weights of shoots and roots. Given the limited knowledge on the effects of PMFs on plants, this study provides empirical insights into the dynamics of plant growth under magnetic stimuli. Our results contribute to filling a gap in understanding the physiological response of pepper plants to PMF influences.

2. Materials and Methods

2.1. Magnetic field application

In this study, we developed a novel system to generate pulsed magnetic fields (PMFs) using two custom-built coils, each assembled around rigid plastic pipes with an 18 cm diameter. The coils differed in their configurations: one had 40 turns, and the other had 80 turns of copper wire, with each wire having a radius of 1.30 mm. As electromagnets, the magnetic field strength generated by these coils was directly proportional to the number of wire turns and the electric current passing through them. The coils' dimensions were chosen to ensure compatibility with the plant pots used in the experiment.

To measure the magnetic field strength, a high-precision HIOKI 3470 Magnetic Field HiTester gaussmeter was positioned at the center of each coil. This measurement process was consistently applied in both the control and experimental areas to establish a reliable baseline for comparison. In order to create two distinct PMF environments, AA Tech AWG-100 signal generators were configured to emit a stable peak-to-peak voltage of 6 volts at a frequency of 1 kHz. Figure 1 provides a visual overview of the experimental setup.

In this configuration, the coils with 40 and 80 turns generated magnetic fields of 17 µT and 34 µT, respectively. Three pots, each containing a pepper plant of identical age and size, were placed at the center of each coil, while the control group was kept without magnetic field exposure. The magnetic fields were applied between 8 am to 2 pm for six hours daily over a 15-day period to assess their impact on the growth and development of the pepper plants. The reasons to select these 17 µT and 34 µT PMFs include (1) closer exposure to Earth's MF, (2) cost efficiency of systems, (3) applicability of these PMFs in practical use in greenhouses and fields and (4) suggestions of preliminary studies with using lower and higher PMFs then the PMFs values used in this report (Unpublished data).

2.2. Plant material

Seeds of commercial VAT 59 F1 pepper (Anamas Seed Company, Türkiye) were surface sterilized in a solution containing sodium hypochlorite (>2.5% active chlorine) and a non-toxic wetting agent, Tween 80 (approximately 0.004%), for 5 minutes. Subsequently, the seeds were rinsed three times with distilled water and left to imbibe overnight in distilled water before being sown in standard seed vials filled with sterilized turf (Kekkila®, China). The germinated seedlings were maintained in a growth chamber set at a temperature of 27±3 ºC, with a relative humidity of 40-50%, and under a 16-hour day/8-hour night light regime providing 230 µmol with 230 µmol m^{-2} sec^{-1} of photosynthetically active radiation. Once the pepper seedlings reached the first real leaf stage, three seedlings were transplanted

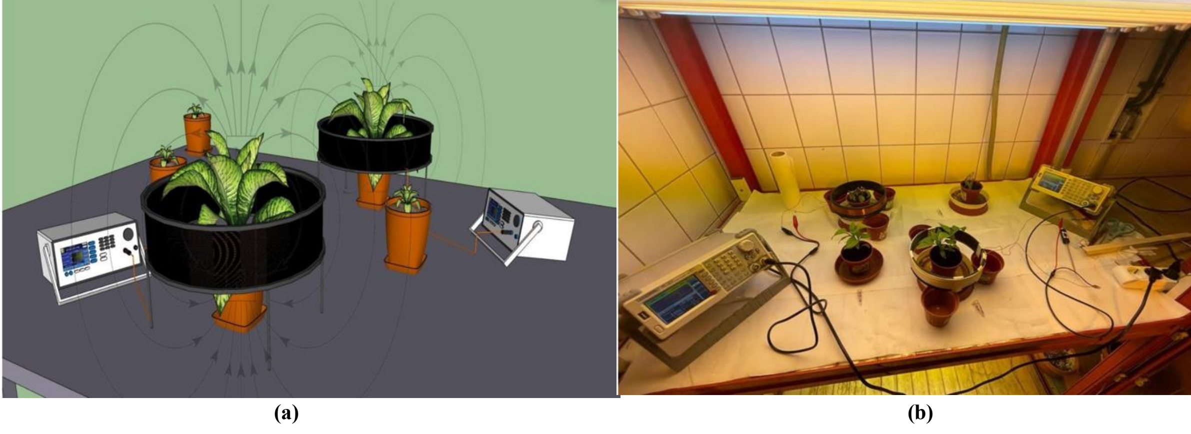

Figure 1. (a) Schematic and (b) actual view of the original experiment.

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from the vials to 0.5 L plastic pots containing sterilized turf (Kekkila®, China). Each pot, housing three seedlings, was positioned in the center of the magnetic fields, with control plants maintained separately. The seedlings, both within the magnetic fields and the control group, were subjected to the previously described growth conditions, including 6 hours of daily magnetic field exposure. Daily irrigation of the pepper seedlings in individual pots was conducted with 50 ml of a nutrient solution containing Peters Professional® ensuring continuous feeding for 15 days until the experiment's conclusion. At the end of the trial, plants were carefully removed from the pots, and their roots were gently rinsed with tap water. Subsequent measurements were conducted to assess the differences among treatments. These included plant length (root and shoot), root length, leaf count, stem diameter, leaf petiole diameter, and the fresh and dry weights of roots, stems, and leaves.

3. Results and Discussion

This study revealed that the two solenoids produced PMFs of 17 and 34 µT, with the direction of the magnetic field alternating due to the applied voltage. In this specialized environment, pepper plants were subjected to various growth measurements including plant length (root and shoot), root length, number of leaves, stem diameter, leaf petiole diameter, and fresh and dry weights of roots, stems, and leaves. The statistical differences among these parameters are presented in Table 1 and Table 2. Although no significant differences were observed in the leaf petiole diameter, and fresh and dry weights of roots, a positive effect of PMFs on the aerial parts of the plants was noted. Despite the alternating direction of the magnetic fields, no side effects were detected. The influence of PMFs on the pepper seedlings showed a favorable interaction with the green parts of the plants. Throughout the experiment, the pepper plants were exposed to PMFs for six hours daily. This rigorous approach allowed for detailed observations and measurements of the plants' growth and development. Comprehensive analysis of various growth parameters, such as root and shoot lengths, leaf count, and stem diameter, revealed significant variations in the green parts of the pepper plants. Notably, marked differences in the number of leaves and both fresh and dry weights of the shoots were observed (Table 1 and Table 2). These PMFs results show that 17 µT had a better affect than 34 µT on leaf petiole diameter, stem fresh weights and stem dry weights.

These findings suggest a pronounced influence of PMFs on the above-ground parts of the plants. In contrast, parameters associated with the root system showed no significant changes. A key observation from our study is that the alternating directions of the magnetic fields had no adverse effects on the plants, suggesting the potential applicability of PMFs, particularly at 34 µT, in agricultural settings. This result matches with the Earth's magnetic field, which typically ranges between 25-65 µT (Martino 2010). Therefore, further studies could explore the effects of PMFs above 25 µT within the Earth's magnetic field range, potentially saving both time and resources for researchers. An intriguing aspect of our findings is the observed increase in plant height due to PMF exposure. However, the mechanisms by which magnetic fields contribute to this growth remain undetermined, as magnetic fields affect various aspects of plant physio-morphological parameters, including enzymes involved in stress physiology and plant metabolism (Radhakrishnan 2019). The application of magnetic fields has been shown to accelerate seed germination and both vegetative and reproductive growth in plants, possibly due to increased energy distribution to biomolecules within the cell (Radhakrishnan 2019). However, detailed molecular or cellular studies are required to elucidate these mechanisms. The absence of any detrimental effects in response to the changing directions of the magnetic fields is noteworthy. This opens possibilities for exploring magnetic fields in enhancing plant resilience against environmental stresses and modulating plant growth patterns for optimized yield. Our study indicates a correlation between PMFs exposure and increased plant height, suggesting promising prospects for further research. Future studies, incorporating advanced molecular and genetic analyses, could provide insights into how magnetic fields influence plant developmental biology. Such research may unveil new aspects of plant growth regulation, leading to innovative strategies in crop management and sustainable agriculture. In summary, this study not only highlights the immediate effects of PMFs on pepper plants but also paves the way for a new paradigm in agricultural science. By integrating concepts from physics, biology, and agronomy, we can develop methods to enhance plant growth and productivity, moving towards more sustainable and efficient agricultural systems.

In conclusion, it is hypothesized that magnetic fields influence many aspects of plant biology, including cell membrane systems, by altering ion transport, affecting cell division and elongation, and modifying hormonal balances. This research lays the groundwork for understanding the effects of PMFs exposure on the cultivation and management of pepper plants, with profound implications for future agricultural practices.

Table 1. Plant tissue measurements after 15 days of magnetic field application on pepper seedlings.

Treatments	Stem Diameter (mm)	Leaf Petiole Dimeter (mm)	Root + Shoot Length (mm)	Number of leaves
Control	2.23 *b	0.89 a	150.7 b	7.08 b
17 µT	2.49 ab	0.88 a	185.4 ab	7.83 ab
34 µT	2.68 a	0.78 b	210.6 a	8.40 a

*Means with a different letter in each column indicate significant differences according to the Duncan comparison test.

Table 2. Plant tissue weights after 15 days of magnetic field application on pepper seedlings.

Treatments	Root Fresh Weights (mg)	Root Dry Weights (mg)	Stem Fresh Weights (mg)	Stem Dry Weights (mg)	Leaf Fresh Weights (mg)	Leaf Dry Weights (mg)
Control	3470 *b	461.51 b	242.5 b	36.38 c	210.27 b	27.97 b
17 µT	4550 ab	605.15 ab	487.5 a	73.13 a	232.55 ab	31.39 a
34 µT	4870 a	647.71 a	345.0 ab	51.75 b	249.48 a	33.68 a

*Means with a different letter in each column indicate significant differences according to the Duncan comparison test.

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Acknowledgement

Authors would like to thank Prof. Dr. Şükrü ÖZEN (Engineering Faculty, Akdeniz University, Türkiye) for providing experimental tools.

References

Atak C, Emiroglu O, Alikamanoglu S, Rzakoulieva A (2003) Stimulation of regeneration by magnetic field in soybean (Glycine max L. Merrill) tissue cultures. Journal of Cell Molecular Biology 2:113-119.

Bajagić M, Đukić V, Cvijanović V, Nedeljković M, Dozet G, Stepić V, Cvijanović G (2021) Effect of low-frequency electromagnetic field treatment of seeds on soybean productivity. Journal of Agricultural Sciences 66: 321-334.

Đukić V, Miladinov Z, Dozet G, Cvijanović M, Tatić M, Miladinović J, Balešević-Tubić S (2017) Pulsed electromagnetic field – a cultivation practice used to increase soybean seed germination and yield. Žemdirbyste Agriculture 104: 345-352.

Florez M, Carbonell MV, Martinez E (2004) Early sprouting and first stages of growth of rice seeds exposed to a magnetic field. Electromagnetic Biology and Medicine 23: 157-166.

Harris SR, Henbest KB, Maeda K, Pannell JR, Timmel CR, Hore PJ (2009) Effect of magnetic fields on cryptochrome-dependent responses in Arabidopsis thaliana. Journal of the Royal Society Interface 6: 1193-1205.

Himoud MS, Lazim SK, Al-Bahadliy AH (2022) Effect of tillage depths and static magnetic seed treatment on growth parameters and yield of maize (Zea mays L.). Indian Journal of Ecology 49: 18-23.

Hozayn MA, Qados MSA (2010) Magnetic water application for improving wheat (Triticum aestivum L.) crop production". Agriculture and Biology Journal of North America 4(1): 677-682.

Hussain MS, Dastgeer G, Afzal AM, Hussain S, Kanwar RR (2020) Eco-friendly magnetic field treatment to enhance wheat yield and seed germination growth. Environmental Nanotechnology, Monitoring Management14: 100299.

Maffei ME (2014) Magnetic field effects on plant growth, development, and evolution. Frontiers in Plant Science5: 445-460.

Martino CF, Portelli L, McCabe K, Hernandez M, Barnes F (2010) Reduction of the Earth's magnetic field inhibits growth rates of model cancer cell lines. Bioelectromagnetics 31(8) 649-655.

Nair RM, Leelapriya T, Dhilip KS, Boddepalli VN, Ledesma DR (2018) Beneficial effects of extremely low frequency (ELF) sinusoidal magnetic field (SMF) exposure on mineral and protein content of mungbean seeds and sprouts. Indian Journal of Agricultural Research 52: 126-132.

Nyakane NE, Markus ED, Sedibe MM (2019) The effects of magnetic fields on plants growth: a comprehensive review. ETP International Journal of Food Engineering 5: 79-87.

Payez A, Ghanati F, Behmanesh M, Abdolmaleki P, Hajnorouzi A, Rajabbeigi E (2013) Increase of seed germination, growth and membrane integrity of wheat seedlings by exposure to static and 10-KHz electromagnetic field. Electromagnetic Biology and Medicine 32: 4 17-429.

Radhakrishnan R (2019) Magnetic field regulates plant functions, growth and enhances tolerance against environmental stresses. Physiology and Molecular Biology of Plants 25: 1107-1119.

Tirono M, Hananto FS (2023) Effective treatment time using a magnetic field to increase soybean (Glycine max) productivity. Jurnal Penelitian Pendidikan IPA 9: 5071-5077.

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Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 115-120
DOI: 10.29136/mediterranean.1530967

Effects of different surface sterilization protocols on fungal load and germination of black henbane seeds

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ARTICLE INFO

Received: August 15, 2024
Received in revised form: October 3, 2024
Accepted: October 10, 2024

***Keywords*:**

Hyacyamus niger
Plant tissue culture
Seed-borne fungi
Sterilization method

ABSTRACT

Black henbane (*Hyoscyamus niger* L.) is an important medicinal plant rich in tropane alkaloids with significant pharmacological effects. To extract these valuable metabolites, seeds are mostly used as the initial material in tissue culture techniques. Due to the intricate surface structure of the seeds, coupled with cultivation of the plant under unsuitable ecological conditions, a high risk of contamination during *in vitro* culture arises. As such, it is important to determine the most suitable sterilization method for successful germination of black henbane seeds under *in vitro* conditions. In this study, the effects of 10 different sterilization protocols with ethyl alcohol (EtOH), sodium hypochlorite (NaOCl), copper sulfate ($CuSO_4$), hydrogen peroxide (H_2O_2), mercury chloride ($HgCl_2$), and silver nitrate ($AgNO_3$), on reduction of the fungal contamination of black henbane seeds was determined by the agar test method. Additionally, the germination rates, as well as shoot lengths and fresh plant weights of the germinated seedlings, were investigated. As a result, it was found that *Alternaria*, *Fusarium*, and *Penicillium* species were the most common fungi on black henbane seeds. Among the chemicals used in the sterilization protocols, $AgNO_3$ was found as the most effective one, completely inhibiting fungal growth. Sterilization protocols with $AgNO_3$ also yielded the highest germination rates.

1. Introduction

Plant tissue culture is a technique where new tissues, plants, or plant products can be regenerated from plant cells, tissues, or organs, under aseptic and controlled conditions using artificial nutrient media. In cases where aseptic conditions are not fully ensured in tissue culture environments, microorganisms are able to proliferate and cause contamination of the nutrient media. Microbial contaminations typically occur due to insufficient sterilization of the working environment and equipment or contamination from the explant, posing a significant problem that threatens the development and sustainability of *in vitro* cultures. These microorganisms are competing with *in vitro* cultures by using and rapidly consuming the nutrient medium, causing insufficient growth, tissue necrosis, decreased shoot proliferation and root growth, and ultimately death (Kane 2000; Oyebanji et al. 2009). In addition to the sterilization of the culture medium and equipment, especially surface sterilization of the plant tissues has great importance in preventing the contamination problem that causes significant damage in plant tissue culture (Misra and Misra 2012). Surface sterilization is defined as the process of completely eliminating the microorganisms from the explant without damaging the explant tissue (Sen et al. 2013). For a successful plant tissue culture system, an effective sterilization method must be selected (Oyebanji et al. 2009). Explant-based infections vary according to the vegetation period, environmental conditions stress factors and the plant organ of the explant. Plant tissues and organs, in contact with the soil, generally pose a higher contamination risk (Battal et al. 2019). Seeds are the plant materials, most frequently used as the initial material for plant tissue culture studies. However, seeds collected from open fields are likely to be highly contaminated with exogenous and endogenous microbial contaminants, including fungi and bacteria. Therefore, seeds, along with tissues and organs in contact with soil are carrying higher risk of contamination than other above-ground explants (Wahyono et al. 2018). The presence of irregularities and protrusions on the seed surface particularly make the surface sterilization of seeds even more challenging (Barampuram et al. 2014).

Fungi are the most important group of seed contaminating microorganisms (Kesho and Abebe 2020). They can contaminate seeds both in the field before harvest and under storage conditions afterward. The most common fungi that contaminate seeds before harvest are; *Alternaria*, *Cladosporium*, and *Fusarium* species, while those found on seeds kept under storage conditions are *Aspergillus*, *Penicillium*, and *Rhizopus* species (Amza 2018). Fungi growing on seeds not only reduce or eliminate the germination capacity of the seeds but also change some physiological and biochemical properties of the seeds (Rao et al. 2014). Elimination of the fungal contamination, which is initially encountered and causes significant losses of plant material, constitutes one of the most crucial stages of an effective tissue culture study. Ethyl alcohol (EtOH), sodium hypochlorite (NaOCl), mercury chloride ($HgCl_2$), hydrogen peroxide (H_2O_2),

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silver nitrate ($AgNO_3$) and nano silver are among the most preferred chemicals in the surface sterilization process (Abdi et al. 2008). The morphological characteristics and growing conditions of the explant source, type of the explant, and duration and concentration of the chemical used for sterilization are critical factors for successful surface sterilization. Furthermore, the chemical used for sterilization must be economical, have a broad spectrum activity, and be easily removed from the plant material and break down without leaving toxic residues that would prevent cell and tissue development of the explant (Tort 1997).

Nowadays, tissue culture studies are intensively carried out on medicinal plants both for *in vitro* propagation and obtaining valuable plant metabolites with pharmacological effects, and seeds are generally used as initial material. Black henbane (*Hyoscyamus niger* L.), rich in tropane alkaloids such as hyoscyamine and scopolamine with high pharmacological effects, is one of the most important medicinal plants. Although seeds are frequently used as initial materials in the *in vitro* production of the valuable tropane alkaloids contained in the plant, the intricate surface structure of its seeds and the cultivation of the plant under unsuitable ecological conditions without disease and pest control, pose a high risk of contamination during *in vitro* culture (Aljibouri et al. 2012; Ghorbanpour et al. 2013). Therefore, it is important to determine the most suitable sterilization method for the successful germination of black henbane seeds under *in vitro* conditions. In this study, the effects of different sterilization protocols, applied to black henbane seeds, on their fungal load and germination rates, as well as the shoot lengths and fresh plant weights of the germinated seedlings, were investigated.

2. Materials and Methods

2.1. Sterilization and culture of the seeds

Black henbane seeds obtained from Istanbul Zeytinburnu Municipality, Medicinal and Aromatic Plants Directorate were used as plant material. In order to break the dormancy of the black henbane seeds and increase their low germination rate under normal laboratory conditions, the seeds were soaked in 250 mg L^{-1} gibberellic acid (GA_3) solution for 48 hours, before being transfrred to a nutrient media (Ghorbanpour et al. 2013). Then the seeds were sterilized using ten different protocols containing EtOH, NaOCl, $CuSO_4$, H_2O_2, $HgCl_2$, and $AgNO_3$ given below:

1. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 20% NaOCl solution with 2-3 drops of Tween 20 for 20 minutes + washing thrice with sterile distilled water, each for 5 minutes,

2. Shaking in 10% NaOCl solution with 2-3 drops of Tween 20 for 15 minutes + washing thrice with sterile distilled water + shaking in 5% NaOCl solution with 2-3 drops of Tween 20 for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,

3. Shaking in 0.05% $CuSO_4$ solution for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,

4. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 0.05% $CuSO_4$ solution for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,

5. Washing with detergent + washing thrice with sterile distilled water + shaking in 0.2% $HgCl_2$ solution for 20 minutes + washing thrice with sterile distilled water, each for 5 minutes,

6. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 0.1% $HgCl_2$ solution with 2-3 drops of Tween 20 for 10 minutes + washing thrice with sterile distilled water, each for 5 minutes,

7. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 20% H_2O_2 (35% stock) solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

8. Shaking in 20% H_2O_2 (35% stock) solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

9. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 1% $AgNO_3$ solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

10. Shaking in 1% $AgNO_3$ solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes.

After surface sterilization, the seeds were transferred to Petri dishes containing 30 ml of MS (Murashige and Skoog 1962) nutrient medium supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar and incubated at 25°C in the dark (Aljibouri et al. 2012). The experiment was set up in 3 replicates, with 5 Petri dishes in each replicate and 10 seeds in each Petri dish.

2.2. Determination of the fungal contamination of the seeds

Black henbane seeds, subjected to 10 different sterilization protocols, were examined for fungal contamination 7 days after being transferred to the nutrient medium. To determine the natural fungal load of the seeds, 100 seeds were placed on the medium without any sterilization treatment, forming the control group. Fungi on the seeds were examined under a stereomicroscope and grouped according to their colony characteristics. Additionally, preparations were made using lactofuchsin and examined under a microscope (Zeiss Axiostar 1061-030). They were identified at the genus level based on morphological characteristics using relevant references (Ellis 1971; Samson et al. 1995; Watanabe 2002). Fungal contamination rates were also determined using the following formula.

Contamination rate (%)= (Number of infected seeds x 100) / Total number of seeds [1]

2.3. Determination of the germination rates of the seeds and seedling development

To determine the effects of different sterilization protocols on the germination rates of black henbane seeds, sterilized seeds were incubated on the medium at 25°C, in the dark for 15 days and their germination rates were determined using the following formula.

Germination rate (%)= (Number of germinated seeds x 100) / Total number of seeds [2]

After germination, seedlings were kept at the same temperature but under 16:8 hours light: dark conditions. After one month of growth following seed sowing, the shoot length of the harvested plants was determined using a ruler. The fresh weights of the plants were determined in grams after being weighed on an analytical balance (Kern PLJ 720-3A).

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2.4. Evaluation of the results

At the end of the experiment, all data were subjected to analysis of variance using the JMP 17 program, and means were compared by Tukey's multiple comparison test. Variance analysis was performed after applying arc sin transformation to percentage values.

3. Results and Discussion

3.1. Effect of surface sterilization protocols on fungal loads of the seeds

It was determined in the study that fungal growth started on the seeds within 2-3 days of being transferred to the nutrient medium following the sterilization protocols. Microscopic examinations after seven days of incubation revealed that the seeds were mostly contaminated with *Alternaria*, *Fusarium*, and *Penicillium* species. These three genera are among the most common seed-borne fungi (Amza 2018). Species belonging to the genera *Cladosporium*, *Mucor*, *Rhizopus*, *Stachybotrys*, *Stemphylium*, and *Trichoderma* were determined at lower rates on the seeds. It was determined that the contamination rate was quite high (86%) on the seeds incubated without sterilization and some seeds were contaminated with more than one fungus. *Penicillium* species were the most common fungi (51%) in the control group, while the contamination rates of other fungi were lower (Table 1). No information was found regarding seed-borne fungi in black henbane seeds in literature. Therefore, the fungi found on black henbane seeds in this study represent the first record in this regard. *Alternaria* species, which are among the most common seed-borne pathogens, have been reported to have negative effects on the physical properties of seeds in addition to causing diseases in plants (Rathod 2012). *Fusarium* species are also considered as important seed-borne plant pathogens, causing serious economic losses by causing diseases such as root rot and wilt on plants grown from contaminated seeds (Blanco and Aveling 2018). Although *Penicillium* species are common on seeds, they are considered as saprops causing no disease in developing plants (Kaygusuz and Coşkuntuna 2022). However, contamination of the seeds with these three groups of fungi is undesirable, not only for their negative effects on seed quality and viability but also for their ability to produce mycotoxins that pose a great health concern to humans and animals (Martin et al. 2022).

The sterilization process with $CuSO_4$ was not effective in reducing the contamination of *Penicillium* species on the seeds, while other chemicals used in the sterilization process completely prevented the growth of this fungus. Similarly, the use of $CuSO_4$ (protocol no 3) and EtOH and NaOCl (protocol no 1) were not sufficient to reduce the development of *Fusarium* species on the seeds, whereas other applications significantly suppressed the development of the fungus (Figure 1). *Alternaria* species were completely inhibited in applications containing $AgNO_3$ (Table 1). Considering the total fungal load on the seeds, the $AgNO_3$ application, which completely eliminated fungal contamination, was determined to be the most effective sterilization protocol, while the effects of EtOH + $AgNO_3$, EtOH + H_2O_2, and H_2O_2 applications were also statistically in the same group (Figure 2). In a similar study, where $AgNO_3$ yielded positive results, it was found that soaking plum (*Prunus domestica* L.) shoots in 1% $AgNO_3$ solution for 20 minutes resulted in 96.67% healthy and 3.3% contaminated buds (Ugur 2020). Nartop (2019), investigating the effects of silver nanoparticles on the sterilization efficiency and germination rates of seeds of different plant species, demonstrated that silver nanoparticles were more effective than NaOCl. Conflicting results were obtained with H_2O_2. Barampuram et al. (2014) reported that H_2O_2 usage in the surface sterilization stage of cotton (*Gossypium hirsutum* L.) seeds resulted in very low levels of fungal contamination. However, in another study, it was found that surface sterilization of peach (*Prunus persica* (L.) Batsch) shoots with H_2O_2 caused high contamination (Al Ghasheem et al. 2018).

It was observed in the study that the sterilization protocols performed with NaOCl, $CuSO_4$, and $HgCl_2$ were not sufficiently effective, although they slightly reduced the fungal contamination on the seeds by inhibiting the development of certain fungi such as *Penicillium* species. There are different findings regarding the effects of NaOCl application. Oyebanji et al. (2009) conducted a study with cowpea (*Vigna unguiculata* (L.) Walp.), rice (*Oryza sativa* L.), and sorghum (*Sorghum bicolor* (L.) Moench) seeds, where seeds were sterilized using three different methods involving various durations of EtOH, NaOCl, and their sequential use. As a result, they found that treating the seeds with 3.5% NaOCl for 20-45 minutes was the most effective method for preventing fungal contamination. In another study, NaOCl at 5% concentration for 5 minutes caused

Table 1. The effects of different surface sterilization protocols on the fungal contamination rates of black henbane seeds

Protocol number	Total contamination*	*Alternaria* spp.	*Fusarium* spp.	*Penicillium* spp.	Other fungi
1	40.00 b**	28.00 abcd	12.00 ab	0.00 b	0.00 c
2	56.00 ab	52.00 a	4.00 b	0.00 b	0.00 c
3	74.00 a	20.00 abcd	14.00 ab	38.00 a	12.00 b
4	30.00 b	28.00 ab	2.00 b	0.00 b	0.00 c
5	36.00 b	32.00 abc	4.00 b	0.00 b	0.00 c
6	26.00 bc	26.00 abc	0.00 b	0.00 b	0.00 c
7	6.00 d	6.00 bcd	0.00 b	0.00 b	0.00 c
8	6.00 cd	2.00 cd	4.00 b	0.00 b	0.00 c
9	2.00 d	0.00 d	2.00 b	0.00 b	0.00 c
10	0.00 d	0.00 d	0.00 b	0.00 b	0.00 c
Control	86.00 a	29.00 ab	37.00 a	51.00 a	37.00 a

*Statistical analysis was performed after applying arc sin transformation to percentage values, but actual values were given in the table. **Means on the same column shown with the same letter are not statistically different from each other according to Tukey's test ($P \leq 0.05$).

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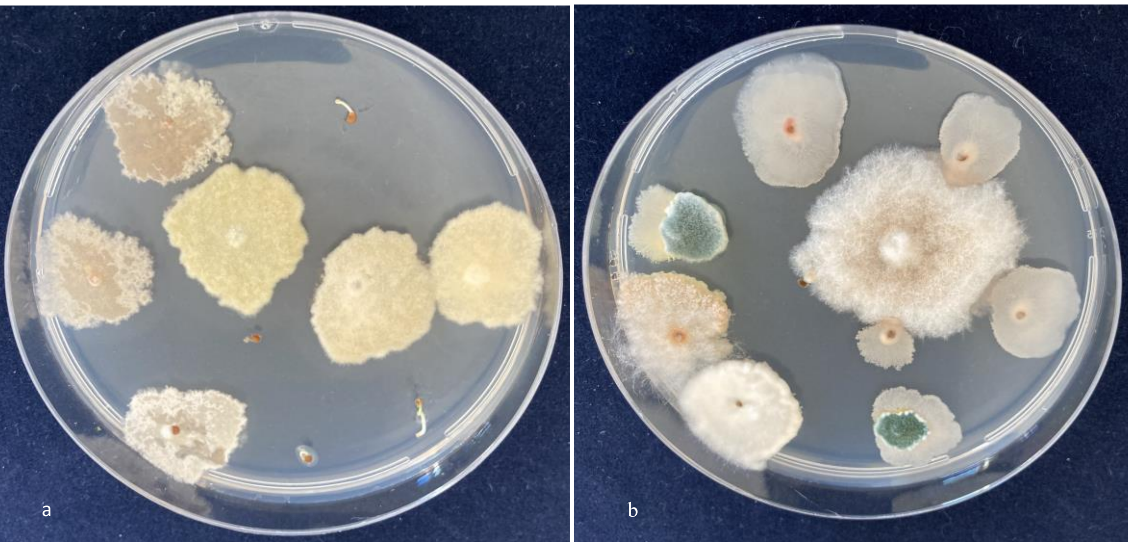

Figure 1. Fungal contamination on the henbane seeds surface sterilized with EtOH + 20% NaOCl (a) and 0.05% $CuSO_4$ (b).

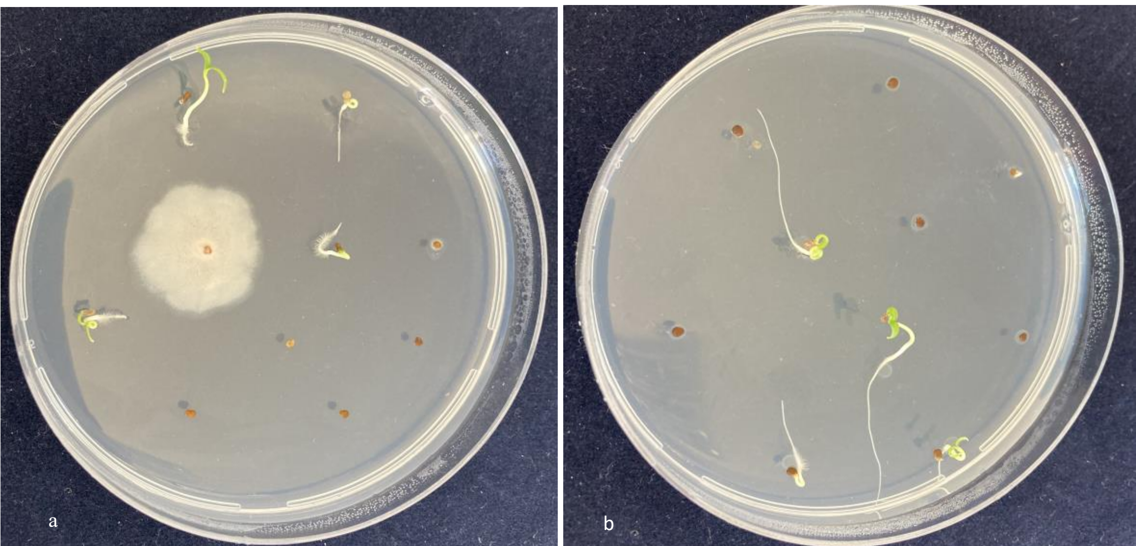

Figure 2. Effects of sterilization protocols with 20% H_2O_2 (a) and 1% $AgNO_3$ (b) on the fungal contamination of black henbane seeds.

100% fungal contamination after a 7-day incubation period and it was concluded that NaOCl was more effective against bacteria than fungi (Pinto et al. 2012). $CuSO_4$ has been used to eliminate microbial contamination on seeds since ancient times (Rai et al. 2018). It was found that $CuSO_4$ treatment of lettuce (*Lactuca sativa* L.) seeds significantly reduced bacterial leaf spot contamination without causing phytotoxicity (Carisse et al. 2000). A study using $HgCl_2$ in the sterilization of *Catharanthus roseus* (L.) G. Don seeds resulted in high contamination (Ramandi et al. 2019).

3.2. Effects of surface sterilization protocols on seed germination and seedling development

It was determined that the different sterilization protocols caused statistically significant differences in the germination rates of the black henbane seeds and the shoot lengths and fresh weights of the seedlings (Table 2). The intense contamination resulting from the applications of NaOCl, $CuSO_4$, and H_2O_2 used in surface sterilization also negatively affected the germination of seeds. Treating plant seeds with NaOCl can affect the germination process by causing changes in seed metabolism. NaOCl has been reported to either promote, inhibit, or have no effect on seed germination in different species depending on the concentrations (Ditommaso and Nurse 2004; Shabana et al., 2021). On the other hand, $CuSO_4$ added to the *in vitro* nutrient medium containing *Piper nigrum* L. shoots significantly increased shoot development with minimal contamination, indicating that the effectiveness of $CuSO_4$ in sterilization may also vary depending on the source of the explant used (Rajmohan et al. 2010). A similar situation was observed for the H_2O_2 applications. In this study, the treatments using H_2O_2 caused complete contamination of the black henbane seeds, thereby preventing their germination. Al Ghasheem et al. (2018) determined that surface sterilization of *P. persica* shoots with H_2O_2 negatively affected plant development by damaging explant tissues. However, Barampuram et al. (2014) reported that the use of H_2O_2 in the surface sterilization of cotton seeds had no significant effect on seed germination. The lowest germination rate of 11.79% was found in the seeds sterilized using protocol

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Table 2. Effects of different sterilization protocols on seed germination rates of black henbane seeds and, shoot lengths and fresh weights of the seedlings

Protocol number	Germination rate (%)	Shoot length (cm)	Seedling fresh weight (g)
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	11.79 c*	5.42 c	0.13 c
6	64.50 b	7.14 b	0.37 b
7	-	-	-
8	-	-	-
9	82.45 a	7.05 b	0.35 b
10	79.27 a	7.44 a	0.41 a

* Means on the same column shown with the same letter are not statistically different from each other according to Tukey's test ($P \leq 0.05$).

number 5 (washing with detergent + distilled water + shaking in 0.2% $HgCl_2$ solution for 20 minutes + rinsing thrice with sterile distilled water, each for 5 minutes). In protocol number 6, $HgCl_2$ was used at a lower concentration, and for a shorter duration, the germination rates of the seeds were significantly higher (64.50%). These results indicate that the use of $HgCl_2$ at a lower concentration for a shorter application time, following ethyl alcohol treatment caused less damage to the seeds. In a similar study, researchers found that $HgCl_2$ used for surface sterilization of *C. roseus* seeds reduced germination rates due to damage to the seed surface (Ramandi et al. 2019). Researchers investigating the effects of $HgCl_2$ on surface sterilization of sugarcane buds found that 55% of the explants died after two weeks of incubation, indicating that $HgCl_2$ was phytotoxic to plant tissues (Danso et al. 2011).

In the present study, the highest germination rates, the highest values for both shoot length, and seedling weight were obtained with protocol number 10, where sterilization was performed by soaking in a 1% $AgNO_3$ solution for 30 minutes. These results showed that $AgNO_3$ had lesser negative effects on plant development compared to other applications. Similarly, it was reported that the shoot lengths of *P. domestica* explants sterilized with $AgNO_3$ were longer (Ugur 2020). Results of another study showed that the effects of $AgNO_3$ on the germination rates of the seeds varied depending on the plant species (Nartop 2019).

4. Conclusion

This study aimed to determine the most effective surface sterilization method for the successful germination of black henbane seeds under *in vitro* conditions. For this purpose, 10 different sterilization methods using various combinations of EtOH, NaOCl, $CuSO_4$, H_2O_2, $HgCl_2$, and $AgNO_3$ were tested. It was found that 1% $AgNO_3$ application to the seeds totally inhibited fungal growth without causing phytotoxicity. However, various studies showed that the effectiveness of chemicals used for surface sterilization varied depending on many different parameters such as; the type of explant, source of the explant, plant species and age, preferred chemical, application time, and concentration. Therefore, these factors should be considered when selecting the chemical to be used for the sterilization of any plant material.

References

Abdi G, Salehi H, Khosh-Khui M (2008) Nanosilver: a novel nanomaterial for removal of bacterial contaminants in valerian (*Valeriana officinalis* L.) tissue culture. Acta Physiologiae Plantarum 30: 709-714.

Al Ghasheem N, Stanica F, Peticila AG, Venat O (2018) *In vitro* effect of various sterilization techniques on peach (*Prunus persica* (L.) Batsch) explants. Scientific Papers, Series B, Horticulture LXII: 227-234.

Aljibouri AMJ, Al-Samarraei KW, Abd AS, Mageed DM, Ali AJA (2012) Alkaloids production from callus of *Hyoscyamus niger* L. *in vitro*. Journal of Life Sciences 6: 874-882.

Amza J (2018) Seed borne fungi; food spoilage, the negative impact and their management: A review. Food Science and Quality Management 81: 70-79.

Barampuram S, Allen G, Krasnyanski S (2014) Effect of various sterilization procedures on the *in vitro* germination of cotton seeds. Plant Cell, Tissue and Organ Culture (PCTOC) 118: 179-185.

Battal A, Görmez G, Türker M (2019) The efficient germination method for marshmallows with medicinal importance: The nicking on seed coat. Bitlis Eren University Journal of Science 8: 843-851.

Blanco R, Aveling TAS (2018) Seed-borne *Fusarium* pathogens in agricultural crops. Acta Horticulturae 1204: 161-170.

Carisse O, Ouimet A, Toussaint V, Philion, V (2000) Evaluation of the effect of seed treatments, bactericides, and cultivars on bacterial leaf spot of lettuce caused by *Xanthomonas campestris* pv. *vitians*. Plant Disease 84: 295-299.

Danso KE, Azu E, Elegba W, Asumeng A, Amoatey HM, Klu GYP (2011) Effective decontamination and subsequent plantlet regeneration of sugarcane (*Sacchrum officinarum* L.) *in vitro*. International Journal of Integrative Biology 11: 90-96.

Ditommaso A, Nurse RE (2004) Impact of sodium hypochlorite concentration and exposure period on germination and radicle elongation of three annual weed species. Seed Science and Technology 32: 377-391.

Ellis MB (1971) Dematiaceous Hyphomycetes. CAB International, Wallingford, UK.

Ghorbanpour M, Hatami M, Khavazi K (2013) Role of plant growth promoting Rhizobacteria on antioxidant enzyme activities and tropane alkaloid production of *Hyoscyamus niger* under water deficit stress. Turkish Journal of Biology 37: 350-360.

Kane ME (2000) Culture indexing for bacterial and fungal contaminants. In: Trigiano RN, Gray DJ (Eds), Plant Tissue Culture Concepts and Laboratory Exercises. CRC Press, Florida, pp. 427-432.

Kaygusuz T, Coşkuntuna A (2022) Determination of seed-bornefungi in some medicinal and aromatic plants. Applied Ecology and Environmental Research 20: 2553-2564.

Kesho A, Abebe W (2020) Detection of seed borne fungi associated with some cereals and legume crops of seeds grown in main season at Holetta Agricultural Research Center. American Journal of Life Sciences 8: 91-95.

Martin I, Galvez L, Guasch L, Palmero D (2022) Fungal pathogens and seed storage in the drystate. Plants 11: 3167.

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Misra AN, Misra M (2012) Sterilization techniques in plant tissue culture. In: Sharma HP, Dogra JVV, Misra AN (Eds), Plant Tissue Culture: Totipotency to Transgenic. Agrobios, India. pp. 31-41.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Nartop P (2019) Silver nanoparticles: ecofriendly surface sterilization of plant seeds in different shapes and sizes. The Journal of Animal & Plant Sciences 29: 453-460.

Oyebanji OB, Nweke O, Odebunmi O, Galadima NB, Idris MS, Nnodi UN, Afolabi AS, Ogbadu GH (2009) Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. African Journal of Biotechnology 8: 5395-5399.

Pinto ACR, Demattê MESP, Creste S, Barbosa JC (2012) Seed and seedling surface-sterilization for *in vitro* culture of *Tillandsia gardneri* (Bromeliaceae). Acta Horticulturae 961: 383-389.

Rai M, Ingle AP, Pandit R, Paralikar P, Shende S, Gupta I, Biswas JK, da Silva SS (2018) Copper and copper nanoparticles: role in management of insect-pests and pathogenic microbes. Nanotechnology Reviews 7: 303-315.

Rajmohan K, Soni KB, Swapna A, Nazeem PA, Suku SS (2010) Use of copper sulphate for controlling systemic contamination in black pepper (*Piper nigrum* L.) cultures. Journal of Food, Agriculture and Environment 8: 569-571.

Ramandi A, Javan IY, Tazehabadi FM, Asl GI, Khosravanian R, Ebrahimzadeh MH (2019) Improvement in seed surface sterilization and *in vitro* seed germination of ornamental and medicinal plant *Catharanthus roseus* (L.). Chiang Mai Journal of Science 46: 1107-1112.

Rao GS, Narayana S, Bhadraiah B, Manoharachary C (2014) Biochemical changes due to fungal infestation in stored seeds of some vegetable crops. Indian Phytopathology 67: 159-163.

Rathod S (2012) Seed borne *Alternaria* species: A review. Current Botany 3: 21-23.

Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (1995) Introduction to Food-borne Fungi. Fourth Edition. Centraalbureau Voor Schimmelcultures, Baarn, Delft.

Sen MK, Jamal MAHM, Nasrin S (2013) Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured *in vitro*. Environmental and Experimental Biology 11: 119-123.

Shabana HA, Mahmoud T, Gairola S, Al Ketbi A, Aljasmi M, Al Sallani M (2021) Effect of storage conditions and sodium hypochlorite treatment on germination of *Cucumis prophetarum* (Cucurbitaceae) seeds from arid Arabian deserts. Research Square. doi: 10.21203/rs.3.rs-201765/v1.

Tort N (1997) Anason (*Pimpinella anisum* L.) ve pamuk (*Gossypium hirsutum* L.) tohumlarının *in vitro* ortamda sterilizasyonu üzerinde bir araştırma. Anadolu Ege Tarımsal Araştırma Enstitüsü Dergisi 7: 41-50.

Ugur R (2020) Development of *in vitro* sterilization protocol for DO-1 (*Prunus domestica*) rootstock. Applied Ecology and Environmental Research 18: 2339-2349.

Wahyono T, Hardani SNW, Sugoro I (2018) Low irradiation dose for sorghum seed sterilization: hydroponic fodder system and *in vitro* study. Buletin Peternakan 42: 215-221.

Watanabe T (2002) Pictorial Atlas of Soil and Seed Fungi, Morphologies of Cultured Fungi and Key to Species. Second Edition. CRC Press, Florida. pp. 486.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 121-127
DOI: 10.29136/mediterranean.1535179

Stepwise membrane filtration for Rebaudioside A and Stevioside enrichment in aqueous and ethanolic stevia extracts

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ARTICLE INFO

Received: August 19, 2024
Received in revised form: November 4, 2024
Accepted: November 5, 2024

Keywords:

Stevia extract
Solvent extraction
Ultrafiltration
Nanofiltration
Enrichment

ABSTRACT

Enriching steviol glycosides derived from the stevia plant is an important step in producing stevia natural sweeteners. This study investigated the enrichment of Rebaudioside A and Stevioside compounds of aqueous and ethanol stevia extracts using cascade membrane filtration techniques. Accordingly, extracts from stevia leaves were processed using membrane filtration techniques such as ultrafiltration and nanofiltration. In particular, the 30 kDa ultrafiltration membrane was highly effective in enriching steviol glycosides. The highest concentrations of Rebaudioside A (21.47 g L^{-1}) and Stevioside (19.97 g L^{-1}) compounds were reached at the 30 kDa ultrafiltration retentate fraction in both aqueous and ethanolic extracts. However, it was found that the permeate fluxes and the extracts obtained after the nanofiltration process had very low concentrations of these compounds. The findings highlight the importance of membrane selection in effectively enriching steviol glycosides.

1. Introduction

Steviol glycosides, which are the natural sweet components of stevia leaves, have recently been the subject of a substantial trend in their extraction. Several studies have advocated for the substitution of ethanol, an industrial process solvent, with water (Das et al. 2015; Díaz-Montes et al. 2020; Zoua Assoumou et al. 2024). This substitution is due to the environment-friendly attribute of water, as well as the non-toxic and non-flammable nature of it. Furthermore, the utilization of water as a solvent may lower the production expenses and enhance the sustainability of the extraction procedure (Díaz-Montes et al. 2021).

Recent developments have focused on the increase of the purity and yield of steviol glycosides by optimizing membrane filtration processes. Díaz-Montes et al. (2020) achieved a significant enrichment of Rebaudioside A by using a two-stage ultrafiltration process to fraction the liquid extracts of *Stevia rebaudiana*. Similarly, Karhan (2020) investigated fractional membrane filtration practices to purify stevia extracts and emphasized the importance of the membrane pore size in achieving desired purity levels. In addition, Zhang et al. (2000) suggested the use of resin or fluctuation agents to improve flow before membrane processing and prevent membrane clogging.

Many studies have been conducted to determine the effectiveness of multiple combinations of different membrane filtration techniques to fractionate target steviol glycosides as much as possible (Liu et al. 1991; Martínez-Alvarado et al. 2017; Díaz-Montes et al. 2020; Karhan 2020). Membrane filtration applications are frequently applied as important enrichment processes in the purification of steviol glycosides. These techniques can selectively separate molecules based on their size and molecular weight. However, testing different membrane filtration combinations is important to determine the change in the steviol glycoside profile and which application will be more successful when specific purification is desired. Therefore, this research was aimed at providing a comprehensive understanding of the factors influencing the enrichment of steviol glycosides, with a focus on the stepwise membrane filtration approach.

2. Materials and Methods

2.1. Material

The stevia plant (*Stevia rebaudiana* var. Levent 93), cultivated in the Department of Field Crops, Akdeniz University trial area, was employed as research material in the study. The freshly harvested leaves were dried in an oven at 70°C until the moisture content reached 5 g 100rte g^{-1} (dry basis). They were then stored in closed containers at room temperature until the time of analysis.

2.2. Production of raw aqueous and ethanolic stevia extracts

The dry stevia leaves were separated from extraneous substances such as stem, flower, waste, etc. then ground using a laboratory grinder (Waring Blender, USA). The dried stevia leaves were mixed with distilled water/ethanol (96% purity) in a ratio of 1:15 (dry ground leaf: water/ethanol) in a laboratory malaxer (Alfa Laval X, Sweden) for 30 min at 20°C. The mixture (Sample: E) was subjected to centrifugation with 2900xg force at 20ºC for 15 minutes. The collected supernatant was passed through a coarse filter paper to remove any solid particles (Sample: C). The same method was used to produce both

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ethanolic stevia extracts as well as aqueous ones. The solvent (ethanol) was removed with a rotary evaporator (IKA RV10 Auto Pro V-C, Germany) at 40°C and 900 rpm only after the coarse filtration stage. Subsequently, distilled water was added to the remaining portion in an amount equivalent to the amount of liquid that had evaporated. Consequently, this mixture was employed as the raw ethanolic stevia extract throughout the analyses.

2.3. Hot clarification

Before the implementation of membrane filtration techniques, a hot clarification procedure was carried out to prevent the membrane filters from becoming fouled. Pre-treatments, such as the usage of lime or flocculating agents, are suggested before membrane processing such as ultrafiltration for improved flux (Zhang et al. 2000).

During the hot clarification phase, clarification aids were added to the stevia extract in a water bath (JeioTech, BS-06/31, Seoul, Korea) kept at 50°C in the quantities determined by preliminary trials (bentonite: 1%, gelatine: 1%, kieselsol: 3%). The clarification was terminated after 3 hours, and the clear part on the upper side (Sample: HC) was filtered through the coarse filter without removing the sediment that had formed at the bottom.

2.4. Stepwise membrane filtration

Polyethersulfone (PESU) membrane with a pore size of 30 kDa (Sartocon Slice, Sartorius Stedim Biotech GmbH, Germany) was used for ultrafiltration, and Hydrosart membrane with a pore size of 5 kDa (Sartocon Cassette, Sartorius Stedim Biotech GmbH, Germany) was used for nanofiltration. Applications of stepwise membrane filtration were implemented at a membrane pressure of 2 bar and a permeate flux of 90%. Accordingly, the translucent fraction that passed through the coarse filter after the hot clarification process was initially filtered by ultrafiltration using a 30 kDa PESU membrane filter. Following that, the permeate was further filtered by nanofiltration using a 5 kDa Hydrosart membrane filter (Figure 1).

2.5. Methods

2.5.1. Total soluble solids and pH analyses

The total soluble solids and pH values of the stevia extracts were measured using a digital refractometer (Isolab, Germany) and a pH meter (FE20-Five, Mettler-Toledo, Ohio, USA) (Cemeroğlu 2007).

2.5.2. Color measurement

Color (L*, a*, b*) values of the samples were measured using an UltraScan-VIS spectrophotometer (Hunterlab, USA) equipped with a CIE-Lab color model. The CIE-Lab model characterizes color by employing three parameters: The L* metric lightness function represents the degree of lightness on a scale from 0 (black) to 100 (white). The a* and b* chromaticity coordinates reflect opposing scales of red–green (+a for reds, -a for greens) and blue-yellow (+b for yellows, -b for blues).

2.5.3. Quantification of Stevioside and Rebaudioside A

An external standard method with HPLC was used to determine the Stevioside and Rebaudioside A amounts in the stevia extracts (Wölwer-Rieck et al. 2010). For the analysis, a High-Performance Liquid Chromatography (HPLC) instrument (Shimadzu, LC 20 AD) was used. The instrument was equipped with a C18 column (Dimensions: 5 µm, 250 x 4.6 mm, ID) along with a photodiode array (PDA) detector system set at a wavelength of 210 nm. A mixture (68:32) of phosphate buffer (10 mmol L^{-1} sodium phosphate, pH 2.6) and acetonitrile (HPLC quality, Merck) was used as the mobile phase and the flow rate was set to 0.8 ml min^{-1} (Wölwer-Rieck et al. 2010).

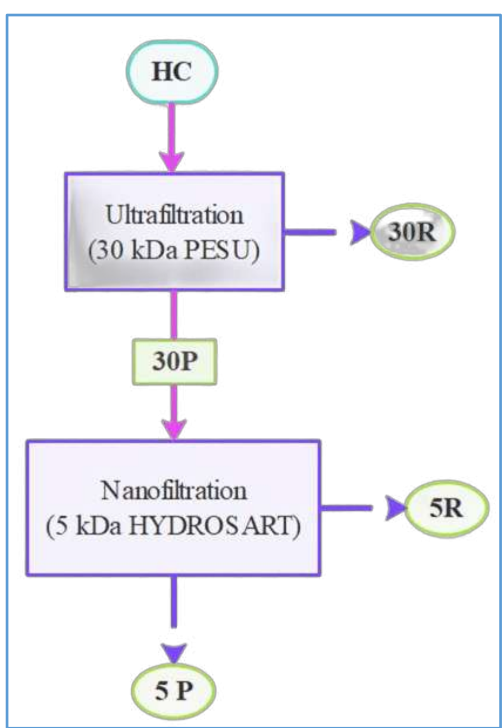

Figure 1. Stepwise membrane filtration procedures.

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2.5.4. Statistical analysis

Statistical analysis of the data (per each sample, three repetitions, and two reading parallels) was performed at the level of 5% significance. The Shapiro-Wilk test was applied to determine the suitability of the data for normal distribution, then the Kruskal-Wallis test (one-way ANOVA), was used. When a significant difference was found between the groups as a result of the Kruskal-Wallis test, Dunn's multiple comparison test was applied (Origin 2019b OriginPro, USA).

3. Results and Discussion

3.1. Variations across extraction and membrane filtration stages

3.1.1. Total soluble solids and pH

The total soluble solids (TSS) contents of the stevia samples are given in Table 1. The findings showed that the large molecules were successfully concentrated by 30 kDa ultrafiltration in the retentate (30R) phase of the aqueous extract, leading to a significant rise in the TSS contents. Iwuozor et al. (2024) found that water extraction had a greater ability to extract soluble solids in comparison to methanol and methanol/water combinations, therefore confirming this observation. The effect of nanofiltration on soluble solids content after ultrafiltration was limited. In the process starting at 30P (0.63 °Bx), which is the permeate part of ultrafiltration, the permeate (5P: 1.39 °Bx) and retentate (5R: 1.80 °Bx) values obtained throughnanofiltration showed a limited concentration increase. This suggests that nanofiltration is not able to distinctly decompose the solution after ultrafiltration but does provide some concentration. In particular, the absence of large differences between the 5P and 5R values compared to 30P, which is the feed solution, reveals that nanofiltration does not provide complete separation.

Table 1. Soluble solids and pH values of aqueous and ethanolic stevia extracts

Parameter	Process	Aqueous	Ethanolic
Total Soluble Solids (°Bx)	E	3.10±0.00^{b}	4.03±0.07^{a}
	C	3.10±0.00^{b}	3.30±0.00^{b}
	HC	4.00±0.06^{b}	2.43±0.09^{c}
	30P	0.63±0.10^{c}	1.63±0.09^{d}
	30R	12.31±1.39^{a}	4.20±0.29^{a}
	5P	1.39±0.52^{c}	1.78±0.12^{d}
	5R	1.80±0.62^{c}	4.92±0.83^{a}
pH	E	5.81±0.00^{b}	6.09±0.02^{a}
	C	4.82±0.02^{d}	5.47±0.04^{c}
	HC	5.93±0.00^{b}	4.63±0.01^{d}
	30P	6.62±0.06^{a}	5.72±0.07^{b}
	30R	5.60±0.19^{c}	4.82±0.06^{d}
	5P	5.57±0.39^{c}	4.65±0.35^{d}
	5R	5.99±0.27^{b}	5.22±0.23^{c}

The values indicated by different letters show a significant difference between different processes under the same extraction method for the same color parameter ($P<0.05$). E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux.

When the ethanolic extract was subjected to membrane filtration, ultrafiltration, and nanofiltration techniques led to a more restricted enrichment, with limited increases in the concentration of soluble solids compared to the aqueous extract. This emphasizes the distinct impacts of ultrafiltration and nanofiltration procedures on the chemical characteristics of the extract, depending on the solvent employed (water or ethanol). For example, in the ethanolic extract, the total soluble solids concentration after ultrafiltration (30R) was 4.20±0.29 °Bx, whereas after nanofiltration (5R), it increased to 4.92±0.83 °Bx, showing a more restricted enrichment compared to the aqueous extract (Table 1).

There were also changes in the pH values of stevia samples extracted in water and alcohol, depending on both extraction and membrane filtration type (Table 1). Samples extracted with ethanol initially have a more alkaline pH value, indicating that ethanol is better able to extract alkaline compounds. Mahl et al. (2010) found that ethanol extraction typically yields a greater quantity of alkaline chemicals, which is consistent with this finding. However, in the process of ultrafiltration, it was observed that alkaline chemicals were concentrated in the permeate phase of the aqueous extract, while acidic compounds were transferred to the permeate phase in the ethanolic extract. The nanofiltration procedure reverses this scenario for both types of extracts, resulting in an augmentation of acidic chemicals in the permeate and a concentration of alkaline compounds in the retentate. Arakawa et al. (2012) demonstrated that pH values significantly influence the adsorption and separation processes during filtration, which helps explain these results. These pH changes can be attributed to the differential permeability of the membrane to alkaline and acidic compounds, as well as the interactions between the membrane material and the solutes in the stevia extracts. During ultrafiltration, larger alkaline molecules may be retained more effectively, leading to their concentration in the retentate, while smaller acidic molecules permeate through the membrane. This selective separation can cause a shift in pH values, as noted by Zhang et al. (2000), who observed similar trends in other plant extract filtration processes.

3.1.2. Color

The color changes that occurred in the stevia extracts with the applied processes areshown in Table 2 and Figure 2. For the aqueous extract, it was observed that the decolorization continued to some extent with nanofiltration (5P) after UF (30P), showing a statistically significant increase in the L* value, and the green tone became slightly more dominant in terms of the a* value. Nevertheless, there were no significant alterations identified in relation to the b* value. Karhan (2020) observed that reducing the size of membrane pores during multi-stage membrane filtration operations led to an elevation in the L* value (representing lightness) of stevia extracts. The author attributed this variation to the removal of pigmented phenolic compounds from the permeate flux. In line with this, Kootstra et al. (2016) observed that the final stevia extract maintained a greenish-brown color even after nanofiltration. This implies that while ultrafiltration and nanofiltration improved transparency, they were not entirely successful in eliminating the color.

For the ethanolic extract, the (5P) a decrease in the L* value was observed after NF and there was a reduction in lightness. Very small changes were observed in the a* and b* values, i.e. no great progress was made in terms of achieving colorlessness. As a result, it can be said that discoloration with NF persists after UF in aqueous extracts, but this increase in lightness is limited.

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Table 2. *L*, a*, b** values of aqueous and ethanolic stevia extracts

Color Parameter	Process	Aqueous	Ethanolic
*L**	E	30.51±0.03[a]	31.46±0.66[a]
	C	26.45±0.02[b]	23.51±0.17[c]
	HC	27.47±0.13[b]	26.99±0.04[b]
	30P	24.63±1.06[c]	26.62±0.48[b]
	30R	24.02±1.81[c]	24.20±1.81[c]
	5P	25.83±0.83[b]	25.54±1.46[b]
	5R	25.42±0.74[b]	26.49±0.33[b]
*a**	E	-0.66±0.06[b]	-1.89±0.7[a]
	C	-0.53±0.03[b]	-0.48±0.07[c]
	HC	-0.53±0.01[b]	-0.25±0.51[d]
	30P	-0.71±0.6[a]	-0.73±0.32[b]
	30R	0.67±0.09[b]	0.74±0.05[b]
	5P	-0.78±0.25[b]	-0.73±0.06[b]
	5R	-0.56±0.74[b]	-0.75±0.07[b]
*b**	E	4.77±0.59[a]	10.28±0.34[a]
	C	0.21±0.02[c]	0.19±0.05[d]
	HC	0.53±0.02[b]	0.45±0.01[b]
	30P	-0.54±0.06[b]	0.36±0.62[c]
	30R	0.45±0.18[b]	0.51±0.59[b]
	5P	-0.53±0.22[b]	0.31±0.37[c]
	5R	-0.49±0.12[b]	0.09±0.09[e]

The values indicated by different letters show a significant difference between different processes under the same extraction method for the same color parameter ($P<0.05$). E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux.

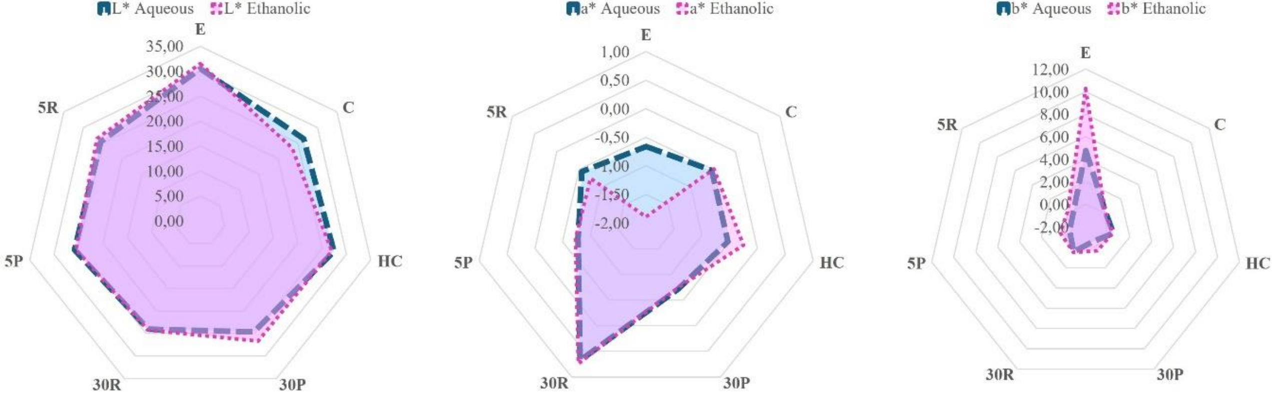

Figure 2. Color changes in aqueous and ethanolic stevia extracts throughout processing stages (E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

In ethanolic extracts, a slight darkening tendency was observed instead of an increase in lightness after NF. This suggests that NF is more effective on aqueous extracts but has a limited effect on ethanolic extracts if colorlessness is the main aim.

While aqueous and ethanolic extracts initially had the lightest color (L*), greenest (a*), and most yellow tones (b*), the ultrafiltration process caused a marked change in color tones, especially in the retentate (30R) stage, with red tones (a*) becoming dominant. After nanofiltration, a tendency to lighten was observed in aqueous extracts, while yellow tones (b*) were somewhat preserved in ethanolic extracts, but there was a shift to blue tones in general (Table 2). Except for the extraction (E) step, aqueous and ethanolic extracts exhibited a fairly similar trend of change in L*, a*, b* values during the processing stages (ultrafiltration and nanofiltration). Both types of extracts wentthrough parallel processes such as color darkening, tonal changes, and partial lightening (Figure 2). However, in the extraction phase (E), the ethanolic extract initially started in lighter, greener, and more yellow tones than the aqueous extract, indicating that the type of solvent had different effects on the color during the extraction phase. That is, although there is similarity in the processing stages, solvent-dependent differences were evident in the initial (E) stage.

3.1.3. Filtration effects on Rebaudioside A and Stevioside enrichment

The variation of steviol glycosides through different membrane filtration steps reveals that ultrafiltration in general (especially the 30 kDa membrane) is highly effective in enriching

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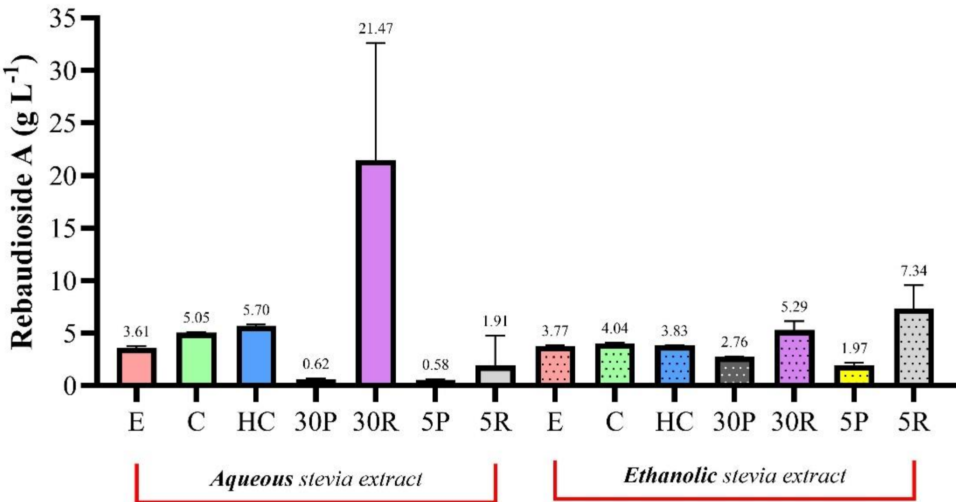
both Rebaudioside A and Stevioside. In both aqueous and ethanolic extracts, it has been observed that the highest concentrations of these compounds are reached at the 30 kDa ultrafiltration retentate fraction. This result is consistent with Das et al.' (2015) findings, who reported that the 30 kDa membrane was the most effective in enriching Rebaudioside A, with minimal fouling behavior. In contrast, the concentrations of these compounds remained markedly low in the permeate fractions and nanofiltration steps. Similarly, Chhaya and Mondal (2012) discovered that nanofiltration had a minimal impact on the enrichment of Rebaudioside A and Stevioside, as a substantial portion of these components was retained by the membrane. This suggests that certain steps of membrane filtration play a critical role in the process of separation and enrichment of steviol glycoside (Figure 3).

When the effect of membrane filtration applications on the enrichment of steviol glycosides was examined in more detail, it was determined that the aqueous extract reached the highest concentrations of 30 kDa ultrafiltration retentate, Rebaudioside A with 21.47 g L^{-1} and Stevioside with 19.97 g L^{-1} (Figure 4).

These values clearly show that steviol glycosides were effectively enriched in this step. On the other hand, in the permeate fractions (30PAq and 5PAq), rebaudioside A and Stevioside concentrations remained at very low levels of 0.62 g L^{-1} and 0.86 g L^{-1}, respectively. The nanofiltration process yielded similarly low concentrations, suggesting that a large proportion of these components were retained in the membrane and did not pass into the permeate fraction. These specific numerical details reveal the effect of each filtration step on the concentration of steviol glycosides.



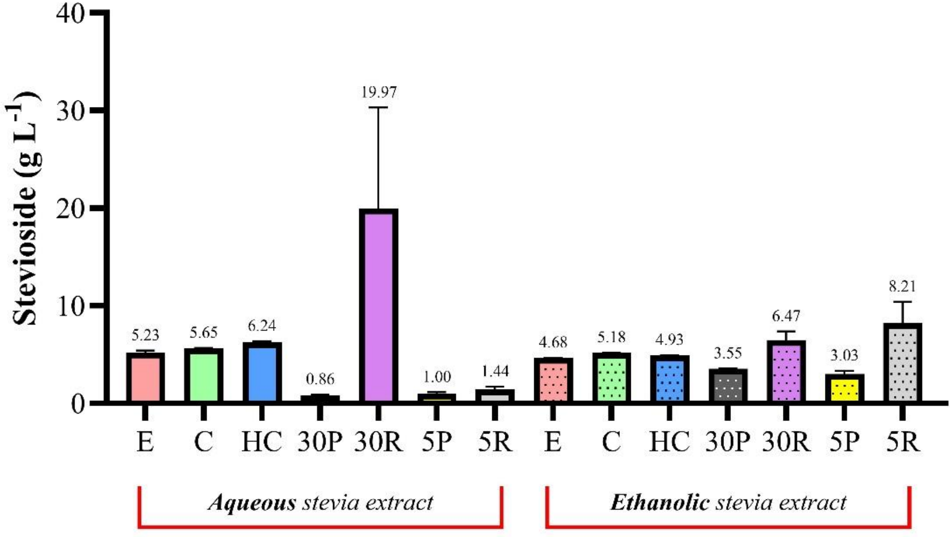


Figure 3. Rebaudioside A and Stevioside concentrations in aqueous and ethanolic stevia extracts across different processing stages (E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

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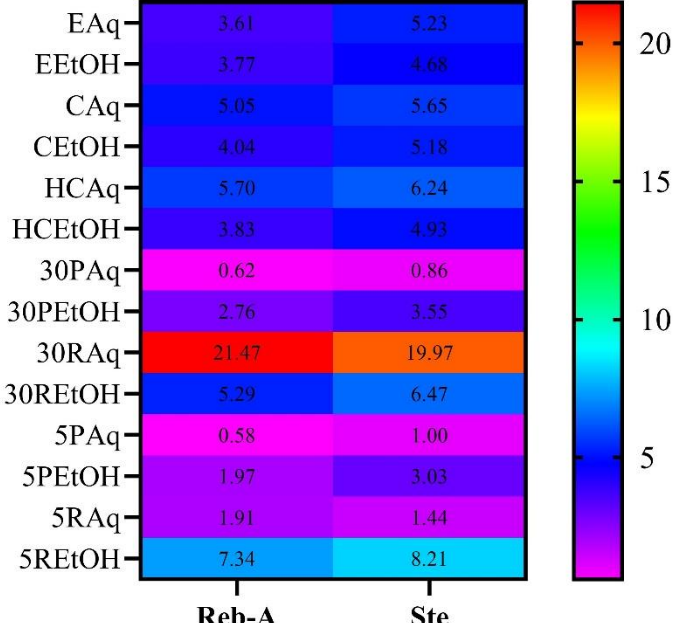

Figure 4. Distribution of rebaudioside A and Stevioside levels in aqueous and ethanolic stevia extracts visualized by heatmap across processing stages (Aq: Aqueous, EtOH: Ethanolic, E: Extraction, C: Centrifugation, HC: Hot clarification, 30P: Ultrafiltration, 30 kDa-permeate flux, 30R: Ultrafiltration, 30 kDa-retentate flux, 5P: Nanofiltration, 5 kDa-permeate flux, 5R: Nanofiltration, 5 kDa-retentate flux).

4. Conclusion

The efficiency of using stepwise membrane filtration procedures during processing of aqueous and ethanolic stevia extracts was investigated in this study. The results indicated that the ultrafiltration (UF) process was highly effective in concentrating the analyzed steviol glycosides. Both Rebaudioside A and Stevioside glycosides were concentrated in the retentate flux of 30 kDa ultrafiltration membrane. However, if nanofiltration process (5 kDa) is to be combined with ultrafiltration, it is thought that using the retentate phase as a feed may be more effective in increasing the purity of the target compounds. The findings of this study indicate that the choice of membrane filtration type is crucial in the separation and concentration of steviol glycosides.

Acknowledgements

This study was supported by the Akdeniz University, Office of Scientific Research Projects (Project No: FYL-2021-5553).

References

Arakawa FS, Mahl CR, de Oliveira SP, da Igreja G, Simões MR, da Silva CF (2012) Clarification of aqueous Stevia extract using alginate beads—evaluation by factorial design methodology. Adsorption Science & Technology 30(2): 147-158. doi: 10.1260/0263-6174.30.2.147.

Cemeroğlu B (2007) Gıda analizleri. Gıda Teknolojisi Derneği Yayınları, Ankara.

Chhaya C, Sharma CS, Mondal SM, Majumdar G, De S (2012) Clarification of stevia extract by ultrafiltration. Food and Bioproducts Processing. doi: 10.1016/j.fbp.2011.10.002.

Das A, Paul D, Golder AK, Das C (2015) Separation of rebaudioside-A from stevia extract. Separation and Purification Technology 144: 8-15. doi: 10.1016/j.seppur.2015.02.004.

Díaz-Montes E, Gutiérrez-Macías P, Orozco-Álvarez C, Castro-Muñoz R (2020) Fractionation of *Stevia rebaudiana* aqueous extracts via two-step ultrafiltration process: towards rebaudioside A extraction. Food and Bioproducts Processing 123: 111-122. doi: 10.1016/j.fbp.2020.06.010.

Díaz-Montes E, García-Depraect O, Castro-Muñoz R (2021) Membrane technologies for the extraction and purification of steviol glycosides. In: Galanakis CM (Ed), Steviol glycosides: production, properties, and applications. Academic Press, pp. 159-199.

Iwuozor KO, Emmanuel SS, Ahmed MO, Idris AM, Emenike EC, Saliu OD, Qudus AH, Adeniyi AG (2024) Technologies for the extraction and post-extraction of *Stevia rebaudiana* leaves. Chemistry Africa 7(2): 539-563. doi: 10.1007/s42250-023-00787-0.

Karhan M (2020) Use of fractional membrane filtration applications in the purification of Stevia extracts (TÜBİTAK 1002 Project Final Report 119O016).

Kootstra A, Elissen H, Huurman S (2016) Extraction of steviol glycosides from fresh Stevia using acidified water; clarification followed by ultrafiltration and nanofiltration. Application Center for Renewable Resources - ACRRES. https://www.acrres.nl/wp-content/uploads/2018/06/PPO-632.pdf. Accessed 9 September 2024.

Liu Y, Chen Y, Wang L, Jiang J (1991) Study of stevioside preparation by membrane separation process. Desalination 83(1-3): 375-382. doi: 10.1016/0011-9164(91)85111-7.

Mahl CR, de Oliveira SP, da Silva BB, da Igreja G, Simões MR, da Silva CF (2010) Effect of pH on the clarification of *Stevia rebaudiana* Bertoni extract using alginate beads. Adsorption Science and Technology 28(2): 189-194. doi: 10.1260/0263-6174.28.2.189.

Martínez-Alvarado JC, Torrestiana-Sánchez B, Aguilar-Uscanga MG (2017) Isolation of steviol glycosides by a two-step membrane process operating under sustainable flux. Food and Bioproducts Processing 101: 223-230. doi: 10.1016/j.fbp.2016.11.013.

Wölwer-Rieck U, Tomberg W, Wawrzun A (2010) Investigations on the stability of stevioside and rebaudioside A in soft drinks. Journal of Agricultural and Food Chemistry 58(23): 12216-12220.

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Zhang SQ, Kumar A, Kutowy O (2000) Membrane-based separation scheme for processing sweeteners from Stevia leaves. Food Research International 33(7): 617-620. doi: 10.1016/S0963-9969(00)00098-3.

Zoua Assoumou U, Öziyci H, Hacıoğlu A, Karhan M (2024) Influence of solvent type and leaf moisture on extraction efficiency of major steviol glycosides in Stevia (var. Levent 93) plant. Acta Alimentaria 53(2): 175-187. doi: 10.1556/066.2023.00245.

Abbreviations

E: Extraction
C: Centrifugation
HC: Hot clarification
30P: Ultrafiltration, 30 kDa-permeate flux
30R: Ultrafiltration, 30 kDa-retentate flux
5P: Nanofiltration, 5 kDa-permeate flux
5R: Nanofiltration, 5 kDa-retentate flux

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AGRICULTURAL SCIENCES
(2024) 37(3): 129-136
DOI: 10.29136/mediterranean.1551068

Quantification and comparison of gliadin proteins in ancient wheats grown in rainfed and irrigated conditions

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ARTICLE INFO

Received: September 17, 2024
Received in revised form: October 23, 2024
Accepted: October 24, 2024

***Keywords*:**

Ancient wheat
BCA Assay
Gliadin
Protein determination
Wheat extraction

ABSTRACT

Protein quantification is crucial for assessing the nutritional and functional qualities of wheat. This study quantified the gliadin content, a major component of wheat storage proteins, in 24 wheat genotypes, including ancient varieties such as einkorn (*Triticum monococcum*), emmer *(Triticum dicoccum),* and spelt *(Triticum spelta).* These varieties were cultivated under rainfed and irrigated conditions in the Konya/Ilgın region of Türkiye. Gliadin proteins were extracted using a 70% ethanol solution to isolate the soluble fractions, which were subsequently analyzed using the Bicinchoninic Acid (BCA) assay. The results revealed significant variation in gliadin content among the genotypes. In general, samples grown under irrigated conditions exhibited higher protein concentrations compared to those grown under rainfed conditions. Among the varieties, Karahan (16339.07 µg mL^{-1}) and Soana (15826.99 µg mL^{-1}) had the highest protein contents under irrigated and rainfed conditions, respectively. These findings demonstrate the impact of both environmental and genetic factors on protein composition, highlighting the importance of ancient wheat varieties in sustainable agriculture and their potential to enhance modern dietary nutrition.

1. Introduction

Food security and nutritional quality have become increasingly critical in the global agriculture landscape. Among staple crops, wheat remains one of the most important worldwide, serving as a primary dietary source for billions across diverse cultures (Shewry and Hey 2015).Within the broad spectrum of wheat varieties, ancient types have garnered renewed attention due to their health benefits and resilience to environmental stressors (Dinu et al. 2018).

Ancient wheat types such as einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), and spelt (*Triticum spelta*) have rich genetic backgrounds and are often distinguished by their lower gluten content, higher micronutrient levels, and greater resilience to stress conditions, such as drought (Shewry 2009). Historically they were cultivated in regions that are now part of modern Türkiye, which is known for its diverse climatic conditions and agricultural practices. The growing interest in ancient wheat varieties is motivated by their potential contributions to sustainable agriculture and human health, particularly in light of rising awareness of gluten-related disorders and the benefits of whole grains (Shewry and Hey 2015).

Mature wheat grains contain approximately 8%–20% proteins, comprising 15%–20% water-soluble albumins and globulins, while the remaining 80%–85% is made up of water-insoluble glutenins and gliadins (Zilić et al. 2011). Glutenins dissolve in dilute acetic acid, while gliadins dissolve in 70% alcohol (Osborne 1924). Due to their prominent levels of proline and glutamine residues, glutenins and gliadins are collectively termed prolamins.

These proteins are not only critical for assessing the nutritional quality of wheat but also for understanding its functional properties, especially in bread-making. Gluten, formed by the interaction between glutenin and gliadin, significantly influences dough elasticity and strength, making protein composition a key factor in evaluating wheat varieties for baking potential (Thanhaeuser et al. 2015). In a related study, 49 emmer lines, 36 einkorn lines, and 3 modern durum wheat cultivars (controls) were analyzed, with specific einkorn lines showing the highest protein levels for these varieties (Akar et al. 2019).

Gliadin proteins play a fundamental role in forming the gluten network and contribute to the nutritional value of wheat, making their accurate quantification vital for both health and food science applications (Thanhaeuser et al. 2015). One commonly used method for quantifying gliadin is the Bicinchoninic Acid (BCA) assay, valued for its simplicity, high sensitivity, and reproducibility (Walker 1994).

In addition to the BCA assay, the Dumas and Kjeldahl methods are widely used for protein quantification though they are based on different principles. The Dumas method, which involves combustion to determine the nitrogen content of a sample, is rapid and requires minimal reagents, making it suitable

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for high-throughput analyses. It also offers an advantage over the Kjeldahl method due to its eco-friendly nature, as it does not require hazardous chemicals such as sulfuric acid. In contrast, the Kjeldahl method is a wet-chemical technique that involves digesting the sample in sulfuric acid, followed by neutralization and distillation to quantify nitrogen. Though labor-intensive, it remains a gold standard for protein determination due to its high accuracy and applicability to a wide range of sample types, including agricultural products. Together, these methods provide complementary insights, especially when analyzing agricultural samples such as wheat. Used alongside the BCA assay, they offer a comprehensive understanding of the total protein and specific protein components such as gliadins and glutenins.

The BCA assay quantifies proteins by reducing Cu^{2+} ions in an alkaline solution to Cu^{1+} ions, in a reaction such as the biuret method but with greater sensitivity. In this assay, proteins reduce Cu^{2+} to Cu^{1+}, which subsequently forms a purple complex with two molecules of bicinchoninic acid (BCA). The complex absorbs light at 562 nm, and the absorbance is directly proportional to the protein concentration in the sample (Smith et al. 1985; Walker 1994).

The BCA assay is favored for its compatibility with various detergents and reducing agents, which can interfere with other protein assays, such as the Bradford assay (Walker 1994; Wiechelman et al. 1988). Its application in wheat protein quantification, particularly for gliadin, provides critical insights into wheat's nutritional and functional properties. By comparing sample absorbance to a standard curve generated from known protein concentrations, accurate quantification of gliadin content can be achieved (Schoel et al. 1995)

Understanding the protein composition of ancient wheat varieties, particularly gliadins, is crucial for evaluating both their nutritional quality and functional capacity in food applications. The BCA assay, in conjunction with methods such as the Dumas and Kjeldahl methods, offers reliable and sensitive tools for this purpose. As interest in ancient wheat varieties grows due to their potential benefits for human health and sustainable agriculture, robust protein quantification methods will play a pivotal role in deepening our understanding of these grains.

The aim of this study was to determine and compare the gliadin protein contents of ancient wheat varieties grown under rainfed and irrigated conditions. Samples were collected from Ilgın/Konya Türkiye and analyzed by BCA assay.

2. Materials and Methods

2.1. Sample Collection and Preparation

The study utilized a trial set of 24 wheat genotypes, which included a variety of candidates from the ancient cultivated species einkorn (*Triticum monococcum*) and emmer (*Triticum dicoccum*). The genotypes also comprised local bread wheat varieties selected through breeding, as well as varieties developed both before and after the Green Revolution (pre- and post-1970). Detailed information on the genotypes used in the study is presented in Table 1.

Table 1. Characteristics of the genetic materials used in the study

Genotypes	Breeding Methods, Registration Years, and Classification	Pedigree
Songar* (Einkorn)	Historical/selection/2019 pre-green revolution	Selection from local population
Mokan* (gernik)	Historical/selection/2019 pre-green revolution	Selection from local population
Ak702	Local variety/selection/1931 pre-green revolution	A mixture of two pure lines obtained from Akbuğday grown around Eskişehir
Sertak	Local variety/selection/1936 pre-green revolution	A mixture of two pure lines numbered 1721 and 1731
Yektay	Old variety/crossbreeding/1968 pre-green revolution	Selection from San Marino wheat population
Bezostaja1	Old variety/crossbreeding/1968 pre-green revolution	LUTESCENS-17/ SKOROSPELKA-2
Bolal	Old variety/crossbreeding/1970 pre-green revolution	CHEYENNE//KENYA-324/MENTANA
Gerek79	Old variety/crossbreeding/1979 post-green revolution	MENTANA/MAYO-48//4-11/3/YAYLA-305
Kırkpınar	Old variety/crossbreeding/1979 post-green revolution	HYSLOP/SIETE-CERROS-66
Sultan95	Old variety/crossbreeding/1995 post-green revolution	AGRI/NACOZARI-76
İkizce96	Old variety/crossbreeding/1996 post-green revolution	ARTHUR*2/SIETE-CERROS-66//BRILL
Pehlivan	Old variety/crossbreeding/1998 post-green revolution	BEZOSTAYA-1/TEVERE/5/CENTRIFEN/BEZOSTAYA-1//SUWEON-92/CI-13645/3/NAINARI-60/4/(SIB)EMU
Karahan	Old variety/crossbreeding/1999 post-green revolution	C-126-15/COLLAFEN/3/NORIN-10-BREVOR/P-14//(P101)PULLMAN-101/4/KIRAC-66
Alparslan	New variety/crossbreeding/2001 post-green revolution	TX-69-A-509-2//BBY2/FOX
Nenehatun	New variety/crossbreeding/2001 post-green revolution	NORD DEPREZ/PULLMAN SELECTION 101//BLUEBOY
Sönmez2001	New variety/crossbreeding/2001 post-green revolution	BEZ//BEZ/TVR/3/KREMENA/LOV29/4/KATIA1
Konya2002	New variety/crossbreeding/2002 post-green revolution	KANRED/ TENMARQ//P-211-6/3/2183/ CO-652643/LANCER
Tosunbey	New variety/crossbreeding/2004 post-green revolution	ECVD-12/KIRAC-66//(SIB) CROW
Ahmetağa	New variety/crossbreeding/2004 post-green revolution	F-885-K-1-1/SIOUXLAND
Esperia	New variety/crossbreeding/2011 post-green revolution	B-16-3/LINEA-RUSSA
Bora	New variety/crossbreeding/2014 post-green revolution	H-31/TRAP-1-F-2//ENESCO
Genesi	New variety/crossbreeding/2014 post-green revolution	COLFIORITO/HEREWARD
Soana	New variety candidate/crossbreeding/2019 post-green revolution	unknown
Galinda	New variety candidate/crossbreeding/2019 post-green revolution	AHMETAĞA/ESPERIA/ //ESPERIA

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The wheat samples were cultivated during 2022-2023 in the Ilgın/Konya region of Türkiye, following a randomized block design with three replications. Samples from ancient wheat varieties were collected from both rainfed and irrigated fields, allowing for a comprehensive analysis of the impact of irrigation on protein content and composition. The wheat grains were harvested at maturity, cleaned, dried, and stored under controlled conditions to minimize degradation. Following standard procedures, the wheat grains were milled into flour to ensure uniformity for subsequent analysis.

2.2. Chemicals, consumables and instrumentation

All chemicals used in the study were of analytical and/or liquid chromatographic grade. Ethanol was sourced from Isolab (Wertheim, Germany). The water for dilutions and preparation of samples was provided by an ultrapure water purification system from MP Minipure (Ankara, Türkiye). Protein extraction was conducted using a Biosan TS-100 type shaker. To determine the constant protein amount, the Pierce BCA Assay Kit (Thermo Fisher Scientific) was used. Absorbance measurements were taken at Thermo Scientific Multiskan GO Microplate Spectrophotometer.

2.3. Protein extraction

For wheat flour extraction, 20 grams of wheat grains were ground and separated into flour and bran to provide sufficient material for the experiments. Initial trials showed that the parameters were optimal for dissolving gliadin proteins at 30°C for 1.5 hours.

According to the protocol (Malalgoda et al. 2018) approximately 250 mg of each flour sample was weighed into sterile 1.5 mL Eppendorf tubes. Then, 750 µL of 70% ethanol was added. Extraction was started at 1400 rpm and 30°C for 1.5 hours on a thermoshaker. After extraction, the samples were then centrifuged at 4550 rpm for 15 minutes. The supernatant containing gliadin was filtered through a 0.45 µm filter into a new tube. Three replicates were prepared for each sample and the extracts were obtained in the same tubes at -18°C. To prevent extract loss during centrifugation, each Eppendorf tube was wrapped in parafilm. Replicates were performed for each sample during extraction, and the replicates were included in the BCA protein analysis in the same manner.

Since multiple measurements were required for determining the protein content in wheat, the multi-sample measurement technique using the microplate procedure was applied.

2.4. BCA protein analysis

The BCA Protein Assay is a widely used method for quantifying total protein content. Pierce BCA Protein Assays have a unique benefit over the Coomassie dye-based methods (Bradford). The methods are compatible with samples that contain up to 5% surfactants and are assumed much less by protein compositional variations, providing greater protein to protein uniformity. The BCA assay was performed according to established protocols, with various concentrations of a bovine serum albumin (BSA). Spectrophotometric measurements were conducted at 562 nm, correlating absorbance values to the protein concentration in the wheat samples. To prepare the protein standard, a 2 mg mL^{-1} albumin was diluted, and each 1 mL was sufficient to prepare the necessary standard. The prepared standards and sample extracts were placed on a microplate for measurement. Extracts containing Gliadin were diluted 50-fold, and then 25 µL of each was added to the microplate wells, followed by 200 µL of reactive BCA agent. The final concentrations of BSA for the standard curve, prepared with different volumes of ultrapure water, are shown in Table 2.

For each standard concentration, 25 µL of the diluted standard was pipetted into a microplate well, and 200 µL of reactive agent was added. The plate was incubated at 37°C for 30 minutes. Absorbance measurements were taken at 562 nm with a spectrophotometer. The plate was shaken for 30 seconds and then incubated at 37°C for 30 minutes before measuring the absorbance at 562 nm. All samples were measured in triplicate, and a standard curve graph (Figure 1) was created based on the average OD values. Both rainfed and irrigated trials were analyzed, and post-harvest physicochemical and rheological properties of the grain products from each plot were determined, as specified in the project proposal.

2.5. Statistical analysis

All chemical analyses were carried out in three replicates per plot and the results obtained were analyzed statistically. Significant differences between genotypes were analyzed by the ANOVA using the Minitab 17.0 statistical program. A test was performed to evaluate the significance of differences between the species means. Differences with $P<0.05$ were considered significant in both tests. The statistical analysis, particularly the ANOVA results, revealed significant differences in gliadin concentrations across both wheat varieties and cultivation conditions at a 1% significance level.

3. Results and Discussion

Gliadin, one of the key proteins in wheat that forms gluten, is essential for understanding the functional properties of flour, particularly in dough quality and elasticity (Shewry et al. 2002). The analysis revealed significant differences in gliadin concentrations between varieties, as well as between cultivation conditions. In the study, using Bicinchoninic Acid (BCA) assay, gliadin concentrations were quantified for 24 different ancient and modern wheat varieties grown under both rainfed and irrigated conditions. The results indicated that ancient wheat varieties, such as Einkorn and Gernik, exhibited higher gliadin concentrations, compared to modern wheat varieties. Notably,

Table 2. Results of BCA protein quantification and albumin standard measurement

Vial	Dilution Volume (µL)	BSA Standard Volume (µL)	Final Con. (µg mL^{-1})
A	0	300 µL BSA Stock	2000
B	125	375 µL BSA Stock	1500
C	325	325 µL BSA Stock	1000
D	175	175 µL from Diluted Vial B	750
E	325	325 µL from Diluted Vial C	500
F	325	325 µL from Diluted Vial E	250
G	325	325 µL from Diluted Vial F	125
H	400	100 µL from Diluted Vial G	25

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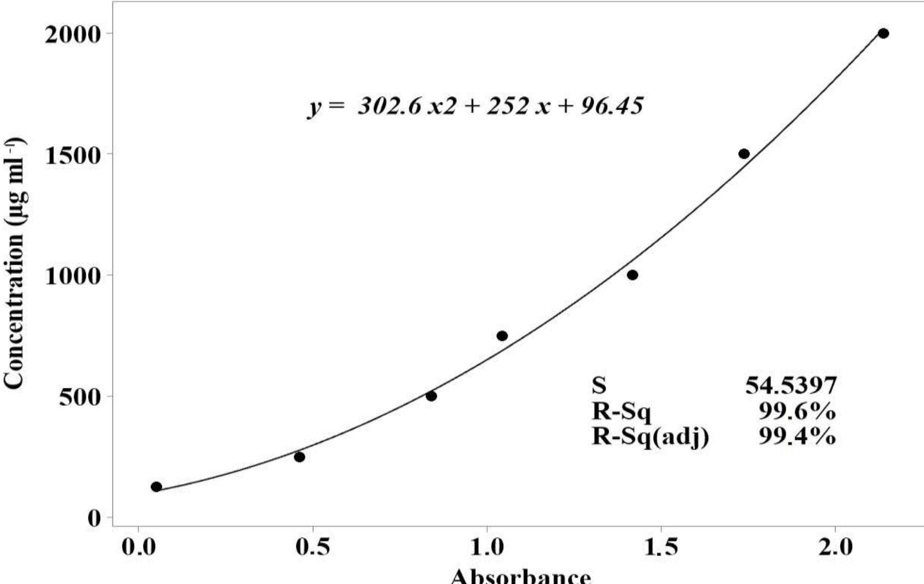

Figure 1. BSA standard concentration.

the Karahan variety, a more recent wheat genotype, displayed the highest gliadin concentration across both cultivation conditions, suggesting its potential use in high-protein applications such as bread-making. Surprisingly, despite their historical reputation for higher nutrient content, ancient varieties such as Einkorn showed moderate gliadin levels. (Dinu et al. 2018).

3.1. Gliadin concentration in ancient and modern wheat varieties

The gliadin concentrations in various ancient and modern wheat genotypes were quantified using the Bicinchoninic Acid (BCA) assay. The gliadin concentrations for the 24 wheat genotypes grown under irrigated conditions are illustrated in the interval plot of gliadin concentration (irrigated varieties) (Figure 1). The data were analyzed for both cultivation environments: irrigated and rainfed. Gliadin, a key protein involved in gluten formation, is a critical component in determining the quality of wheat flour for bread-making. The concentration of gliadin was compared between the different genotypes to assess the impact of both genotype and cultivation conditions on protein levels.

The results, presented in Figure 1, revealed substantial variability in gliadin content across the different genotypes. The Alparslan variety exhibited the highest gliadin concentration at approximately 16946 µg mL^{-1}, while Sönmez2001 had the lowest gliadin concentration at 10745 µg mL^{-1}. Among ancient wheat varieties, Einkorn had a higher gliadin concentration compared to other ancient varieties such as Yektay, Bezostaja1, Bolal, Gerek79, Kırkpınar, Sultan95, İkizce, and Pehlivan under irrigated conditions.

Einkorn's higher gliadin levels among older cultivars, especially under irrigated conditions, can be attributed to its genetic heritage and historical adaptation to less favorable growing conditions. When grown with regular irrigation systems, Einkorn can have an improved ability to synthesize considerable amounts of protein compared to other varieties that are selectively bred for different purposes.

Both Karahan and Alparslan, classified as old varieties developed through crossbreeding, stand out for their high gliadin concentrations. These varieties, developed after the Green Revolution (post-1970), exemplify how modern wheat breeding can benefit from the robustness of ancient varieties to enhance protein content. Karahan had a notable gliadin concentration (16339.07 µg mL^{-1}), making it one of the top performers. In contrast, varieties such as Bezostaja1, Bolal, and Gerek79, also developed through crossbreeding methods, displayed comparatively lower gliadin levels.

The results of the gliadin concentrations under rainfed conditions (Figure 2) provide further insights into the adaptability and protein synthesis capabilities of various wheat genotypes.

The interval plot of gliadin concentration for rainfed varieties (Figure 3) illustrates that gliadin concentrations were generally lower under rainfed conditions compared to irrigated conditions. On average, the gliadin concentration across all varieties decreased by approximately 15% under rainfed conditions. This trend aligns with existing research that highlights the importance of water availability in protein synthesis in wheat. Water stress can hinder nutrient uptake and metabolic activities essential for protein formation (Blum 2011).

Despite the overall reduction, certain varieties maintained high gliadin concentrations under rainfed conditions. Notably, Alparslan and Karahan recorded gliadin concentrations of 16946 µg mL^{-1} and 16343 µg mL^{-1}, respectively, values close to their levels under irrigated conditions, indicating a strong resilience to water stress. Alparslan, registered in 2001, is a new variety developed through crossbreeding post-Green Revolution. Its pedigree is TX-69-A-509-2//BBY2/FOX. The breeding methods utilized advanced selection techniques focusing on both yield and stress tolerance, which likely contributed to its high gliadin content even under rainfed conditions. Karahan registered in 1999 as an old variety but developed post-Green Revolution through crossbreeding. Its complex pedigree (C-126-15/COLLAFEN/3/NORIN-10-BREVOR/P-14//(P101) PULLMAN -101/4/KIRAC-66) includes contributions from drought-resistant lines such asNorin-10. This genetic background may enhance its ability to sustain protein synthesis under limited water availability (Lumpkin 2015).

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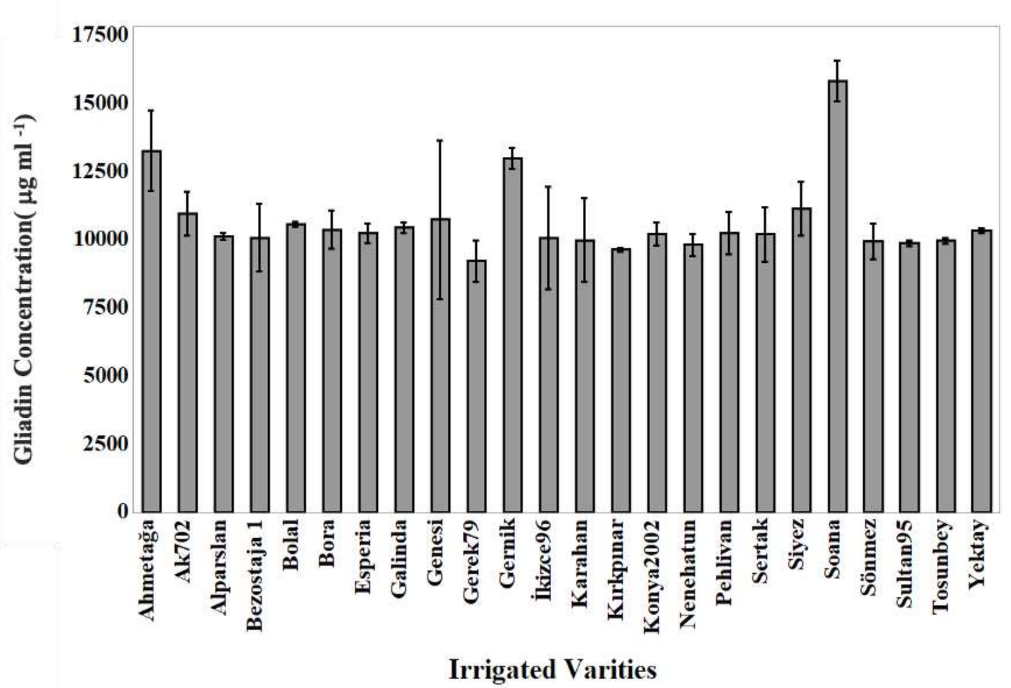

Figure 2. Interval plot of gliadin concentration (irrigated varieties).

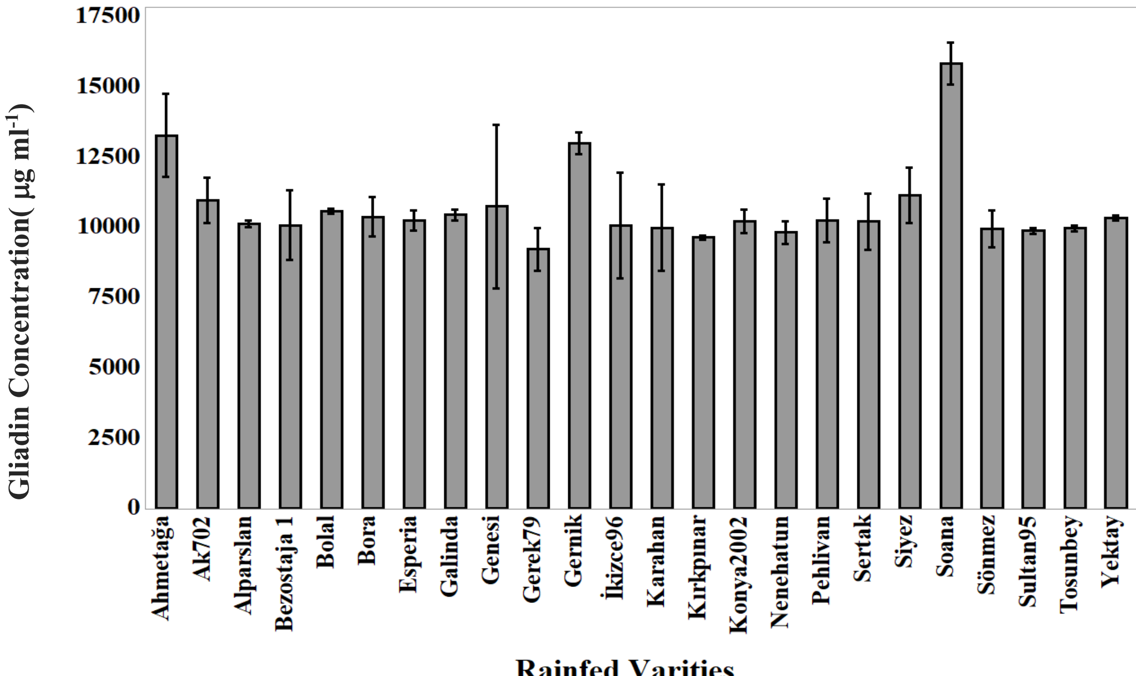

Figure 3. The interval plot of gliadin concentration (rainfed varieties).

Conversely, Sönmez2001, a new variety also registered in 2001 and developed through crossbreeding, displayed the lowest gliadin concentration at 10745 µg mL^{-1} under rainfed conditions. Its pedigree (BEZ//BEZ/TVR/3/KREMENA/LOV29/4/KATIA1) may lack specific traits associated with drought tolerance, making it more susceptible to water stress in terms of protein synthesis. Interestingly, the ancient variety Soana demonstrated a significant gliadin concentration of 13696 µg mL^{-1} under rainfed conditions, outperforming many modern varieties. Soana is classified as a new variety candidate registered in 2019 and developed through crossbreeding post-Green Revolution, with a pedigree of AQUILANTE/SOLEHIO.

The success of varieties such as Alparslan and Karahan demonstrates the potential of modern breeding to achieve high protein content even under sub-optimal conditions such as water scarcity.

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The Interval plot comparing irrigated and rainfed conditions (Figure 4) demonstrates that gliadin concentrations were significantly higher under irrigated conditions than under rainfed conditions. The mean gliadin concentration for irrigated varieties was approximately 12500 µg mL^{-1}, while that for rainfed varieties was around 11500 µg mL^{-1}. This statistically significant difference ($P<0.05$) highlights the importance of water availability in enhancing protein accumulation in wheat.

The grouping information from the Tukey test (Table 3 and Table 4) provided additional statistical insights into the differences between genotypes. Genotypes such as Alparslan, Karahan, and Ahmetağa were consistently grouped in the highest category (Group A), whereas Sönmez2001 was placed in the lowest group (Group G) across both conditions. This clear separation of genotypes is based on their gliadin concentrations, with significant differences observed between groups.

The post-hoc Tukey test further validated these differences, with varieties such as Karahan and Alparslan consistently grouped into higher gliadin concentration categories, while varieties such as Bezostaja1 and Tosunbey were grouped into lower categories. This highlights the distinct protein profiles of wheat varieties studied, underscoring the influence of their genetic backgrounds and breeding histories.

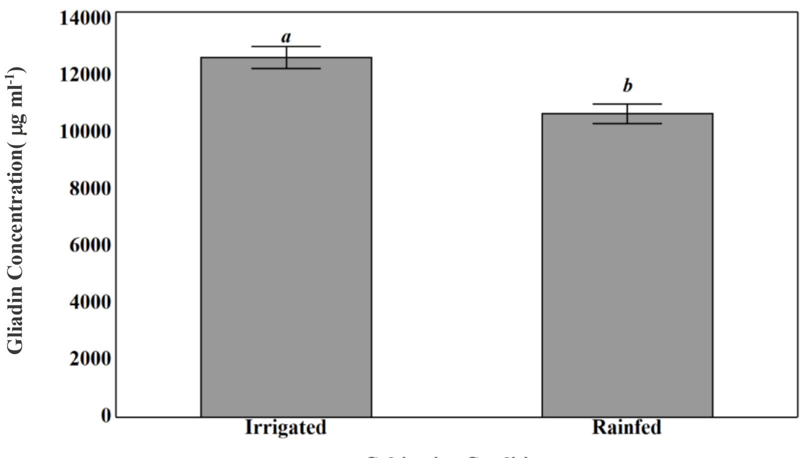

Figure 4. The interval plot comparing irrigated and rainfed conditions.

Table 3. Irrigated varieties grouping information using the Tukey method and 95% confidence

Varieties	Mean	Grouping						
Alparslan	16946±717	A						
Karahan	16343±595	A						
Ahmetağa	15646±190	A						
Einkorn	13714±137		B					
Soana	13697±145		B					
Kırkpınar	13115±154		B	C				
Pehlivan	12936±116		B	C	D			
Konya2002	12758±134		B	C	D	E		
Galinda	12565±095		B	C	D	E	F	
Gerek79	12524±225		B	C	D	E	F	G
Sultan95	12443±398		B	C	D	E	F	G
Tosunbey	12343±109		B	C	D	E	F	G
Ak702	12341±051		B	C	D	E	F	G
Gernik	12268±261		B	C	D	E	F	G
İkizce96	11770±651			C	D	E	F	G
Esperia	11749±315			C	D	E	F	G
Genesi	11735±484			C	D	E	F	G
Bezostaja1	11631±126			C	D	E	F	G
Yektay	11580±171			C	D	E	F	G
Bolal	11429±167				D	E	F	G
Bora	11306±258					E	F	G
Sertak	11186±058					E	F	G
Nenehatun	11016±627						F	G
Sönmez2001	10745±979							G

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Table 4. Rainfed varieties grouping information using the Tukey method and 95% confidence

Varieties	Mean	Grouping					
Soana	15828±298	A					
Ahmetağa	13267±595		B				
Gernik	12992±152		B				
Einkorn	11135±397			C			
Ak702	10965±328			C	D		
Genesi	10745±117			C	D	E	
Bolal	10576±034			C	D	E	
Galinda	10441±078			C	D	E	F
Bora	10376±280			C	D	E	F
Yektay	10341±034			C	D	E	F
Esperia	10253±144			C	D	E	F
Pehlivan	10243±314			C	D	E	F
Konya2002	10220±171			C	D	E	F
Sertak	10208±403			C	D	E	F
Alparslan	10130±047			C	D	E	F
Bezostaja1	10078±503			C	D	E	F
İkizce96	10078±758			C	D	E	F
Karahan	9987±620			C	D	E	F
Tosunbey	9964±043			C	D	E	F
Sönmez2001	9944±263			C	D	E	F
Sultan95	9878±309			C	D	E	F
Nenehatun	9821±163				D	E	F
Kırkpınar	9635±032						F
Gerek79	9226±307						F

4. Conclusion

The results obtained revealed significant variation in protein content among ancient wheat varieties, with notable differences between rainfed and irrigated growing conditions. The investigation into protein quantification in ancient wheat varieties collected from Türkiye under contrasting cultivation conditions underscores the significance of both genetic diversity and environmental influences on agricultural outputs. The application of the BCA assay provides a reliable method for protein quantification, highlighting its utility in assessing the nutritional quality of ancient grains. As the global agricultural landscape evolves, the preservation and promotion of ancient wheat varieties may play a key role in addressing food security, enhancing human health, and fostering sustainable agricultural practices. This study emphasizes the critical importance of detailed agricultural and nutritional informing strategies for the utilization and conservation of these invaluable genetic resources.

Acknowledgments

The authors thank to the Turkish National Science Foundation (TUBITAK) which provided financial support for this research under grant number 123O333.

References

Akar T, Cengiz M, Tekin M (2019) A comparative study of protein and free amino acid contents in some important ancient wheat lines. Quality Assurance and Safety of Crops & Foods 11: 191-200.

Blum A (2011) Genetic Resources for Drought Resistance. Plant Breeding for Water-Limited Environments. Springer New York, New York, NY, pp. 217-234.

Dinu M, Whittaker A, Pagliai G, Benedettelli S, Sofi F (2018) Ancient wheat species and human health: Biochemical and clinical implications. The Journal of Nutritional Biochemistry 52: 1-9.

Lumpkin TA (2015) How a Gene from Japan Revolutionized the World of Wheat: CIMMYT's Quest for Combining Genes to Mitigate Threats to Global Food Security. In: Ogihara Y, Takumi S, Handa H (eds) Advances in Wheat Genetics: From Genome to Field. Springer Japan, Tokyo, pp. 13-20.

Malalgoda M, Ohm J-B, Meinhardt S, Simsek S (2018) Association between gluten protein composition and breadmaking quality characteristics in historical and modern spring wheat. Cereal Chemistry 95: 226-238.

Osborne TB (1924) The vegetable proteins. Longmans, Green and Company.

Schoel B, Welzel M, Kaufmann SH (1995) Quantification of protein in dilute and complex samples: modification of the bicinchoninic acid assay. Journal of Biochemical Biophysical Methods 30: 199-206.

Shewry PR (2009) Wheat. Journal of Experimental Botany 60: 1537-1553.

Shewry PR, Halford NG, Belton PS, Tatham AS (2002) The structure and properties of gluten: an elastic protein from wheat grain. Philosophical Transactions of the Royal Society of London. Series B Biological Science 357: 133-142.

Shewry PR, Hey SJ (2015) The contribution of wheat to human diet and health. Food and Energy Security 4: 178-202.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150: 76-85.

Thanhaeuser SM, Wieser H, Koehler P (2015) Spectrophotometric and fluorimetric quantitation of quality-related protein fractions of wheat flour. Journal of Cereal Science 62: 58-65.

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Walker JM (1994) The Bicinchoninic Acid (BCA) Assay for Protein Quantitation. In: Walker JM (ed) Basic Protein and Peptide Protocols. Humana Press, Totowa, NJ, pp. 5-8.

Wiechelman KJ, Braun RD, Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. Analytical Biochemistry 175: 231-237.

Zilić S, Barać M, Pešić M, Dodig D, Ignjatović-Micić D (2011) Characterization of proteins from grain of different bread and durum wheat genotypes. International Journal of Molecular Science 12: 5878-5894.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 137-142
DOI: 10.29136/mediterranean.1540898

Cold stress impairs nitrogen uptake and enhances translocation through *AMT1* and *NRT2* gene regulation in tomato

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ARTICLE INFO

Received: August 30, 2024
Received in revised form: September 30, 2024
Accepted: October 2, 2024

Keywords:

Nitrogen metabolism
Cold stress
AMT1
NRT2
Tomato

ABSTRACT

Nitrogen is a vital nutrient for plant growth, playing a crucial role in various physiological processes. Cold stress significantly impacts plant physiology, including nitrogen uptake and translocation. This study investigates the effects of cold stress on nitrogen dynamics in tomato plants by examining the expression of ammonium (*AMT1*) and nitrate (*NRT2*) transporter genes. Under normal conditions, *AMT1* and *NRT2* genes are predominantly expressed in the roots, with varying levels of expression in other tissues. However, following exposure to cold stress, a significant downregulation of most *AMT1* and *NRT2* genes in the roots was observed, indicating a reduced capacity for nitrogen uptake and assimilation. Conversely, there was a notable upregulation of these genes in the leaves, suggesting an enhanced capacity for nitrogen translocation and metabolism under cold conditions. This differential expression between roots and leaves highlights the plant's adaptive mechanisms to cope with environmental stress. It indicates a strategy to conserve energy in the roots while increasing nutrient transport in the leaves to support metabolic adjustments. These insights into the molecular basis of nitrogen management under cold stress can inform strategies to enhance crop resilience and productivity.

1. Introduction

Nitrogen (N) is a crucial macronutrient and an essential component of key cellular molecules, including amino acids, nucleic acids, and chlorophyll (Hawkesford et al. 2012). Plants require nitrogen in larger quantities than any other mineral nutrient for optimal growth. They utilize various sources of nitrogen from the soil, including inorganic forms such as ammonium (NH_4^+) and nitrate (NO_3^-), as well as organic complexes like amino acids (Williams and Miller 2001). Among these, ammonium is often preferred due to its lower energy requirement for assimilation compared to nitrate (Bloom et al. 1992). However, nitrate (NO_3^-) is the predominant form of inorganic nitrogen in soil, therefore its uptake and translocation within plants exert a significant influence on their nitrogen use efficiency (Jin et al. 2015).

Nitrate (NO_3^-) is absorbed through the roots and leaves, and transported within the plant by several nitrate transporters, each with distinct features (Bai et al. 2013; Guan 2017). Nitrate assimilation is energy-intensive, requiring substantial amounts of ATP, reducing equivalents, and carbon (C) skeletons (Nunes-Nesi et al. 2010). It is regulated based on nitrogen availability in the environment and the plant's developmental needs. Environmental factors, such as drought and salinity, also influence the activation and deactivation of these systems (Yao et al. 2008). The first identified eukaryotic *NRT2* gene, *crn*A was isolated from *Aspergillus nidulans*, a filamentous fungus, approximately 35 years ago (Johnstone et al. 1990; Unkles et al. 1991). Based on their sequence homologies with *crn*A, a number of barley (Trueman et al. 1996), tobacco (Quesada et al. 1994), soybean (Amarasinghe et al. 1998) and tomato (Ono et al. 2000) *NRT2* genes have since been identified and functionally characterized.

The nitrogen assimilation process starts with the reduction of nitrate to ammonium, which is then incorporated into amino acids (Masclaux-Daubresse et al. 2010). Specific transporters in the plasma membrane are essential for the uptake of these ions by root cells (Shelden et al. 2001). To avoid toxicity, the uptake and metabolism of ammonium are tightly regulated (Sonoda et al. 2003a). The *AMT1* gene family, the first known ammonium transporter family, comprises high-affinity NH_4^+ transporters in Arabidopsis (Ninnemann et al. 1994). The expression of the *AMT1;1* gene in Arabidopsis roots was found to increase approximately four-fold under nitrogen deprivation (Shelden et al. 2001). It was reported that *OsAMT1;1* is the most consistently expressed *AMT1* gene in both roots and shoots, while *OsAMT1;2* and *OsAMT1;3* are primarily expressed in roots (Ninnemann et al. 1994). The transcription factor OsDOF18 regulates ammonium transport and nitrogen distribution by modulating the *OsAMT1;1*, *OsAMT1;3*, *OsAMT2;1*, and *OsAMT4;1* genes (Wu et al. 2017). In tomatoes, *LmAMT1;1*, *LmAMT1;2*, and *LmAMT1;3* exhibit different expression patterns in leaves and root hairs under nitrogen deficiency, varying CO_2 levels, and different light conditions (von Wiren et al. 2000).

Tomato (*Solanum lycopersicum*, *Sl*) is the second most widely consumed vegetable worldwide, following the potato, and plays a critical role in the food industry (FAO 2022). Due to its extensive cultivation, tomato crops require significant amounts

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of nitrogenous fertilizers. However, cold stress can disrupt nitrogen accumulation in tomatoes by impairing their ability to absorb and utilize nitrogen efficiently. This disruption can lead to reduced growth, poor fruit production, and inefficient nitrogen use, ultimately affecting overall plant health and productivity (Bhattacharya 2022; Soualiou et al. 2022). Three *AMT1* and four *NRT2* genes have been recently identified and characterized in the tomato genome, with their expression profiles thoroughly analyzed under conditions of drought and salinity stress (Akbudak et al. 2022; Filiz and Akbudak 2020). The present study aimed to expand on this by examining the expression profiles of these genes under cold stress conditions.

2. Material and Methods

2.1. Plant materials and stress treatment

S. lycopersicum (Istek F1; Istanbul Agriculture Co.) plants were grown in a 3:1 peat-to-perlite mixture at 25°C with 50% humidity under a 16-hour photoperiod in a greenhouse for four weeks. Control plants remained in the greenhouse, while treatment plants were exposed to 4°C in a growth chamber for 24 hours. Following the cold treatment, leaves and roots were harvested for RNA isolation.

2.2. RNA isolation and gene expression analysis

RNA was isolated from leaf and root tissues using the RNA Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions. The RNA samples were then treated with RQ1 RNase-Free DNase (Promega, USA). Gel electrophoresis was used to verify the RNA's integrity and check for DNA contamination. RNA quantities were measured with a Qubit fluorometer (Invitrogen, USA). RT-qPCR was performed on a CFX384 Real Time PCR System (Bio-Rad, USA). Gene expression was quantified using 10 ng of RNA per sample with the Luna Universal One-Step RT-qPCR Kit (NEB, USA). Forward and reverse primers (Table 1) were utilized for the RT-qPCR analysis (Akbudak et al. 2022; Filiz and Akbudak 2020). The *actin isoform B* (*Actin*) gene served as an endogenous reference control (Goupil et al. 2009).

2.3. Chromosomal distribution

The locations of the *SlAMT1* and *SlNRT2* genes on each chromosome were obtained from the tomato genome database (Ensembl Plants), and their chromosomal distribution was illustrated using the Mapgene2chrom 2.1 (MG2C v2.1) online tool (http://mg2c.iask.in/mg2c_v2.1/) (Chao et al. 2015; Chao et al. 2021).

2.4. Digital expression pattern

Data from the Tomato Genome Consortium (2012) was obtained to analyze the expression patterns of *SlAMT1* and *SlNRT2* genes. The expression profiles of these genes were examined across different anatomical parts and developmental stages. The heatmap was drawn using the Heatmap program in TBtools software.

3. Results

3.1. Chromosomal distribution of *SlAMT1* and *SlNRT2* genes

In *S. lycopersicum*, the ammonium transporter 1 (*AMT1*) gene family consists of three members (Filiz and Akbudak 2020), while the nitrate transporter 2 (*NRT2*) gene family has four members (Akbudak et al. 2022). Fig. 1 shows their chromosomal distribution, revealing that the *SlAMT1* and *SlNRT2* genes are spread across six different chromosomes. Notably, aside from *SlNRT2.2* and *SlNRT2.3*, none of the genes are located on the same chromosome.

Table 1. Primers used in the RT-qPCR analysis of *SlAMT1* and *SlNRT2* genes

Gene	Phytozome ID	Forward Primer (5′➔3′)	Reverse Primer (5′➔3′)
AMT1.1	Solyc09g090730	TCGCTAAAGGGGAGTTTGTG	GATTATATGCGCCCCGAGTA
AMT1.2	Solyc04g050440	CAGCAATCACGTCAGGTTGT	AGCTGCCAATGCGTTAAATC
AMT1.3	Solyc03g045070	CCTGTTGTTGCTCATTGGCT	ATCCCACCAACCAAATGCAC
SlNRT2.1	Solyc02g067790	TGGGCTTGCTAATGGATTCG	ATCGCTGGGAAAATGAACGC
SlNRT2.2	Solyc06g010250	GGCAGAGCAGAAACACTTCC	TCAATTGCGTTAAACCACCA
SlNRT2.3	Solyc06g074990	TAGTGAACGGAACGGCTGCT	CACGATTTCCGTCGGGTAAA
SlNRT2.4	Solyc11g069750	TTTTGCTGCTGCCCCTTTAG	TACTACCGGAAACTGAGGCAAC
SlActin	Solyc03g078400	GGGATGGAGAAGTTTGGTGGTGG	CTTCGACCAAGGGATGGTGTAGC

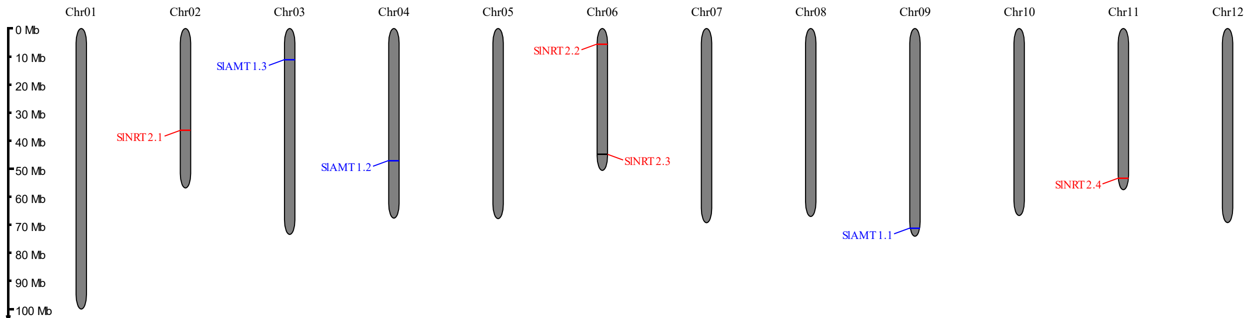

Figure 1. Chromosomal positions of *AMT1* and *NRT2* genes in the tomato genome generated in the MG2C tool. The chromosome number is shown at the top of each chromosome. The genome scale in megabases (Mb) is given on the left.

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3.2. Digital expression profile of SlAMT1 and SlNRT2 genes

The expression data of *SlAMT1* and *SlNRT2* genes were retrieved using the RNASeq Expression Browser. A heat map of the gene FPKM values was then constructed using TBtools software. The expression data indicates that all *SlAMT1* and *SlNRT2* genes are predominantly expressed in roots under regular conditions, except for *SlAMT1-3* and *SlNRT2-1* (Fig. 2). Among the *SlAMT1* and *SlNRT2* genes, *SlNRT2-3* exhibits the highest expression, followed by *SlAMT1-1*. In most tissues other than roots, the expression of *SlAMT1* and *SlNRT2* genes is either barely detectable or absent. However, *AMT1-3* is predominantly and robustly expressed in leaf tissues. *SlNRT2-1* shows relatively consistent low expression across tissues and stages, with the highest expression observed in buds. *SlNRT2-2* has a higher expression in buds and roots. *NRT2-3* stands out with extremely high expression in roots. *SlNRT2-4* generally displays very low or zero expression across most conditions, suggesting minimal activity.

For the *SlAMT1* genes, *SlAMT1-1* exhibits high expression in flowers and roots, indicating significant involvement in these tissues. *SlAMT1-2* shows higher expression in buds and roots. *SlAMT1-3* shows significantly high expression in leaves compared to other tissues, indicating a potential key role in leaves. Fig. 2 highlights that, except for *SlNRT2-3* in roots, *SlNRT2* genes are generally less active across all organs and developmental stages compared to *SlAMT1* genes in the tomato genome. The figure also highlights the specific tissues in which certain genes are highly or minimally expressed, with *SlNRT2-3* having dominant expression in roots, *SlAMT1-1* showing notable expression in flowers and roots, and *SlAMT1-3* having high expression in leaves. *SlNRT2-4* generally exhibits low expression.

3.3. Expression profiles of AMT1 and NRT2 genes in tomato under cold stress

Under cold stress, the expression patterns of *AMT1* and *NRT2* genes in tomato root and leaf tissues show significant variations, highlighting their unique physiological responses to the cold stress (Fig. 3). In root tissues, the expression of *SlAMT1.1* shows a modest upregulation with a fold difference of 0.61. In contrast, the other SlAMT family genes, specifically *SlAMT1.2* and *SlAMT1.3*, are downregulated with fold differences of -0.45 and -2.39, respectively. This pattern suggests that cold stress may selectively inhibit certain ammonium transporters in roots. The SlNRT family genes also exhibit pronounced downregulation, with *SlNRT2.1* showing the most significant decrease at -3.92-fold difference. *SlNRT2.2*, *SlNRT2.3*, and *SlNRT2.4* follow this trend with fold differences of -0.93, -0.50, and -0.28, respectively. This widespread downregulation in roots indicates a potential reduction in nitrate transport and assimilation capacity under cold stress.

Conversely, in leaf tissues, the response to cold stress differs. *SlAMT1.1* shows slight upregulation with a fold difference of 0.98, and *SlAMT1.2* is significantly upregulated with a 4.02-fold difference, suggesting an increased demand for ammonium

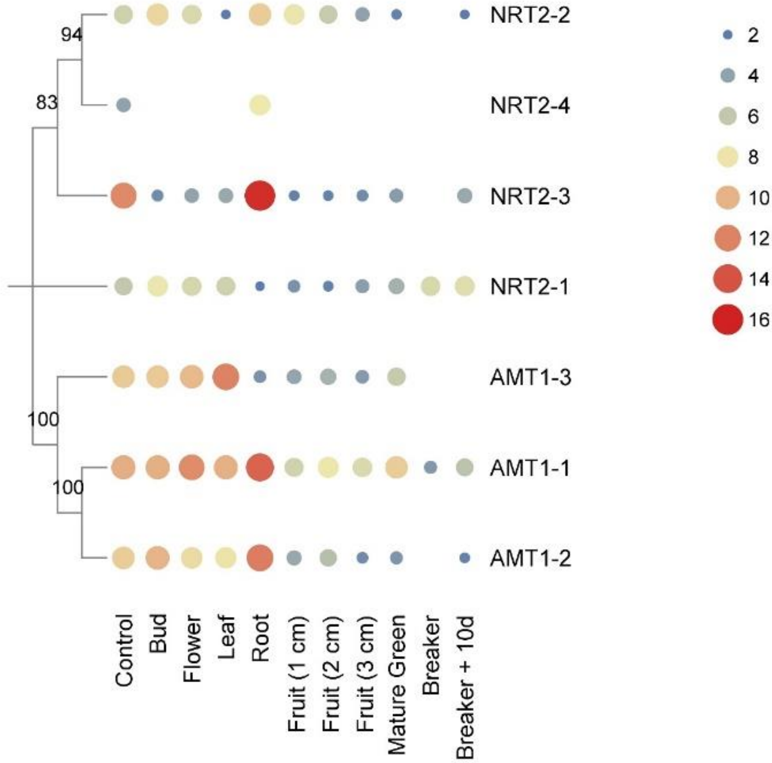

Figure 2. Heatmap of the expression profiles of *AMT1* and *NRT2* genes across various organs and developmental stages in tomato. Hierarchical clustering was employed to generate the heatmap, which was visualized using TBtools software. Expression values were log2-transformed and normalized. In the heatmap, blue elements indicate low relative expression levels, while red elements indicate high relative expression levels.

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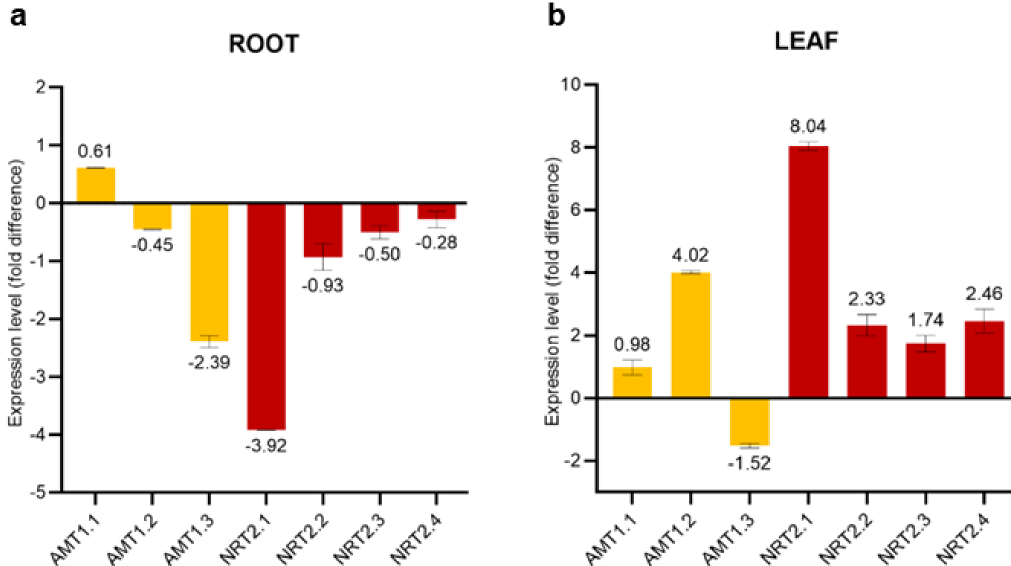

Figure 3. Expression profiling of *AMT1* (a) and *NRT2* (b) genes in tomato leaves and roots subjected to cold treatment, measured by RT-qPCR. Bars above the x-axis indicate upregulation, while bars below the x-axis represent downregulation. Gene expression values are shown on a log2 scale to effectively display the magnitude of both upregulated and downregulated genes. Error bars represent the standard deviation of the mean (SDOM; n= 3).

transport in leaves under cold conditions. *SlAMT1.3*, however, is downregulated with a fold difference of -1.52. The SlNRT family genes in leaves exhibit strong upregulation under cold stress, with *SlNRT2.1* showing a dramatic increase at 8.04-fold difference. *SlNRT2.2*, *SlNRT2.3*, and *SlNRT2.4* are also upregulated, with fold differences of 2.33, 1.74, and 2.46, respectively. This upregulation suggests an enhanced capacity for nitrate transport in leaves, likely to support metabolic adjustments and stress responses under cold conditions.

4. Discussion

The ability of plants to adapt to abiotic stresses, such as cold, is crucial for their survival and productivity. Among the various genes involved in stress responses, AMT1 and NRT2 are essential for nutrient uptake and assimilation (Goel and Singh 2015). Investigating their expression profiles under cold stress can shed light on the adaptive mechanisms of plants and potentially guide agricultural practices to enhance crop resilience.

Several plant species have been found to contain multiple ammonium transporters (AMTs) and nitrate transporters (NRTs). Specifically, Arabidopsis has six AMTs (Gazzarrini et al. 1999), rice has 10 (Sonoda et al. 2003b), soybean has 16 (Kobae et al. 2010), and poplar also has 16 (Wu et al. 2015). In the genome of *Saccharum spontaneum*, researchers have identified 178 *NRT1*, 20 *NRT2*, and six *NRT3* genes distributed across all eight chromosomes (Wang et al. 2019). Furthermore, in wild soybean (*Glycine soja*), 120 *NRT1* and five *NRT2* genes have been discovered (You et al. 2020). In the potato genome, there are 33 *NRT1*, four *NRT2*, and two *NRT3* genes, which show a closer similarity to Arabidopsis *NRT* genes than to those of rice (Zhang et al. 2021). These genes, along with their protein motifs, are conserved in both genomic and peptide sequences, playing essential roles in plant growth, development, and stress adaptation.

AMT gene expression is tightly regulated by the plant's nitrogen status. Under nitrogen-deficient conditions, *AMT* genes are upregulated to boost ammonium uptake (Loqué et al. 2006). Conversely, the availability of ammonium or nitrate can differentially influence *AMT* gene expression, with some genes responding more strongly to ammonium than to nitrate (Dechorgnat et al. 2019). This regulation is crucial for maintaining nitrogen homeostasis and supporting plant growth across varying nutrient conditions.

AMTs are integral to ammonium uptake, translocation, and overall nitrogen management in plants. Beyond their primary role in ammonium uptake, AMTs also play a role in plant responses to abiotic stresses. For instance, overexpression of certain AMTs in Arabidopsis has been shown to enhance root growth under salt stress, indicating that these transporters may mitigate ammonium toxicity under stress (Yi et al. 2020). Similarly, *AMT* genes are upregulated in response to drought stress in species like *Populus simonii* and *Malus prunifolia*, suggesting their involvement in improving nitrogen uptake and metabolism under adverse conditions (Huang et al. 2018). In rice, a key staple crop cultivated in flooded conditions, there are at least 12 *AMT* genes categorized into four subfamilies: *OsAMT1*, *OsAMT2*, *OsAMT3*, and *OsAMT4* (Al-Tawaha et al. 2020). *OsAMT1* subfamily members primarily function as high-affinity transporters, while the other subfamilies mostly consist of low-affinity transporters. Research indicates that knockout of certain *OsAMT1* genes significantly reduces ammonium uptake in rice, highlighting their critical role in nitrogen acquisition (Li et al. 2016). These genes are expressed in various tissues, including the root stele, vascular bundles, and mesophyll cells, and are involved in translocating ammonium from roots to shoots.

Ammonium is considered a superior nitrogen source for plants because its absorption and utilization require less energy compared to nitrate (Li et al. 2013). Filiz and Akbudak (2020) found that all *SlAMT1* genes were mainly downregulated (up to 6-fold) in leaf and root tissues under drought and salt stresses. In this study, under cold stress, the expression of all *NRT* genes, as well as *SlAMT1.2* and *SlAMT1.3*, was downregulated, while *SlAMT1.1* was the only gene upregulated, showing a 0.61-fold increase. This result aligns with literature indicating that under

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cold stress, tomato plants reduce nitrogen accumulation, especially in nitrate form, to conserve energy.

Understanding AMTs' roles and regulation in different crops, particularly under stress conditions, can offer valuable insights for improving crop productivity and stress resilience. Comprehensive characterization of *AMT* genes in crops presents potential strategies for enhancing nitrogen use efficiency and developing varieties with better tolerance to nutrient deficiencies and environmental stresses. Abiotic stresses such as salinity and drought significantly alter plant transcriptomes. Previous research has demonstrated that *SlNRT* genes exhibit gene- and tissue-specific responses under salt and drought conditions (Pu et al. 2023). However, these genes follow similar expression patterns in response to cold stress. Although their expression levels vary, all *SlNRT* genes are consistently downregulated in roots and upregulated in leaves. Diverse expression patterns of *NRT2* genes have also been observed in other plants such as potato, cassava, rapeseed, wild sugarcane, and apple, often showing tissue-specific and stress-responsive regulation. Pu et al. (2023) analyzed the expression profile of seven cotton *NRT2* genes under salt, drought, cold, and heat stresses and found no significant differences in the regulation of five genes, while two were downregulated compared to the control.

Overall, the expression results from this study reveal a clear divergence in gene regulation between roots and leaves under cold stress. Roots generally downregulate both ammonium and nitrate transport genes, possibly to conserve energy and resources under adverse conditions. In contrast, leaves upregulate these genes, likely to enhance nutrient transport and support cold stress mitigation processes. This differential expression underscores the adaptive strategies of plants, where distinct tissues modulate their genetic responses to optimize survival and function under environmental stress.

5. Conclusion

The analysis of gene expression under cold stress in tomato root and leaf tissues reveals several key insights into how plants adapt to environmental challenges. There is a clear difference in how genes are regulated in roots compared to leaves under cold stress. Roots tend to downregulate the expression of most ammonium and nitrate transporter genes, while leaves show an upregulation of these genes. This suggests that different parts of the plant prioritize different strategies to cope with cold stress.

The downregulation of both *SlAMT* and *SlNRT* genes in roots implies a strategy focused on conserving energy. Since nutrient uptake and transport require significant metabolic energy, reducing the expression of these transporters could help the plant minimize energy expenditure in roots during stressful conditions. This energy conservation could be critical for maintaining root viability when overall metabolic activity is compromised due to cold stress. On the other hand, the upregulation of *SlAMT* and *SlNRT* genes in leaves indicates a response aimed at enhancing nutrient transport. Leaves, being the site of photosynthesis and other metabolic activities, may require increased nutrient uptake to sustain these processes and to support the synthesis of stress-related proteins and metabolites. By boosting the expression of these transporters, leaves can maintain their metabolic functions and potentially improve cold tolerance.

The contrasting expression patterns underscore the importance of tissue-specific responses to environmental stress. While roots focus on reducing metabolic load, leaves ramp up their nutrient transport capabilities. This division of labor highlights the complex and coordinated nature of plant responses to stress, ensuring that different tissues contribute optimally to the overall survival strategy. The differential expression of genes also points to the adaptive significance of such regulatory mechanisms. By selectively modulating gene expression, plants can fine-tune their physiological responses to meet the demands of different tissues under stress. This ability to differentially regulate gene activity is crucial for plants to thrive in varying environmental conditions.

In summary, the research suggests that under cold stress, plants employ a nuanced approach where roots conserve energy by downregulating nutrient transporters, while leaves enhance their capacity to transport nutrients, thereby supporting essential metabolic activities. This adaptive strategy reflects the plant's need to balance resource allocation and metabolic demands across different tissues to optimize survival and function under adverse conditions.

Acknowledgements

MAA is thankful for the financial support provided by the Fulbright Visiting Scholar Grant from the Turkish Fulbright Commission.

Authors' Contribution

DC: Investigation and Data curation, MAA: Conceptualization, Writing- Original Draft, Writing- Review & Editing, Supervision.

References

Akbudak MA, Filiz E, Çetin D (2022) Genome-wide identification and characterization of high-affinity nitrate transporter 2 (NRT2) gene family in tomato (Solanum lycopersicum) and their transcriptional responses to drought and salinity stresses. Journal of Plant Physiology 272: 153684.

Al-Tawaha ARMS, Singh S, Singh V, Kafeel U, Naikoo MI, Kumari A, Imran, Amanullah, Al-Tawaha AR, Qaisi AM (2020) Improving water use efficiency and nitrogen use efficiency in rice through breeding and genomics approaches. In: Roychoudhury, A. (eds) Rice Research for Quality Improvement: Genomics and Genetic Engineering. Springer, Singapore. 307-337.

Amarasinghe BHRR, de Bruxelles GL, Braddon M, Onyeocha I, Forde BG, Udvardi MK (1998) Regulation of GmNRT2 expression and nitrate transport activity in roots of soybean (Glycine max). Planta 206: 44-52.

Bai H, Euring D, Volmer K, Janz D, Polle A (2013) The Nitrate Transporter (NRT) Gene Family in Poplar. Plos One 8 (8): e72126.

Bhattacharya A (2022) Low-temperature stress and nitrogen metabolism in plants: A review. Physiological processes in plants under low temperature stress. Springer Singapore 299-407.

Bloom AJ, Sukrapanna SS, Warner RL (1992) Root respiration associated with ammonium and nitrate absorption and assimilation by barley. Plant Physiol 99: 1294-1301.

Chao J-T, Kong Y-Z, Wang Q, Sun Y-H, Gong D-P, Lv J, Liu G-S (2015) MapGene2Chrom, a tool to draw gene physical map based on Perl and SVG languages. Yi chuan= Hereditas 37: 91-97.

Chao J, Li Z, Sun Y, Aluko OO, Wu X, Wang Q, Liu G (2021) MG2C: A user-friendly online tool for drawing genetic maps. Molecular Horticulture 1: 1-4.

Dechorgnat J, Francis KL, Dhugga KS, Rafalski JA, Tyerman SD, Kaiser BN (2019) Tissue and nitrogen-linked expression profiles of ammonium and nitrate transporters in maize. Bmc Plant Biology 19: 1-13.

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FAO (2022) Food and Agriculture Organization of the United Nations. Rome.

Filiz E, Akbudak MA (2020) Ammonium transporter 1 (AMT1) gene family in tomato (Solanum lycopersicum L.): Bioinformatics, physiological and expression analyses under drought and salt stresses. Genomics 112: 3773-3782.

Gazzarrini S, Lejay L, Gojon A, Ninnemann O, Frommer WB, von Wiren N (1999) Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into arabidopsis roots. Plant Cell 11: 937-947.

Goel P, Singh AK (2015) Abiotic Stresses Downregulate Key Genes Involved in Nitrogen Uptake and Assimilation in Brassica juncea L. Plos One 10(11): e0143645.

Goupil P, Souguir D, Ferjani E, Faure O, Hitmi A, Ledoigt G (2009) Expression of stress-related genes in tomato plants exposed to arsenic and chromium in nutrient solution. Journal of Plant Physiology 166: 1446-1452.

Guan PZ (2017) Dancing with Hormones: A Current Perspective of Nitrate Signaling and Regulation in Arabidopsis. Frontiers in Plant Science 8.

Hawkesford M, Horst W, Kichey T, Lambers H, Schjoerring J, Møller IS, White P (2012) Chapter 6 - Functions of Macronutrients. In: Marschner P (ed) Marschner's Mineral Nutrition of Higher Plants (Third Edition). Academic Press, San Diego, pp: 135-189.

Huang L, Li M, Zhou K, Sun T, Hu L, Li C, Ma F (2018) Uptake and metabolism of ammonium and nitrate in response to drought stress in Malus prunifolia. Plant Physiology and Biochemistry 127: 185-193.

Jin Z, Zhu YJ, Li XR, Dong YS, An ZS (2015) Soil N retention and nitrate leaching in three types of dunes in the Mu Us desert of China. Scientific Reports 5.

Johnstone IL, Mccabe PC, Greaves P, Gurr SJ, Cole GE, Brow MAD, Unkles SE, Clutterbuck AJ, Kinghorn JR, Innis MA (1990) Isolation and Characterization of the Crna-Niia-Niad Gene-Cluster for Nitrate Assimilation in Aspergillus-Nidulans. Gene 90: 181-192.

Kobae Y, Tamura Y, Takai S, Banba M, Hata S (2010) Localized Expression of Arbuscular Mycorrhiza-Inducible Ammonium Transporters in Soybean. Plant and Cell Physiology 51: 1411-1415.

Li S-X, Wang Z-H, Stewart BA (2013) Responses of crop plants to ammonium and nitrate N. Advances in agronomy 118: 205-397.

Li C, Tang Z, Wei J, Qu H, Xie Y, Xu G (2016) The OsAMT1. 1 gene functions in ammonium uptake and ammonium–potassium homeostasis over low and high ammonium concentration ranges. Journal of Genetics and Genomics 43: 639-649.

Loqué D, Yuan L, Kojima S, Gojon A, Wirth J, Gazzarrini S, Ishiyama K, Takahashi H, Von Wirén N (2006) Additive contribution of AMT1; 1 and AMT1; 3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. The Plant Journal 48: 522-534.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany 105: 1141-1157.

Ninnemann O, Jauniaux JC, Frommer WB (1994) Identification of a High-Affinity Nh4+ Transporter from Plants. Embo Journal 13: 3464-3471.

Nunes-Nesi A, Fernie AR, Stitt M (2010) Metabolic and Signaling Aspects Underpinning the Regulation of Plant Carbon Nitrogen Interactions. Molecular Plant 3: 973-996.

Ono F, Frommer WB, von Wiren N (2000) Coordinated diurnal regulation of low- and high-affinity nitrate transporters in tomato. Plant Biology 2: 17-23.

Pu Y, Wang P, Abbas M, Khan MA, Xu J, Yang Y, Zhou T, Zheng K, Chen Q, Sun G (2023) Genome-wide identification and analyses of cotton high-affinity nitrate transporter 2 family genes and their responses to stress. Frontiers in Plant Science 14: 1170048.

Quesada A, Galvan A, Fernandez E (1994) Identification of Nitrate Transporter Genes in Chlamydomonas-Reinhardtii. Plant Journal 5: 407-419.

Shelden MC, Dong B, de Bruxelles GL, Trevaskis B, Whelan J, Ryan PR, Howitt SM, Udvardi MK (2001) Arabidopsis ammonium transporters, AtAMT1;1 and AtAMT1;2, have different biochemical properties and functional roles. Plant and Soil 231: 151-160.

Sonoda Y, Ikeda A, Saiki S, Yamaya T, Yamaguchi J (2003a) Feedback regulation of the ammonium transporter gene family AMT1 by glutamine in rice. Plant and Cell Physiology 44: 1396-1402.

Sonoda Y, Ikeda A, Saiki S, von Wiren N, Yamaya T, Yamaguchi J (2003b) Distinct expression and function of three ammonium transporter genes (OsAMT1;1-1;3) in rice. Plant and Cell Physiology 44: 726-734.

Soualiou S, Duan F, Li X, Zhou W (2022) Crop production under cold stress: An understanding of plant responses, acclimation processes, and management strategies. Plant Physiology and Biochemistry 190: 47-61.

Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485: 635.

Trueman LJ, Richardson A, Forde BG (1996) Molecular cloning of higher plant homologues of the high-affinity nitrate transporters of Chlamydomonas reinhardtii and Aspergillus nidulans. Gene 175: 223-231.

Unkles SE, Hawker KL, Grieve C, Campbell EI, Montague P, Kinghorn JR (1991) Crna Encodes a Nitrate Transporter in Aspergillus-Nidulans. Proceedings of the National Academy of Sciences of the United States of America 88: 204-208.

von Wiren N, Lauter FR, Ninnemann O, Gillissen B, Walch-Liu P, Engels C, Jost W, Frommer WB (2000) Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. Plant Journal 21: 167-175.

Wang J, Li YX, Zhu F, Ming R, Chen LQ (2019) Genome-Wide Analysis of Nitrate Transporter (NRT/NPF) Family in Sugarcane Saccharum spontaneum L. Tropical Plant Biology 12: 133-149.

Williams LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annual Review of Plant Physiology and Plant Molecular Biology 52: 659-+.

Wu XY, Yang H, Qu CP, Xu ZR, Li W, Hao BQ, Yang CP, Sun GY, Liu GJ (2015) Sequence and expression analysis of the AMT gene family in poplar. Frontiers in Plant Science 6.

Wu Y, Yang W, Wei J, Yoon H, An G (2017) Transcription Factor OsDOF18 Controls Ammonium Uptake by Inducing Ammonium Transporters in Rice Roots. Mol Cells 40: 178-185.

Yao J, Shi WM, Xu WF (2008) Effects of salt stress on expression of nitrate transporter and assimilation-related genes in tomato roots. Russian Journal of Plant Physiology 55: 232-240.

Yi LI, Jinyan Z, Dongli HAO, Shunying Y, Yanhua SU (2020) Arabidopsis under ammonium over-supply: Characteristics of ammonium toxicity in relation to the activity of ammonium transporters. Pedosphere 30: 314-325.

You HG, Liu YM, Minh TN, Lu HR, Zhang PM, Li WF, Xiao JL, Ding XD, Li Q (2020) Genome-wide identification and expression analyses of nitrate transporter family genes in wild soybean (Glycine soja). Journal of Applied Genetics 61: 489-501.

Zhang JY, Han ZJ, Lu Y, Zhao YF, Wang YP, Zhang JY, Ma HR, Han YZ (2021) Genome-wide identification, structural and gene expression analysis of the nitrate transporters (NRTs) family in potato (Solanum tuberosum L.). Plos One 16 (10): e0257383.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 143-146
DOI: 10.29136/mediterranean.1551310

Comparative analysis of some multiple sequence alignment tools using *Gallus gallus* COX1 sequences

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ARTICLE INFO

Received: September 16, 2024
Received in revised form: November 4, 2024
Accepted: November 14, 2024

***Keywords*:**

Multiple sequence alignment
ClustalW
Clustal Omega
MUSCLE
MAFFT

ABSTRACT

Multiple Sequence Alignment (MSA) is an essential method in bioinformatics for detecting conserved sequence regions and deducing evolutionary relationships. However, performance variability exists among MSA tools, and different tools yield varying results depending on the dataset. This study conducts a comparative evaluation of four widely used MSA tools: ClustalW, Clustal Omega, MUSCLE, and MAFFT. The alignment quality and processing efficiency of these tools were assessed using 40 randomly selected *Gallus gallus* cytochrome c oxidase subunit 1 (COX1) DNA sequences. The findings offer valuable insights into the specific contexts in which these tools may be most effective. MAFFT demonstrated a notable advantage in processing speed, while Clustal Omega and MAFFT excelled in Column Score (CS). For Total Consensus (TC) score, ClustalW and MUSCLE showed superior performance, and Clustal Omega exhibited the highest performance based on Root Mean Square Deviation (RMSD) values. No significant difference was observed between the tools in terms of the Sum-of-Pairs (SP) score. This study serves as a valuable resource for researchers seeking to optimize the use of MSA tools for their specific applications.

1. Introduction

Multiple Sequence Alignment (MSA) is a cornerstone technique in bioinformatics, widely employed for identifying conserved sequence regions, elucidating evolutionary relationships, and predicting the structure and function of biological macromolecules (Levasseur et al. 2008; Pervez et al. 2014). With the advent of advanced sequencing technologies, the accumulation of vast amounts of DNA data has made MSA an indispensable tool across various research fields. In particular, MSA has become increasingly important in plant and animal biotechnology, where it aids in studying genetic diversity and enhancing disease resistance in plant and animal genomes (Ferrer-Costa et al. 2005; Prykhozhij 2015; Chowdhury and Garai 2017; Park et al. 2017).

However, the effectiveness of MSA heavily depends on the performance of the alignment tools used. Over time, several MSA algorithms have been developed, each employing different strategies to align sequences. One of the earliest and most widely used tools, ClustalW (www.clustal.org), follows a progressive alignment approach (Thompson et al. 1994), though it can produce suboptimal alignments due to the “once a gap, always a gap” issue, particularly with highly divergent sequences. Clustal Omega (www.clustal.org), an improved version, addresses some of these limitations by offering faster and more accurate alignments, especially for large datasets (Sievers and Higgins, 2018). MUSCLE (www.drive5.com) utilizes an iterative strategy that refines the guide tree and alignment to improve accuracy progressively, while MAFFT incorporates Fast Fourier Transform (FFT) to balance speed and accuracy (Katoh et al. 2002).

Despite the diversity of available tools, comparative studies reveal that no single method consistently outperforms others across all datasets and scenarios (Nuin et al. 2006; Aniba et al. 2010; Thompson et al. 2011; Pais et al. 2014). This variability necessitates a careful evaluation and selection of MSA tools based on the specific requirements of each study. To address this need, our study conducts a comprehensive evaluation of four prominent MSA tools: ClustalW, Clustal Omega, MUSCLE, and MAFFT, using COX1 DNA sequences from 40 randomly selected *Gallus gallus* specimens. By assessing alignment accuracy and computational efficiency across various metrics, including Time, Sum-of-Pairs (SP) score, Column Score (CS), Total Consensus (TC) score, and Root Mean Square Deviation (RMSD), we aim to provide a detailed analysis of each tool's strengths and weaknesses. This comparison will offer valuable guidance for researchers, helping them select the most appropriate MSA tool for their specific applications, thereby enhancing the reliability and reproducibility of bioinformatics analyses.

2. Material and Method

2.1. Dataset selection

In this study, DNA sequences from *Gallus gallus* were utilized. A dataset of 40 randomly selected COX1 (1548 bp)

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(Gene ID: 807639) DNA sequences was obtained from the NCBI (National Center for Biotechnology Information) database. These sequences, which vary in length and genetic variation, were chosen to provide a comprehensive evaluation of multiple sequence alignment (MSA) tool performance.

2.2. Alignment tools and parameters

The DNA sequences were aligned using four MSA tools: ClustalW, Clustal Omega, MUSCLE, and MAFFT, all of which were integrated into the Python environment through relevant libraries and packages. ClustalW and MUSCLE were implemented using the Biopython (v1.78) library, while Clustal Omega and MAFFT were accessed via Bioconda.

ClustalW: Alignments were performed using the stepwise alignment method, with default parameters set for gap opening and extension costs at 10 and 0.1, respectively.

Clustal Omega: This tool utilized the incremental alignment method, designed for fast and accurate alignments. The gap opening and extension costs were also set to 10 and 0.1, respectively.

MUSCLE: Iterative alignment was employed, with 16 iterations by default. The gap opening cost was set to 1.0.

MAFFT: Fast Fourier Transform (FFT)-based alignment was used, with gap opening and extension costs set to 1.0 and 0.1, respectively.

2.3. Alignment process and evaluation

To assess the alignment quality, several metrics were calculated using custom Python scripts.

Sum-of-Pairs (SP) Score: SP scores were computed by summing the pairwise sequence similarities, and these were compared against reference alignments to evaluate accuracy.

Column Score (CS): This metric assessed the accuracy of each aligned column by determining whether the reference alignment columns were correctly aligned.

Total Column (TC) Score: The TC score was calculated by determining the proportion of sequences aligned in the same position across each column.

Root Mean Square Deviation (RMSD): RMSD was calculated using the scipy.spatial.distance module from SciPy (v1.10.0), measuring the average squared structural deviations between aligned sequences.

Computation Time: The completion time for each alignment and analysis process was recorded in minutes using the “time” and “timeit” Python modules. The efficiency of each tool was assessed by comparing these processing times.

All performance evaluations were automated through Python scripts, and the resulting data was analyzed directly to compare the efficiency and accuracy of each alignment tool.

3. Results and Discussion

In this study, Time, SP, CS, TC, and RMSD metrics were compared to assess the performance of multiple sequence alignment (MSA) tools, including ClustalW, Clustal Omega, MUSCLE, and MAFFT. The "Time" metric, measured in minutes, represents the duration of the alignment process for each tool. Among the tools, MAFFT demonstrated the fastest processing time at 0.6 minutes, while ClustalW, MUSCLE, and Clustal Omega required 6.62, 5.97, and 4.05 minutes, respectively. The SP score, which measures alignment accuracy, was identical across all tools at 966.250 indicating similar performance in this aspect. The CS metric, which evaluates alignment quality, indicated that Clustal Omega and MAFFT had the lowest CS values (0.03), whereas ClustalW and MUSCLE exhibited slightly higher values (0.04). Lower CS values generally correspond to better alignment quality. The TC metric, reflecting the total number of columns in the alignment, was 641 for both Clustal Omega and MAFFT, while ClustalW and MUSCLE yielded 630 columns, suggesting variations in the granularity of alignment results across tools. Overall, MAFFT stood out for its rapid processing time, and both Clustal Omega and MAFFT delivered superior alignment quality with lower CS values. While the tools showed comparable performance in terms of alignment accuracy, as indicated by identical SP scores, differences were observed in processing time and the detail of alignment results (Figure 1).

When comparing the tools based on RMSD, the performances of ClustalW, Clustal Omega, MUSCLE, and MAFFT varied significantly (Figure 2). RMSD is a metric that quantifies the differences between aligned sequences, where lower values indicate better alignment quality. The results revealed an RMSD value of 0 between ClustalW and Clustal Omega, indicating identical alignment results for these two tools. In contrast, ClustalW produced higher RMSD values compared to MUSCLE and MAFFT, with an RMSD of 9.59 between ClustalW and MUSCLE, and 15.98 between ClustalW and MAFFT. The RMSD between MUSCLE and MAFFT was 18.42, indicating that MAFFT introduces more alignment discrepancies compared to MUSCLE. Overall, these findings suggest that Clustal Omega delivers superior alignment quality, exhibiting the most consistent and lowest RMSD values across the tools. While ClustalW also demonstrated low RMSD values in certain cases, comparable to Clustal Omega, it generally showed higher values compared to other tools. The higher RMSD values observed for MUSCLE and MAFFT suggest that their alignments deviate more from the others, reflecting lower alignment quality relative to Clustal Omega.

In general, the findings from our study indicate that each multiple sequence alignment (MSA) tool presents distinct advantages. MAFFT excels in terms of processing time, while Clustal Omega and MAFFT perform better in terms of CS scores. On the other hand, ClustalW and MUSCLE perform well in terms of TC scores, with Clustal Omega also demonstrating superior performance based on RMSD. These results align with previous studies that report varying performances for different tools depending on the dataset used. Mohamed et al. (2018) compared six well-known MSA tools, including Clustal Omega, MAFFT, BROBCONS, KALIGN, RETALIGN, and MUSCLE. They found that BROBCONS outperformed the others in terms of both TC and SP scores, as well as processing time. MAFFT ranked third across these metrics, while Clustal Omega ranked lowest in terms of TC and SP scores, and fifth in processing time. This contrasts with our findings, where Clustal Omega and MAFFT performed better in terms of CS and RMSD, although not in SP scores. Sievers and Higgins (2018) classified MSA tools into two categories: those optimized for fast processing and large alignments, and those optimized for higher accuracy with fewer sequences. MUSCLE and MAFFT were cited as examples of the first group, while T-Coffee and MAFFT L-INS-i were placed in the second group. Our findings, that MAFFT is the fastest tool, are consistent with Katoh et al. (2005), who also identified MAFFT as the fastest in terms of processing time. However,

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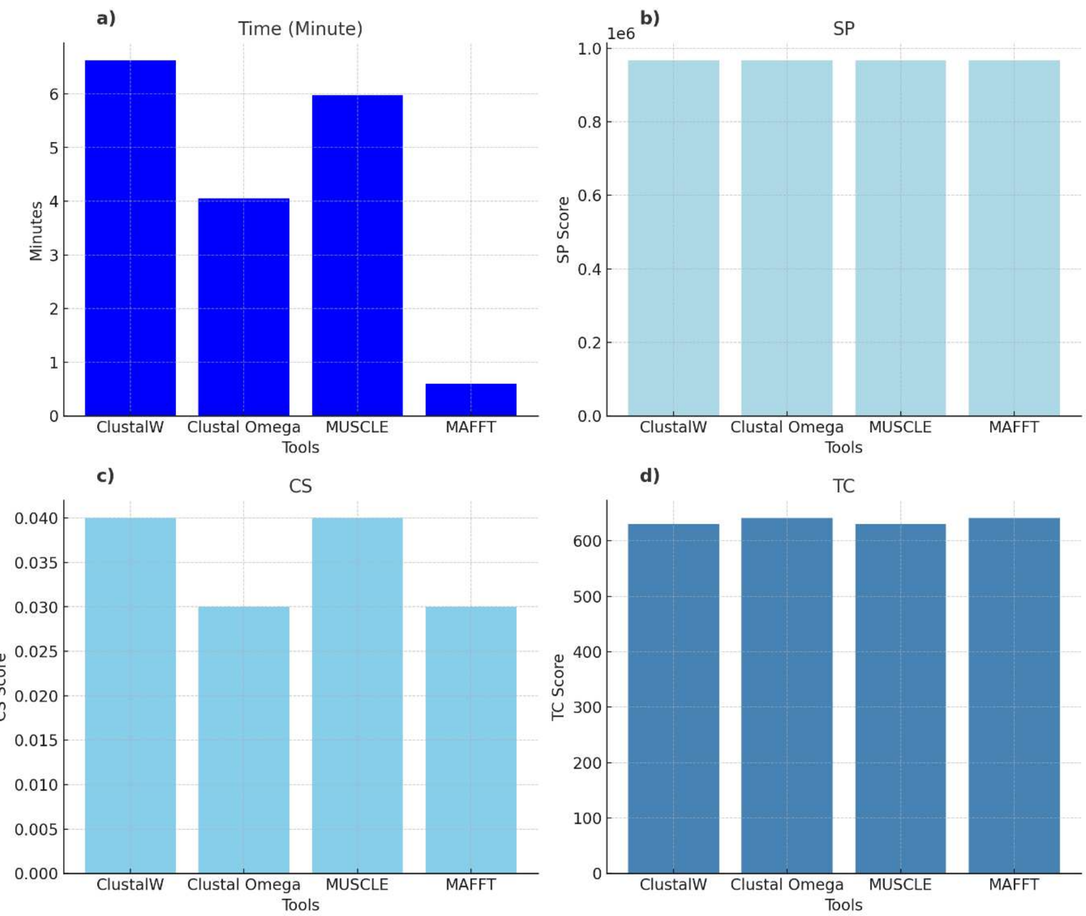

Figure 1. Performance comparison of alignment tools: a) Time b) Sum-of-Pairs (SP) c) Column Score (CS) d) TC scores (Total Consensus).

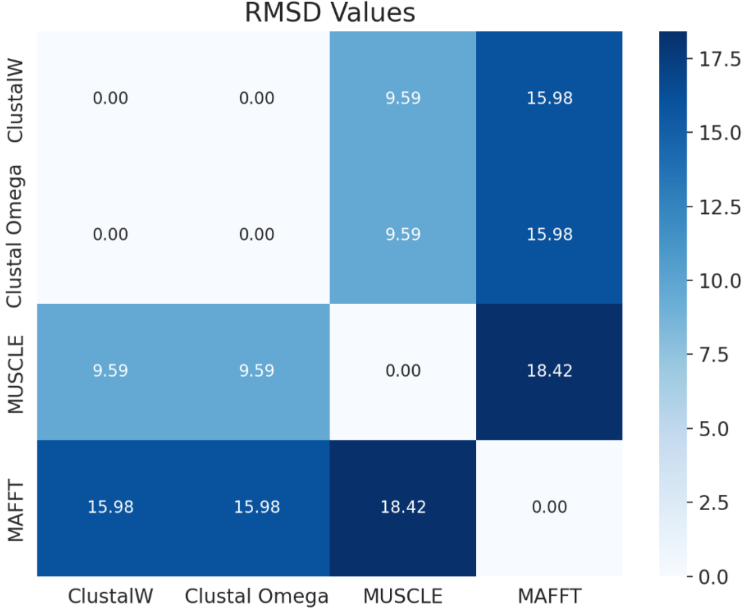

Figure 2. Pairwise values of the alignment tools.

Sievers and Higgins (2018) also noted that ClustalW2, MAFFT, and MUSCLE exhibited lower performance in terms of SP scores, while Clustal Omega slightly outperformed these tools with default settings, which is consistent with our results for CS and RMSD, though not for SP scores. In another study, Pais et al. (2014) compared the performance of ClustalW, Clustal Omega, DIALIGN-TX, MAFFT, MUSCLE, POA, Probalign, PROBCONS, and T-Coffee. They found that PROBCONS, T-

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Coffee, Probalign, and MAFFT had superior accuracy, while ClustalW and MUSCLE were identified as the fastest tools. These findings reinforce the hypothesis proposed by Nuin et al. (2006), Aniba et al. (2010), Thompson et al. (2011), and Pais et al. (2014) that the performance of MSA tools is context dependent. In conclusion, the selection of an appropriate tool should be based on the specific requirements of the dataset and analysis to achieve optimal results, as each tool has its own strengths and limitations.

4. Conclusion

In this study, we evaluated the performance of four multiple sequence alignment (MSA) tools: ClustalW, Clustal Omega, MUSCLE, and MAFFT. Our findings demonstrate that each tool offers distinct advantages depending on the dataset and research context, underscoring the importance of selecting the appropriate tool for specific research needs. MAFFT emerged as the fastest tool in terms of processing time, while Clustal Omega and MAFFT outperformed the others in terms of CS score, and ClustalW and MUSCLE excelled in terms of TC score. Additionally, Clustal Omega showed superior alignment quality based on RMSD scores. In conclusion, the optimal choice of MSA tool should be made based on the characteristics of the dataset, the goals of the research, and the computational resources available. This study provides valuable insights into the comparative performance of these widely used MSA tools, helping researchers make informed decisions in tool selection. Continued development and optimization of these tools can further enhance their applicability, benefiting a wide range of fields from fundamental biological research to applied biotechnology, ultimately contributing to the more efficient utilization of biological data.

References

Aniba MR, Poch O, Thompson JD (2010) Issues in bioinformatics benchmarking: the case study of multiple sequence alignment. Nucleic Acids Research 38: 7353-7363.

Chowdhury B, Garai G (2017) A review on multiple sequence alignment from the perspective of genetic algorithm. Genomics 109: 419-431.

Ferrer-Costa C, Orozco M, de la Cruz X (2005) Use of bioinformatics tools for the annotation of disease-associated mutations in animal models. Proteins: Structure, Function, and Bioinformatics 61: 878-887.

Katoh K, Misawa K, Kuma KI, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30: 3059-3066.

Katoh K, Kuma KI, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33(2): 511-518.

Levasseur A, Pontarotti P, Poch O, Thompson JD (2008) Strategies for reliable exploitation of evolutionary concepts in high throughput biology. Evolutionary Bioinformatics 4: EBO-S597.

Mohamed EM, Mousa HM, Keshk AE (2018) Comparative analysis of multiple sequence alignment tools. International Journal of Information Technology and Computer Science 10: 24-30.

Nuin PA, Wang Z, Tillier ER (2006) The accuracy of several multiple sequence alignment programs for proteins. BMC Bioinformatics 7: 1-18.

Pais FSM, Ruy PDC, Oliveira G, Coimbra RS (2014) Assessing the efficiency of multiple sequence alignment programs. Algorithms for Molecular Biology 9: 1-8.

Park D, Park SH, Ban YW, Kim YS, Park KC, Kim NS, Kim Choi IY (2017) A bioinformatics approach for identifying transgene insertion sites using whole genome sequencing data. BMC Biotechnology 17: 1-8.

Pervez MT, Babar ME, Nadeem A, Aslam M, Awan AR, Aslam N, Hussain T, Naveed N, Qadri S, Waheed U, Shoaib M (2014) Evaluating the accuracy and efficiency of multiple sequence alignment methods. Evolutionary Bioinformatics, 10: EBO-S19199.

Prykhozhij SV, Rajan V, Gaston D, Berman JN (2015) CRISPR multitargeter: a web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. PloS one 10: e0119372.

Sievers F, Higgins DG (2018) Clustal Omega for making accurate alignments of many protein sequences. Protein Science 27: 135-145.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

Thompson JD, Linard B, Lecompte O, Poch O (2011) A comprehensive benchmark study of multiple sequence alignment methods: current challenges and future perspectives. PloS one, 6: e18093.

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Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 147-154
DOI: 10.29136/mediterranean.1556782

Overexpression of the tomato pathogenesis-related gene *SlPR-1.9* confers increased tolerance to salt stress in *Arabidopsis thaliana*

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ARTICLE INFO

Received: September 27, 2024
Received in revised form: October 7, 2024
Accepted: October 24, 2024

***Keywords*:**

SlPR-1.9
Salt stress
Arabidopsis thaliana
Pathogenesis-related proteins
Galactolipase

ABSTRACT

Pathogenesis-related (PR) proteins are essential components of plant defense mechanisms, responding to both biotic and abiotic stresses. Among these, PR-1 proteins feature a CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1) domain, which is crucial for immune responses and pathogen defense due to its ability to stabilize protein structures and interact with various molecules. This study investigated the role of the tomato PR-1 gene *SlPR-1.9* in enhancing salt tolerance in *Arabidopsis thaliana*. The gene's coding sequence was cloned and transferred into *Arabidopsis* to create *SlPR-1.9* overexpression lines. These transgenic lines, alongside wild-type plants, were exposed to salt stress (150 mM NaCl) to assess their tolerance. Morphological analysis revealed that the transgenic lines demonstrated greater resilience to salt stress compared to wild-type plants, with less severe leaf curling and color changes. Additionally, lower proline accumulation, a stress marker, in the transgenic lines indicated an enhanced adaptive response. Bioinformatics analysis of the protein encoded by *SlPR-1.9*, A0A3Q7HSC4, suggested a strong interaction with galactolipase. Expression analysis showed that SlPR-1.9 was mainly expressed in roots and during early fruit development, suggesting a significant role in root physiology and stress response. These findings indicate that overexpression of *SlPR-1.9* can improve plant tolerance to salt stress, offering potential applications for enhancing crop resilience to environmental challenges.

1. Introduction

Plants employ various strategies to manage environmental threats, including adapting growth habits and developing mechanisms to sustain essential functions (Singhal et al. 2016). Gene expression related to stress responses occurs at both the transcriptional and translational levels (Cushman and Bohnert 2000). Understanding these mechanisms is vital for safeguarding agricultural productivity and quality. Comprehensive analyses of stresses are facilitated by combining molecular biology with morphological, physiological, and biochemical methods (Roca Paixão et al. 2019; Liu et al. 2019). Advances in functional genetics and modern genetic technologies have greatly enhanced our ability to identify and characterize genes involved in stress responses (Wani et al. 2017; Khan et al. 2019; Prihatna et al. 2018).

Plant pathogenesis-related (PR) proteins are key components of the plant defense mechanism against microbial pathogens and insects, encompassing seventeen well-characterized families (van Loon et al. 2006; Sels et al. 2008). These proteins are typically low molecular weight, acid-soluble, and protease-resistant, attributes that enhance their stability and efficacy in the plant defense system. Notably, PR proteins can be synthesized in both infected and uninfected tissues, ensuring a broad-spectrum defensive response (Ahuja et al. 2012; Ali et al. 2017). Among these, PR-1 proteins were the first to be identified and remain the most abundantly produced, underscoring their pivotal role in plant immunity.

It has been shown that pathogenesis-related 1 (PR-1) proteins respond not only against biotic stress elements but also response in abiotic stress tolerance (Hong and Hwang 2005; Liu et al. 2022). PR-1 proteins consist of a CAP domain, found in proteins such as Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1. It is characterized by its high cysteine content, which forms disulfide bonds, stabilizing the protein structure. This domain is involved in immune responses, pathogen defense, and other biological processes due to its ability to interact with various molecules. The PR-1 superfamily proteins are found in a wide range of organisms, including prokaryotes (Yeats et al. 2003) and non-vertebrate eukaryotes (Milne et al. 2003).

PR-1 genes are crucial for systemic acquired resistance (SAR) and respond to both biotic and abiotic stress factors (Almeida-Silva and Venancio 2022). Their expression increases in response to drought (Akbudak et al. 2020), freezing (Goyal et al. 2016), and salinity (Wang et al. 2019a). It has been shown that overexpressing PR-1 genes can enhance plant resistance to various harmful environmental conditions. For example, overexpression of the pepper CABPR1 gene, a basic PR-1

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homologue, not only enhanced defense against pathogens such as *Ralstonia solanacearum*, *Phytophthora nicotianae*, and *Pseudomonas syringae* pv. Tabaci but also improved tobacco plants' tolerance to heavy metal stress (Sarowar et al. 2005).

The primary enzyme co-expressed with *SlPR-1.9* in the tomato genome, known as galactolipase or galactolipid acyl-hydrolase, plays a crucial role in lipid metabolism by catalyzing the hydrolysis of galactolipids. These galactolipids are integral components of thylakoid membranes in plants, particularly within chloroplasts (Douce and Joyard 1980). Belonging to the Lipolytic Acyl Hydrolases (LAH) family, galactolipases contribute to the remodeling of membrane lipids, a vital process for maintaining membrane fluidity and integrity, especially under challenging environmental conditions such as cold stress or drought (Yu et al. 2021). The enzymatic activity of galactolipase breaks down galactolipids into fatty acids and glycerol (Bhattacharya 2022). The fatty acids released by galactolipase activity serve, not only as energy sources but also as signaling molecules. For example, jasmonic acid, a plant hormone crucial for stress responses and developmental processes, is derived from linolenic acid, which is released through lipolytic activity. Additionally, during pathogen attacks, LAH enzymes play a role in the production of defense-related molecules, with free fatty acids acting as precursors for antimicrobial compounds (Lee and Park 2019; Wang et al. 2019b).

LAH enzymes are also involved in the synthesis of secondary metabolites such as cutin and suberin, which are components of the plant cuticle that help protect against environmental stresses. The expression and activity of LAH enzymes are tightly regulated by developmental cues and environmental factors, ensuring that lipid metabolism is precisely modulated to meet the plant's physiological needs. In plants, galactolipase activity is particularly important during stress conditions such as drought, freezing, or pathogen attack, where membrane remodeling and the release of fatty acids are crucial for survival. This enzyme also plays a significant role during leaf senescence and other developmental stages where lipid turnover is essential (Moellering and Benning 2011). *SlPR-1.9* (*Solyc08g068990*) is one of thirteen PR-1 genes identified in the tomato genome (Akbudak et al. 2020). Although its expression is notably upregulated under drought (Akbudak et al. 2020) and cold stress conditions (Kasap and Akbudak 2024), its specific role in these responses is still not fully understood. In this study, we further investigated *SlPR-1.9* through bioinformatics analyses, followed by its cloning and functional assessment in *Arabidopsis* under salt stress. Our findings reveal that *SlPR-1.9* enhances salt tolerance in transgenic *Arabidopsis* plants.

2. Material and Methods

2.1. Obtaining SlPR-1.9 OE arabidopsis lines

The coding sequence of *SlPR1.9* was amplified from the genomic DNA (gDNA) of tomato plants using PCR and subsequently cloned into the pIPKb004 vector (Himmelbach et al. 2007) through the Gateway cloning method. Successful integration of *SlPR1.9* into the plasmid was confirmed by PCR and restriction enzyme analysis. The resulting transformation vector, designated pPR1.9, was then introduced into *Arabidopsis thaliana* using a refined floral dip method. Floral-dipped plants were grown in a growth chamber at 23°C during the day and 21°C at night, with a 16-hour light/8-hour dark photoperiod and 60% relative humidity for one month. When the seed pods started turning brown, they were left to dry for about a week before the seeds were carefully harvested. After 3-4 days of drying, approximately ¼ of the seeds were surface sterilized and sown on ½ MS medium containing 25 mg l^{-1} Hygromycin B. The selection plates were placed in the growth chamber under the same conditions. Three plants which tested positive through antibiotic selection were transferred to a 2:1 mixture of peat and perlite. DNA was isolated from these plants and the presence of T-DNA having *SlPR1.9* gene was confirmed by PCR. The PCR-verified transgenic plants were grown normally, then dried, and their seeds collected.

2.2. Stress application

Three transgenic *SlPR1.9* OE plants and three wild-type (WT) plants were sown in a 2:1 mixture of peat and perlite and grown under standard conditions for three weeks, with watering every two days. After this period, the plants were subjected to a 150 mM NaCl treatment. During the two-week stress application, the same concentration of NaCl was applied at each watering, while the control plants received only tap water. Following the stress treatment, the plants were bulk harvested, flash-frozen in liquid nitrogen, and stored at -80°C for further analysis.

2.3. Proline analysis

Leaf tissue samples weighing 0.5 g from each plant group were ground in liquid nitrogen and then homogenized in 10 ml of 3% sulfosalicylic acid. The extracts obtained were centrifuged at 5000 rpm for 5 minutes. A 2 ml portion of the supernatant was combined with 2 ml of an acid ninhydrin solution (prepared by dissolving 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) and 2 ml of glacial acetic acid. This mixture was then incubated at a temperature of 95-100°C for 1 hour. To stop the reaction, the samples were cooled on ice, and 4 ml of cold toluene was added. The phase containing the chromophore was measured at 520 nm using a spectrophotometer. Each measurement was conducted in triplicate. The proline content was determined as micromoles of proline per gram of fresh weight using the formula provided by Bates et al. (1973).

2.4. Analysis of conserved domain and protein interaction network

The amino acid sequences, conserved domains and the protein structures of the proteins were retrieved from UNIPROT (https://www.uniprot.org), InterPro (https://www.ebi.ac.uk/interpro/) and Phytozome (https://phytozome-next.jgi.doe.gov), respectively. The interaction networks were generated using the STRING 11 database and can be accessed at http://string-db.org/ (Szklarczyk et al. 2021).

2.5. Digital expression pattern

Data from the Tomato Genome Consortium (2012) was obtained to analyze the expression patterns of *SlPR-1.9* and tomato *galactolipase* genes. The expression profiles of these genes were examined across different anatomical parts and developmental stages. The heatmap was drawn using the Heatmap program in TBtools software.

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3. Results

3.1. Morphological analysis of SlPR1.9 OE lines

Under stress-free conditions, the WT (wild-type) and the *SlPR1.9* OE line (1, 2, and 3) plants displayed healthy, well-expanded leaves with symmetrical growth and a robust appearance (Fig. 1). Under salt stress conditions, WT plants experienced significant reduction in leaf size, noticeable color change, and pronounced curling, indicating severe stress symptoms. In contrast, the *SlPR-1.9* OE lines demonstrated better tolerance to salt stress. SlPR1.9 (1) maintained its green color better and exhibited less curling compared to WT. SlPR1.9 (2) experienced some curling but retained its leaf area and green color more effectively than WT. SlPR1.9 (3) showed leaf size reduction but less color change compared to WT. Overall, SlPR1.9 lines, especially SlPR1.9 (1) and SlPR1.9 (2), exhibited better resilience to salt stress compared to WT, with less severe morphological changes and better maintenance of leaf color.

3.2. Proline Accumulation in SlPR-1.9 OE Lines

Prolonged or excessive accumulation of proline could indicate that the plant is experiencing sustained or severe stress (Ghosh et al. 2022). In such cases, while the plant is managing stress, ongoing or severe conditions might still impact its overall health and growth. High proline levels in plants typically indicate that the plant is under stress. Proline accumulates in response to various environmental challenges such as drought, salinity, or extreme temperatures. This accumulation helps the plant manage stress by maintaining osmotic balance, protecting cellular structures from oxidative damage, and stabilizing proteins and membranes. While high proline levels do signal stress, they also reflect the plant's adaptive mechanisms to cope with and survive adverse conditions.

Under the salt treatment, the SlPR1.9 (1) and SlPR1.9 (2) lines showed lower proline levels compared to the wild-type (WT), indicating a potentially less robust stress response. In contrast, the SlPR1.9 (3) line exhibited proline similar to the WT, suggesting it was affected more by the salt stress compared to the other two lines (Fig. 2).

3.3. Analysis of A0A3Q7HSC4 encoded by SlPR-1.9

SlPR-1.9 (*Solyc08g068990*) encodes the A0A3Q7HSC4 protein, consisting of 181 amino acids (Fig. 3). This protein is localized extracellularly. Domain analysis using the InterPro tool revealed that A0A3Q7HSC4 contains a CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1) domain. The PPI (Protein-Protein Interaction) network analysis revealed that protein A0A3Q7HSC4 (Solyc08g068990) in tomato had a strong interaction with protein A0A3Q7F1E6 (Solyc02g065090), with an interaction score of 0.720 (Fig. 4). A0A3Q7F1E6 was identified as galactolipase, a patatin-like protein, belonging to the Lipolytic Acyl Hydrolases (LAH) family. Patatin proteins are known for their lipid acyl hydrolase (lipase) activity, which allows them to hydrolyze fatty acids from membrane lipids, thus playing a key role in lipid metabolism in plants. Beyond their metabolic functions, patatin proteins are also crucial for plant defense. Their lipase activity can compromise the membrane integrity of pathogens, thereby contributing to the plant's immune response. In addition to A0A3Q7F1E6, A0A3Q7HSC4 interacts with two other uncharacterized proteins, A0A3Q7HKV2 and A0A3Q7HYQ6, with interaction scores of 0.441 and 0.452, respectively (represented by blue and green dots in Fig. 4). The UniProt BLAST analysis identified F3L17.40 (AT4G31470) as the primary ortholog of A0A3Q7HSC4 in *Arabidopsis thaliana*. This protein, F3L17.40, is another pathogenesis-related protein consisting of a CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1) domain, similar to A0A3Q7HSC4. It is composed of 185 amino acids (Fig. 5). At high confidence level (0.700), STRING protein network analysis revealed that there were five predicted functional partners of F3L17.40 in *Arabidopsis* (Fig. 6, Table 1). CXE3 acts on esters with varying acyl chain lengths and is likely involved in the breakdown or modification of esters within the cell, potentially influencing metabolic pathways or detoxification

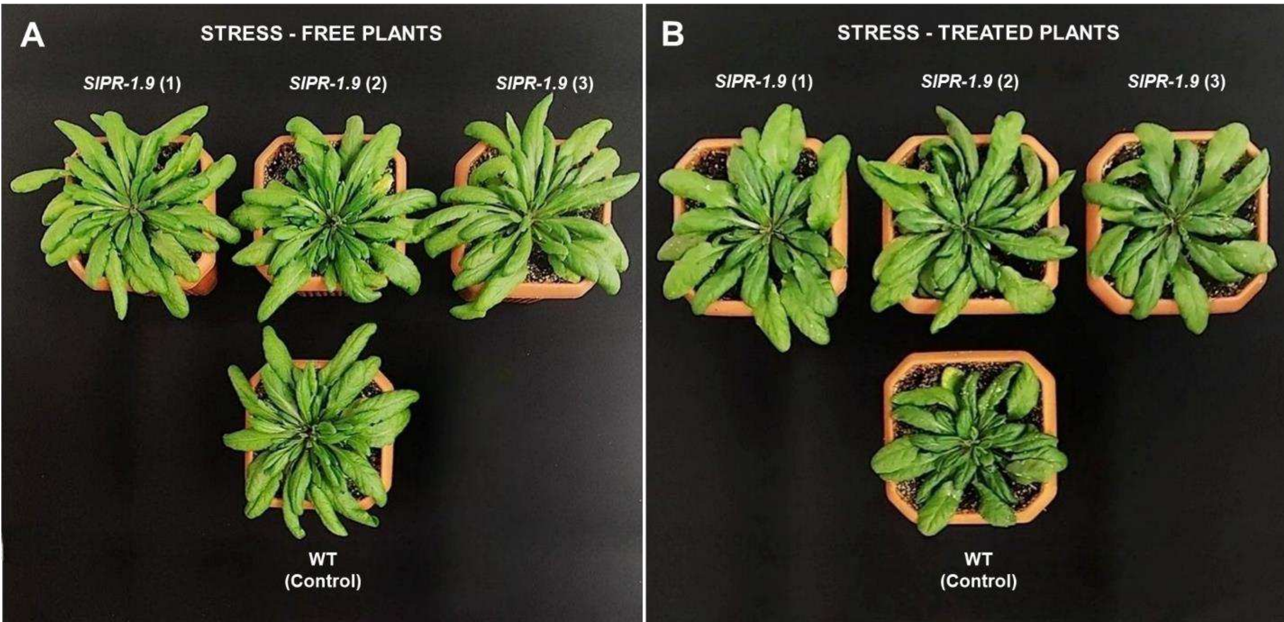

Figure 1. Increased NaCl stress tolerance in transgenic plants overexpressing *SlPR-1.9*. (A) Phenotypes of wild-type (WT) and T2 transgenic *SlPR-1.9* overexpression (OE) lines under stress-free conditions. (B) Phenotypes of WT and transgenic lines following NaCl treatment.

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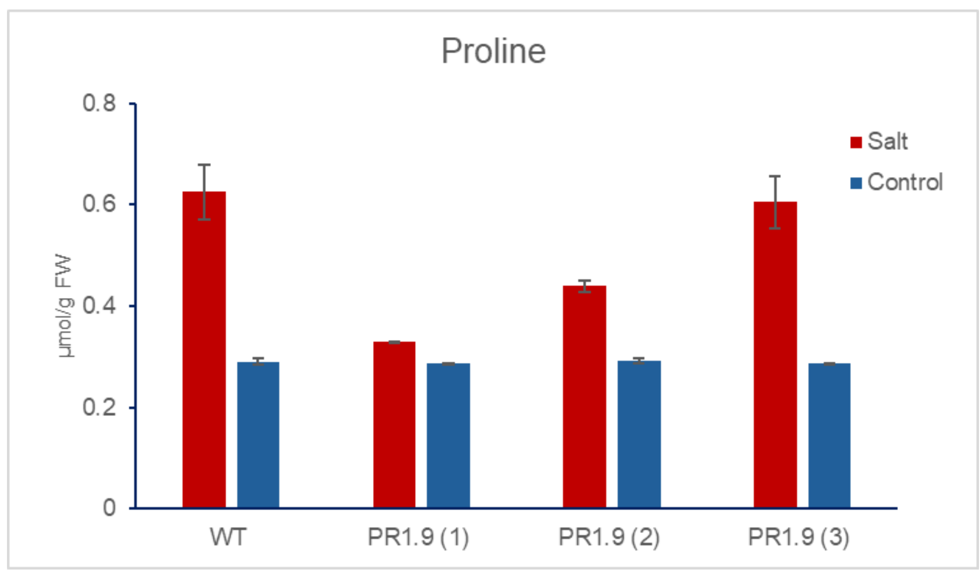

Figure 2. Proline accumulation in Arabidopsis leaves. Control: Stress-free, Salt: 150 mM NaCl treatment. Error bars represent the standard error of the mean (n= 3).

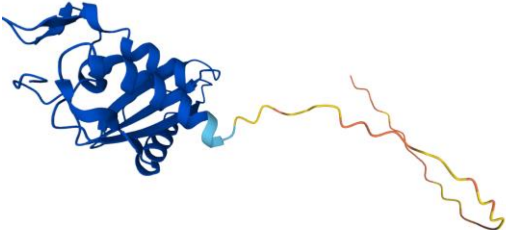

Figure 3. Protein structure of A0A3Q7HSC4. Modeled using data from the UniProt database (https://www.uniprot.org).

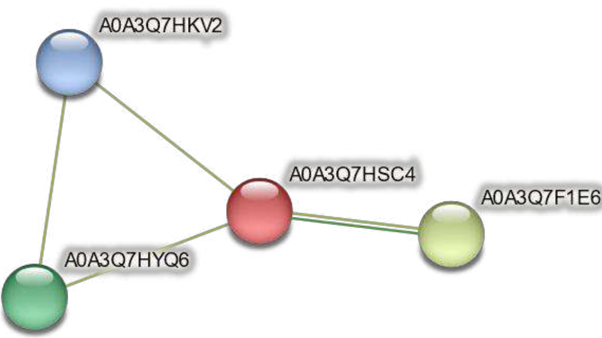

Figure 4. Protein-Protein Interaction network of A0A3Q7HSC4 in tomato. The dark green line represents evidence of direct protein–protein interactions and while the light green lines indicates associations identified through textmining.

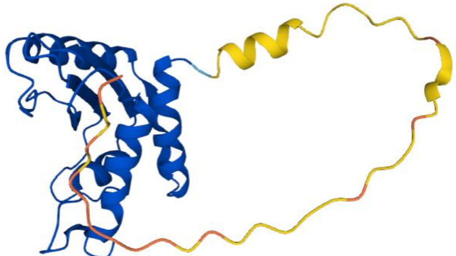

Figure 5. Protein structure of F3L17.40. Modeled using data from the UniProt database (https://www.uniprot.org).

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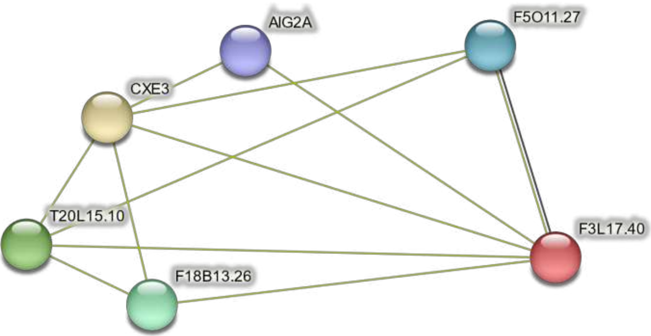

Figure 6. Protein–Protein Interaction Network of F3L17.40 in Arabidopsis. Green lines represent evidence of direct protein–protein interactions, while black lines denote co-expression relationships.

Table 1. Predicted functional partners of F3L17.40

Protein	Description	Conf. Score
CXE3	Probable carboxylesterase 3	0.794
T20L15.10	Nuclear transport factor 2 (NTF2) family protein	0.791
F18B13.26	MAPK kinase substrate protein At1g80180	0.779
F5O11.27	Uncharacterized protein	0.759
AIG2A	Protein AIG2 A; Putative gamma-glutamylcyclotransferase	0.718

processes. T20L15.10 is a Nuclear Transport Factor 2 Family Protein that plays a critical role in the transport of molecules between the nucleus and cytoplasm, affecting gene regulation and signal transduction. F18B13.26, also referred to as MAPK Kinase Substrate Protein (At1g80180), may play a role in the regulation of stomatal patterning and is likely involved in signaling pathways that regulate stomatal development, which is crucial for plant respiration and water regulation. AIG2A, is a putative gamma-glutamylcyclotransferase and belongs to the gamma-glutamylcyclotransferase family, potentially involved in the modification or degradation of peptides, impacting amino acid metabolism and cellular homeostasis. F3L17.40 is co-expressed with F5O11.27, an uncharacterized protein with an unknown function, highlighting a promising area for further investigation. The interaction network of F3L17.40 in *Arabidopsis* suggests a potential involvement in a complex, which could be crucial for understanding how pathogenesis response genes regulate the plant's response to environmental stresses.

3.4. Expression profiles of SlPR-1.9 and tomato galactolipase genes

Digital expression analysis showed that *SlPR-1.9* is highly expressed in root tissue (Fig. 7). Additionally, the gene exhibits significant expression during the early stages of fruit development, particularly in 1 cm and 2 cm fruits, where its expression is notably high. This expression pattern suggests that *SlPR-1.9* may play a crucial role in the initial phases of fruit formation. In contrast, the expression level of *SlPR-1.9* is substantially lower in other tissues, including the bud, flower, and leaf. The minimal expression in these tissues suggests that *SlPR-1.9* is less involved in the physiological processes occurring in these parts of the plant or at these stages of development. Overall, the expression profile of *SlPR-1.9* highlights its likely significance in root physiology and early fruit development. The expression analysis revealed that the galactolipase gene expressed across different plant tissues and developmental stages shows a highly specific pattern (Fig. 8). The most significant expression of galactolipase is found in the root tissue. This suggests that the galactolipase gene is highly active in the roots, likely contributing to its well-known roles in lipid metabolism and storage, which are critical for root function and energy reserves.

In addition to its high expression in the roots, moderate expression was observed in leaf tissues. This implies that while galactolipase's primary role might be in the root, it also has some level of activity in the leaves, possibly contributing to lipid metabolism or defense mechanisms within these tissues. In contrast, the expression of the galactolipase gene is minimal to negligible in other plant parts. Little to no activity of galactolipase indicates that the gene is not significantly involved in the physiological processes of these tissues, especially those related to reproductive development.

4. Discussion

The overexpression of the *SlPR-1.9* gene in *A. thaliana* significantly enhances tolerance to salt stress, as demonstrated by improved morphological characteristics and reduced proline accumulation in transgenic lines compared to wild-type (WT) plants. These findings contribute to the growing body of evidence supporting the role of PR-1 proteins in both biotic and abiotic stress tolerance, expanding our understanding of their functional diversity. The PR-1 protein family, to which *SlPR-1.9* belongs, is characterized by the presence of the CAP domain, known for its role in stabilizing protein structures and mediating immune responses (Gibbs et al. 2008). This domain is highly conserved across various species, indicating its fundamental role in defense mechanisms (Yeats et al. 2003; Milne et al. 2003). The ability of PR-1 proteins to interact with diverse molecules, as evidenced by their involvement in systemic acquired resistance (SAR) and responses to abiotic stresses like drought, freezing, and salinity (Akbudak et al. 2020; Wang et al. 2019a), underscores their multifaceted role in plant stress responses.

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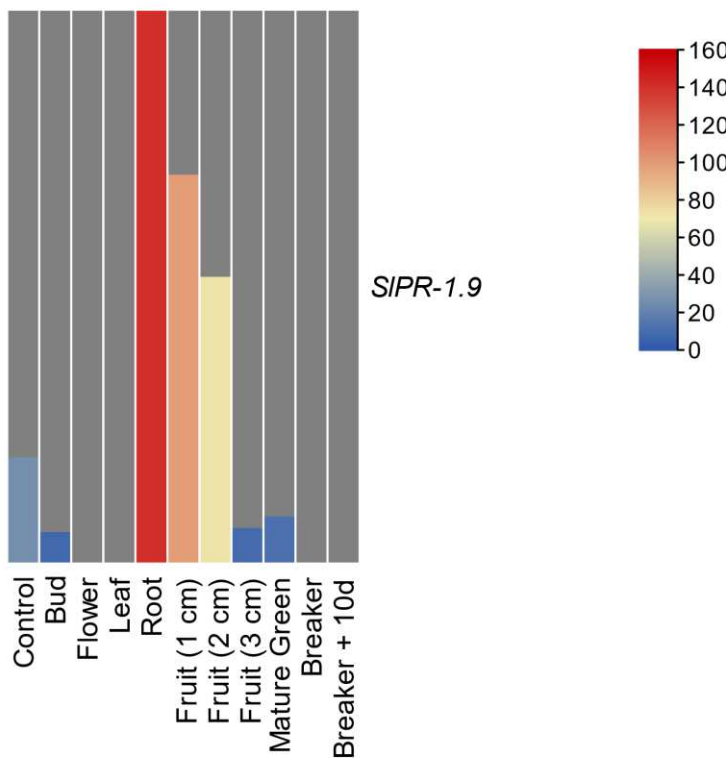

Figure 7. Heatmap of the expression profile of *SlPR-1.9* gene across various organs and developmental stages in tomato. In the heatmap, blue elements indicate low relative expression levels, while red elements indicate high relative expression levels.

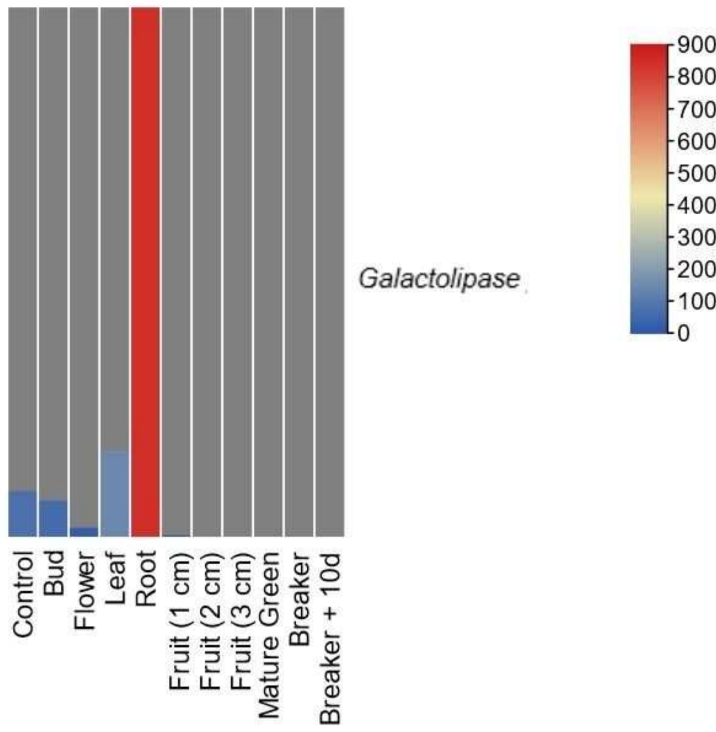

Figure 8. Heatmap of the expression profile of *Galactolipase* gene across various organs and developmental stages in tomato. In the heatmap, blue elements indicate low relative expression levels, while red elements indicate high relative expression levels.

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The morphological analysis in this study revealed that *SlPR-1.9* overexpression lines exhibited less severe leaf curling and color changes under salt stress compared to WT plants, which is consistent with the role of PR-1 proteins in maintaining cellular integrity under stress conditions (Sarowar et al. 2005). The reduced proline accumulation in transgenic lines further supports the idea that *SlPR-1.9* helps mitigate the effects of osmotic stress, a common consequence of high salinity. Proline acts as an osmoprotectant and is a marker of stress severity; thus, lower levels in transgenic plants suggest a more efficient stress adaptation mechanism (Cushman and Bohnert 2000).

Bioinformatics analysis revealed a significant interaction between the protein encoded by *SlPR-1.9* and galactolipase, an enzyme involved in lipid metabolism (Moellering and Benning 2011). Galactolipase plays a critical role in the hydrolysis of galactolipids, which are major components of thylakoid membranes in chloroplasts. Under stress conditions, such as drought or salinity, membrane remodeling is essential for maintaining cellular homeostasis (Moellering and Benning 2011). The interaction between *SlPR-1.9* and galactolipase suggests that *SlPR-1.9* may enhance salt tolerance by influencing lipid metabolism and membrane stability, which are crucial for plant survival under adverse environmental conditions. Furthermore, the expression pattern of *SlPR-1.9* predominantly in root tissues and during early fruit development, as shown in this study, aligns with the findings of Almeida-Silva and Venancio (2022), who highlighted the tissue-specific expression of PR-1 genes under stress conditions. Roots, being in direct contact with soil, are particularly vulnerable to abiotic stresses such as salinity, making the high expression of *SlPR-1.9* in roots a likely adaptive response to such challenges. The involvement of PR-1 proteins in both biotic and abiotic stress responses, as reported by Ahuja et al. (2012) and Ali et al. (2017), further supports the notion that *SlPR-1.9* plays a critical role in the plant's defense mechanisms.

The ability of *SlPR-1.9* to interact with proteins involved in lipid metabolism, such as galactolipase, also highlights the potential for these interactions to influence metabolic pathways that are crucial for stress tolerance. This is particularly relevant in the context of systemic acquired resistance (SAR), where the coordination of multiple stress response pathways is essential for plant survival (Loon et al. 2006).

In conclusion, the overexpression of *SlPR-1.9* in *A. thaliana* enhances salt stress tolerance through mechanisms that likely involve both direct effects on stress-responsive pathways and interactions with key metabolic processes, such as lipid metabolism. These findings not only expand our understanding of the functional roles of PR-1 proteins but also suggest potential applications in developing crops with improved resistance to environmental stressors, thereby contributing to agricultural sustainability in challenging climates. Future research should focus on elucidating the detailed molecular pathways through which *SlPR-1.9* and its interacting partners, such as galactolipase, confer stress tolerance, as well as exploring the applicability of these findings to other economically important crops.

Acknowledgments

This study was supported by Akdeniz University Scientific Research Projects Coordination Unit grant no. FYL-2022-5900. MAA is thankful for the financial support provided by the Fulbright Visiting Scholar Grant from the Turkish Fulbright Commission.

Authors' Contribution

KY: Investigation and Data curation, MAA: Conceptualization, Writing- Original Draft, Writing- Review & Editing, Supervision.

References

Ahuja I, Kissen R, Bones AM (2012) Phytoalexins in defense against pathogens. Trends in Plant Science 17(2): 73-90.

Akbudak MA, Yildiz S, Filiz E (2020) Pathogenesis related protein-1 (PR-1) genes in tomato (*Solanum lycopersicum* L.): Bioinformatics analyses and expression profiles in response to drought stress. Genomics 112(6): 4089-4099.

Ali S, Mir ZA, Tyagi A, Bhat JA, Chandrashekar N, Papolu PK, Rawat S, Grover A (2017) Identification and comparative analysis of Brassica juncea pathogenesis-related genes in response to hormonal, biotic and abiotic stresses. Acta Physiol Plant 39(12): 268.

Almeida-Silva F, Venancio TM (2022) Pathogenesis-related protein 1 (PR-1) genes in soybean: Genome-wide identification, structural analysis and expression profiling under multiple biotic and abiotic stresses. Gene 809: 146013.

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39(1): 205-207.

Bhattacharya A (2022) Lipid Metabolism in Plants Under Low-Temperature Stress: A Review | SpringerLink. Physiological processes in plants under low temperature stress: 409-516.

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Current Opinion in Plant Biology 3(2): 117-124.

Douce R, Joyard J (1980) 12 - Plant Galactolipids. In: Lipids: Structure and Function (Stumpf PK, Ed.). Academic Press, pp. 321-362.

Ghosh UK, Islam MN, Siddiqui MN, Cao X, Khan M a. R (2022) Proline, a multifaceted signalling molecule in plant responses to abiotic stress: understanding the physiological mechanisms. Plant Biology 24(2): 227-239.

Gibbs GM, Roelants K, O'Bryan MK (2008) The CAP Superfamily: Cysteine-Rich Secretory Proteins, Antigen 5, and Pathogenesis-Related 1 Proteins—Roles in Reproduction, Cancer, and Immune Defense. Endocrine Reviews 29(7): 865-897.

Goyal RK, Fatima T, Topuz M, Bernadec A, Sicher R, Handa AK, Mattoo AK (2016) Pathogenesis-Related Protein 1b1 (PR1b1) Is a Major Tomato Fruit Protein Responsive to Chilling Temperature and Upregulated in High Polyamine Transgenic Genotypes. Front Plant Sci 7: 901.

Himmelbach A, Zierold U, Hensel G, Riechen J, Douchkov D, Schweizer P, Kumlehn J (2007) A Set of Modular Binary Vectors for Transformation of Cereals. Plant Physiology 145(4): 1192-1200.

Hong JK, Hwang BK (2005) Functional Characterization of PR-1 Protein, β-1,3-Glucanase and Chitinase Genes During Defense Response to Biotic and Abiotic Stresses in Capsicum annuum. The Plant Pathology Journal 21(3): 195-206.

Kasap M, Akbudak MA (2024) Domates Pr-1 Genlerinin Soğuk Stresi Altındaki İfade Profilleri. In: 8th International Cukurova Agriculture and Veterinary Congress.

Khan R, Zhou P, Ma X, Zhou L, Wu Y, Ullah Z, Wang S (2019) Transcriptome Profiling, Biochemical and Physiological Analyses Provide New Insights towards Drought Tolerance in Nicotiana tabacum L. Genes 10(12): 1041.

Lee H-J, Park OK (2019) Lipases associated with plant defense against pathogens. Plant Science 279: 51-58.

Liu Y, Qi Y, Zhang A, Wu H, Liu Z, Ren X (2019) Molecular cloning and functional characterization of AcGST1, an anthocyanin-related glutathione S-transferase gene in kiwifruit (Actinidia chinensis). Plant Mol Biol 100(4): 451-465.

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Liu T, Chen T, Kan J, Yao Y, Guo D, Yang Y, Ling X, Wang J, Zhang B (2022) The GhMYB36 transcription factor confers resistance to biotic and abiotic stress by enhancing PR1 gene expression in plants. Plant Biotechnology Journal 20(4): 722-735.

Loon LC van, Martijn Rep, Corné MJ Pieterse (2006) Significance of Inducible Defense-related Proteins in Infected Plants. Annu Rev Phytopathol 44(1): 135-162.

Milne TJ, Abbenante G, Tyndall JDA, Halliday J, Lewis RJ (2003) Isolation and Characterization of a Cone Snail Protease with Homology to CRISP Proteins of the Pathogenesis-related Protein Superfamily *. Journal of Biological Chemistry 278(33): 31105-31110.

Moellering ER, Benning C (2011) Galactoglycerolipid metabolism under stress: a time for remodeling. Trends in Plant Science 16(2): 98-107.

Prihatna C, Barbetti MJ, Barker SJ (2018) A Novel Tomato Fusarium Wilt Tolerance Gene. Front Microbiol 9.

Roca Paixão JF, Gillet F-X, Ribeiro TP, Bournaud C, Lourenço-Tessutti IT, Noriega DD, Melo BP de, Almeida-Engler J de, Grossi-de-Sa MF (2019) Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with a Histone AcetylTransferase. Sci Rep 9(1): 8080.

Sarowar S, Kim YJ, Kim EN, Kim KD, Hwang BK, Islam R, Shin JS (2005) Overexpression of a pepper basic pathogenesis-related protein 1 gene in tobacco plants enhances resistance to heavy metal and pathogen stresses. Plant Cell Rep 24(4): 216-224.

Sels J, Mathys J, De Coninck BMA, Cammue BPA, De Bolle MFC (2008) Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiology and Biochemistry 46(11): 941-950.

Singhal P, Jan AT, Azam M, Haq QMR (2016) Plant abiotic stress: a prospective strategy of exploiting promoters as alternative to overcome the escalating burden. Frontiers in Life Science 9(1): 52-63.

Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, Doncheva NT, Legeay M, Fang T, Bork P, Jensen LJ, von Mering C (2021) The STRING database in 2021: customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Research 49(D1): D605-D612.

Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485(7400): 635-641.

Wang J, Mao X, Wang R, Li A, Zhao G, Zhao J, Jing R (2019a) Identification of wheat stress-responding genes and TaPR-1-1 function by screening a cDNA yeast library prepared following abiotic stress. Sci Rep 9(1): 141.

Wang K, Durrett TP, Benning C (2019b) Functional diversity of glycerolipid acylhydrolases in plant metabolism and physiology. Progress in Lipid Research 75: 100987.

Wani SH, Dutta T, Neelapu NRR, Surekha C (2017) Transgenic approaches to enhance salt and drought tolerance in plants. Plant Gene 11: 219-231.

Yeats C, Bentley S, Bateman A (2003) New Knowledge from Old: In silico discovery of novel protein domains in Streptomyces coelicolor. BMC Microbiol 3(1): 3.

Yu L, Zhou C, Fan J, Shanklin J, Xu C (2021) Mechanisms and functions of membrane lipid remodeling in plants. The Plant Journal 107(1): 37-53.

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Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 155-164
DOI: 10.29136/mediterranean.1455528

Determination of water-retention and physicochemical properties of selected media as related to tomato seedling quality parameters

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ARTICLE INFO

Received: March 21, 2024
Received in revised form: November 12, 2024
Accepted: November 13, 2024

Keywords:

Diatomite
Growing medium
Peat
Vermicompost
Zeolite

ABSTRACT

In this study, it was aimed to determine the relationships between changes in water retention properties and some physicochemical properties of different growth media used in seedling cultivation and tomato (*Solanum lycopersicon* cv.) seedling quality parameters. Growing media were prepared from eight different mixtures of peat (P), diatomite (D), zeolite (Z) and vermicompost (V). KAYRA F1 tomato variety was used for the seedling. At the end of 45 days of incubation, the water retention characteristics of medias were determined at different matric potential (0, -2, -4, -5, -8, -10, -33 and -1500 kPa). The highest available water capacity was realized in M1 (100% peat) and the highest saturation value was realized in M5 (70% peat + 15% zeolite + 15% vermicompost). Nutrient content and chemical and physical properties of the media were important for tomato seedling yield and quality parameters. Especially the increase in the ratio of vermicompost with a high EC value in the mixture caused a decrease in the germination rate. In the mixtures with a vermicompost ratio not exceeding 15%, significant improvement was achieved in seedling quality parameters. The best medium for tomato seedling yield and the quality parameters were obtained in M8 (70% peat + 10% zeolite + 10% diatomite + 10% vermicompost), and it was also observed that favorable results may be obtained in terms of quality seedling cultivation in M6 (70% peat + 15% diatomite + 15% vermicompost), M5 (70% peat + 15% zeolite + 15% vermicompost) and M4 (80% peat + 20% vermicompost) mediums.

1. Introduction

The most important factors affecting the success of soilless agriculture are ecology, greenhouse structure, climate, growing system planning, seedling type, variety and growing environment. The world population is expected to reach 9.7 billion in 2050 and 11 billion by 2100 (United Nations 2021). Novel and improved agricultural systems should be introduced in urban areas due to limited arable land, soil degradation and water scarcity (Lehman et al. 1993). In vegetables grown in the field and under greenhouse conditions, ready-made seedlings are generally used as starting material in production. While ready-to-use seedling production companies in Türkiye produce aproximately 2 billion seedlings annually, tomato ranks first among the seedlings produced with 41.2% (Yelboğa 2014; Tüzel et al. 2015). The use of ready-made seedlings in Türkiye was 100% in greenhouse agriculture and 70% in field agriculture (Yelboğa 2014). In 2020, the area of greenhouse agriculture in Türkiye was 805159 ha. Among the species grown in greenhouses, tomato ranks first in terms of both area and production. Of the total 7771766 tons of vegetables grown in greenhouses, 4099129 tons are tomatoes, with a share of approximately 52% (TÜİK 2021).

Cultivation carried out in soilless agriculture is divided into two, substrate and hydroponic culture (Gül 2012). Various substrates, both inorganic and organic, can be used as growth media and inorganic substrates include expanded clay, glass wool, gravel, perlite, pumice, rock wool, sand sepiolite, vermiculite, volcanic tuff and zeolite. The organic substrates include bark, coconut coir, coco soil, fleece, marc, peat, ruffia bark, and rice husk, saw dust and wood chips (Mariyappillai and Arumugam 2021). The main objective in crop production is to produce healthy and high-quality products with high yields. The growing medium must meet different criteria such as physical, chemical, hydrological and biological properties to meet the growth requirements of the plant (Nelson 2012). The medium used in soilless media should be cheap and readily available, have good drainage, low volume-to-weight ratio, good rehydration properties after drying, stable structure, good pH-buffering capacity, low micro-organism activity, good aeration, free from diseases and pests, non-toxic, low soluble salt content, and should not lose its chemical and biological properties after sterilization (Sevgican 1999; Gruda et al. 2013). Due to environmental issues, the search for alternative components to remove or reduce peat, traditionally the main component of most seedling growing media, has been the subject of numerous studies in recent years (Abad et al. 2001). In this sense, special attention has been paid to the use of composts, municipal waste and other residual materials of organic origin (Barral et al. 2007; Paradelo et al. 2012, 2016).

The media to be used in seedling cultivation should be an ideal mixture that can meet the demands of vegetables. Prepared

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seedling media mixtures should be specific to the plant species. For this reason, it is important to determine the ideal media mixtures that can meet the requirements of many vegetables (Uzun 2001; Doğan 2003). It has been reported that the volume of media available for use by tomato plants grown on a soil bed in a greenhouse is about 200 L, whereas the corresponding volume size for production in soilless culture (substrate media) is smaller (Sonneveld 1981).

Many researchers have tested and modeled many properties of materials that can be used as growing media in soilless culture (Pokorny and Henny 1984; Pokorny et al. 1986; Bures et al. 1993a, b). Any media used in soilless culture can have some effect on plant growth, as well as the fertilizers added. In addition, the porosity of the medium and the state of water are also very important for plant growth. The physical and hydraulic properties of most substrates are superior to those of soils. However, the hydraulic properties of substrates used in soilless agriculture have not been adequately characterized to date. In addition, each of the media components used is unique in terms of air/water properties (Raviv and Leith 2008). These definitions are limited to only a few parameters or to limited soil water matric potential (up to -10 kPa) (Wallach et al. 1992). Understanding the water retention characteristics of the growing medium can help develop best management practices for containerized farming systems for efficient management of irrigation water (Kumar et al. 2010).

In this study, it was aimed to a) determine the differences in physicochemical properties and water retention ability of growing media consisting of different mixtures of peat, diatomite, zeolite and vermicompost b) assess differences in tomato seedling quality parameters as a response to different growing media.

2. Material and Methods

Four different materials were used to create diverse growing media: zeolite, peat, diatomite and vermicompost. The materials were obtained from different commercial companies. The Kekkilä brand peat obtained from peat deposits in Finland was used in the study. The main components of the peat are white and brown Sphagnum peat. Vermicompost is an organic fertilizer that is obtained as a result of the processing of food consisting entirely of organic wastes (such as cattle manure, vegetable and fruit wastes, fruit pulp, etc.) by worms in a period of 10-12 months. The zeolite used in the study is a natural soil conditioner called Agro-Clino and is produced from zeolite-clinoptilolite. Diatomite was obtained from AGRIPOWER commercial company. The product, commercially produced under the name Agripower Silica, is a material composed of skeletal remains (diatoms) of freshwater algae (Melosira Granulata species). Some analytical data of the materials declared by the manufacturers are given in Table 1.

The research was conducted in the greenhouses of the Faculty of Agriculture of Akdeniz University (Fig 1). The greenhouse used was a double-span, north and south oriented Venlo-type greenhouse, covered with ethylene-tetra fluoroethylene film, equipped with air conditioning for winter heating, and supported by natural ventilation through the roof and side windows. Experiments were carried out on 4 x 1 m tables inside the greenhouse. Indoor temperature values given in Table 2 were obtained from the greenhouse thermometer.

In the study, KAYRA F1 hybrid (*Solanum lycopersion* cv.) tomato seeds were used as a plant material to determine the effects of different media on seedling development. The tomato variety used is a high-yielding variety suitable for spring and summer planting, with high aroma, long shelf life, tolerant to Fusarium radici (For) and Fusarium 0.1 (L2) and resistant to cracking.

Table 1. Physicochemical properties of substrates used in the preparation of media*

Parameters	Substrates			
	Peat	Vermicompost	Zeolite	Diatomite
Size	-	-	<50mm	<50mm
pH	5.9	7.0	-	-
EC (dS m^{-1})	0.24	3.2	-	-
Organic matter (%)	90	39.96	-	-
Bulk density (g cm^{-3})	0.85	-	0.45-0.55	-
Grain density (g cm^{-3})	-	-	-	1.9-2.1
Porosity (%)	-	-	35-40	35-40
Moisture (%)		30.39	120-125	120-125
SiO_2 (%)	-	-	68.8	-
Al_2O_3 (%)	-	-	14.6	-
Total potassium (K) (mg kg^{-1})	700-1200	9600	-	-
Total phosphorus(P) (mg kg^{-1})	150-450	14000	-	-
Total nitrogen (N) (%)	0.04-0.12	3.2	-	-
Organic nitrogen (N) (%)	-	2.5	-	-
$CaCO_3$ (%)	0.48	-	-	-
C/N	-	8.42	-	-
Total humic + fulvic acid (%)	-	25.07	-	-

*: Values declared by the commercial company.

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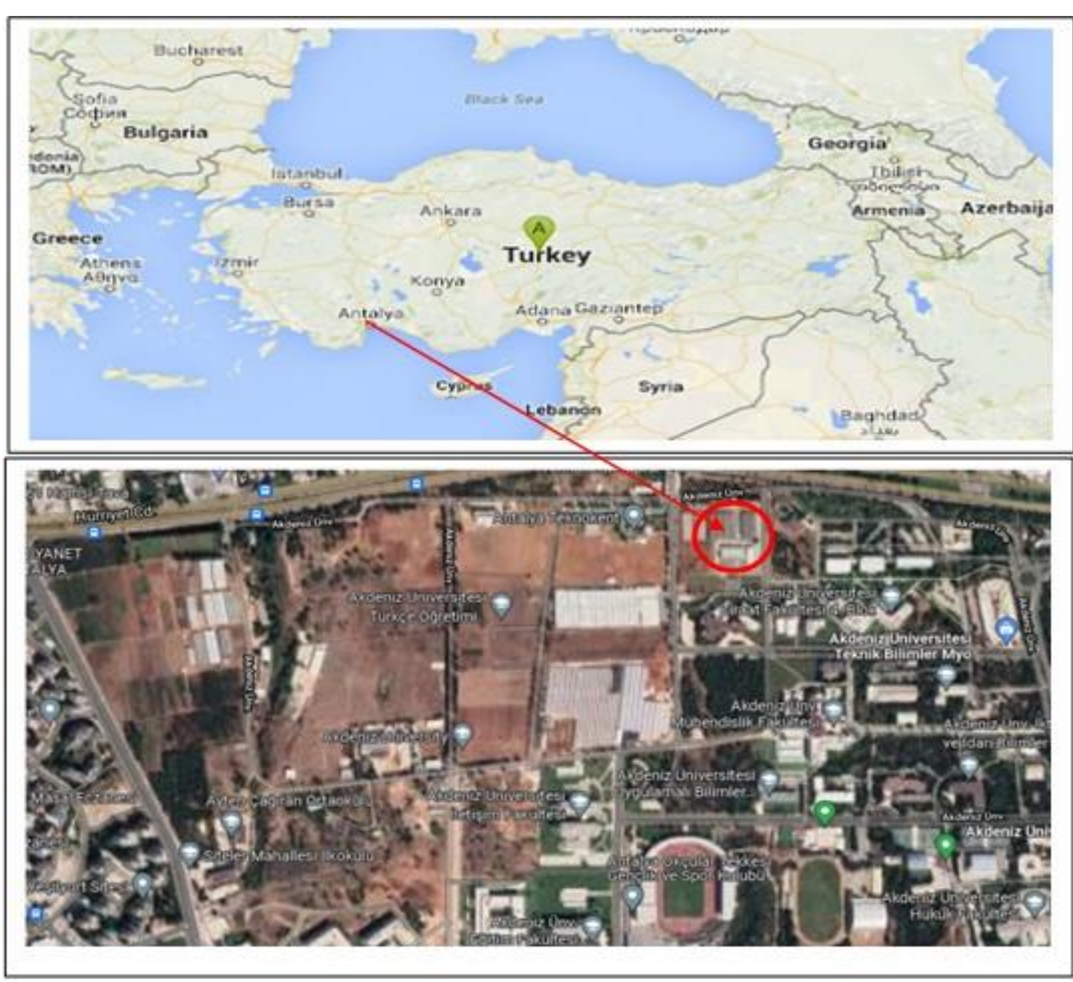

Figure 1. The location of the research area.

Table 2. Temperature values inside the greenhouse during the experiment*

	Trial duration (week)						
Temperature (°C)	1	2	3	4	5	6	7
Min.	14.0	15.4	13.3	11.3	12.4	14.7	14.5
Max.	28.9	31.5	27.6	23.4	28.3	32.3	29.6
Mean	21.5	23.5	20.4	17.4	20.3	23.2	22.1

*: Temperature data are the mean of measurements made four times a day.

The experiment was established according to the randomized plot design and consisted of a total of 40 pots including 8 growing media with 5 replications. The mixtures of growth media given in Table 3 were prepared and placed in 13.5 L pots with dimensions of 15 cm x 57 cm x 17 cm. In each pot, 20 tomato seeds were planted 1 cm below the surface and a total of 800 seedlings were used. No nutrients were added to the media during the experiment and only irrigation was made to each medium according to the plant's requirement. Irrigation was applied to all seedlings in equal amounts, considering the turgor of the seedlings. Some chemical and physical properties of the growing media are given in Table 4.

In the study, the amount of water retained by the medium was determined at matric potential values of 0, -2, -4, -5, -8 and -10 kPa in the Eijkelkamp Sand-Box device. The same samples were then placed in the pressure membrane and the amount of water retained at -33 kPa and -1500 kPa was determined (Demiralay 1993). The total porosity of the media was determined by calculating the volume of water retained at 0 kPa and the macro-pore percentages were determined by calculating the difference between the volume of water retained at 0 kPa and -5 kPa (Demiralay 1993).

The bulk density of the media was determined using the cylinder method (Demiralay 1993), and pH and EC values were determined using a pH and EC meter in a 1:5 mixture of media:water (Jackson 1967). The cation exchange capacity of the media was determined according to 1 N ammonium acetate method (Kacar 1995).

The effects of different growing media on the development of tomato seedlings were evaluated using seedling height (cm), germination percentage (%), stem diameter (mm), root length (cm), fresh root weight (g), fresh stem weight (g). The chlorophyll content of the seedlings was measured 40 days after planting with a portable chlorophyll meter (Minolta SPAD-502, Osaka, Japan). SPAD measurements were carried out by selecting 5 seedlings from each pot and 3 leaves for each seedling in the open air before noon (11:00-12:00). The measurements

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Table 3. Growing media prepared using four different substrates

Growing media	Description
1. %100 Peat	M1
2. %80 Peat + %20 Zeolite	M2
3. %80 Peat + %20 Diatomite	M3
4. %80 Peat + % 20 Vermicompost	M4
5. %70 Peat + %15 Zeolite + %15 Vermicompost	M5
6. %70 Peat + %15 Diatomite + %15 Vermicompost	M6
7. %70 Peat + %15 Zeolite + %15 Diatomite	M7
8. %70 Peat + %10 Zeolite + %10 Diatomite + %10 Vermicompost	M8

Table 4. Some measured analytical data of growing media

Growing media	pH (1:5)	EC (µS cm^{-1})	CEC (cmol kg^{-1})	Bulk density (g cm^{-3})
M1	5.67	249	280	0.49
M2	5.60	167	75	0.66
M3	6.99	398	75	0.73
M4	6.66	1623	50	0.56
M5	6.54	967	21	0.64
M6	6.79	1313	215	0.53
M7	6.40	294	190	0.74
M8	6.82	614	165	0.72

were made on the secondary and tertiary leaves after the cotyledon leaves. Each leaf SPAD value obtained was given as the average of 3 readings on each side of the midrib.

Descriptive statistics of mean and standard deviation were calculated, and the ANOVA technique was used to infer the effects of different growing media on tomato seedling variables. The Duncan's mean comparison test was used to group the statistically significant data. The SAS 9.4 program was used in statistical analysis and the differences in the variables $P<0.05$ were considered statistically significant.

3. Results

The amount of water retained in the growing media at different matric potentials (0, -2, -4, -5, -8, -10, -33 and -1500 kPa) is given in Fig 2. The highest moisture content at saturation point (0 kPa) occurred in M4 with 59.41% and the lowest moisture content in M7 with 44.64%. Except for the highest matric potential value (-1500 kPa), M4 had the highest moisture content of all matric potential values. In addition, when the difference between the moisture contents of the media at 0 kPa (saturation point) and -1500 kPa was examined, the highest moisture content was in M4 (41.54%), and the environments were ranked as M4>M1>M5>M8>M2>M6>M3>M7 in terms of water contents. On the other hand, when the difference between the moisture contents of the media at -33 kPa and -1500 kPa was considered, the media were ranked as M1>M4>M4>M8>M5>M3>M7>M2>M6>M3>M6 from the highest to the lowest moisture content (Fig. 2).

Measurements of some quality parameters of tomato seedlings grown in different growing medium are given in Fig. 3 a–ı. It was determined that all parameters, except the seedling root length (cm), were statistically significant depending on the difference in the growing medium. Differences between germination percentages in different growing medium were found to be statistically significant ($P<0.0001$) (Fig 3a). The highest germination percentage was obtained in M_1 (79%) medium. M_2 (76%), M_8 (67%), M_3 (63%) and M_7 (59%) mediums took place in the same group in terms of germination percentage. The lowest germination percentage was determined in M_4 (17%) medium.

The changes in stem diameter values of tomato seedlings grown in different growing media are shown in Fig. 3b. The differences between the stem diameter values of tomato seedlings were statistically significant ($P<0.0001$). The highest stem diameter was obtained in M8 (3.56 mm) and M4 (3.54 mm), while the lowest stem diameter was obtained in M7 (2.35 mm).

The changes in the height of tomato seedlings in different growing media are shown in Fig. 3c. The differences between the seedling heights in the growing media were statistically significant ($P<0.0001$). The highest seedling height values were obtained in M4 (22.21 cm), M5 (22.12 cm) and M8 (19.5 cm) environments and these environments were statistically in the same group. The lowest seedling height value was obtained in M7 (10.85 cm).

Changes in the root length of tomato seedlings in different growing media are shown in Fig. 3d. The differences between the root lengths of seedlings in different growing media were not statistically significant. The changes in fresh root weight of tomato seedlings in different growing media are shown in Fig. 3e. The differences between fresh root weights were statistically significant ($P<0.0001$) and the highest fresh root weight was obtained in M8 (19.12 g) and the lowest fresh root weight was obtained in M2 (3.31 g).

Changes in dry root weight of tomato seedlings in different growing media are shown in Fig. 3f. The differences between seedling root dry weights in different growing media were statistically significant ($P<0.0001$). The highest seedling root dry weights were obtained in M8 (1.32 g) and M5 (1.13 g) and these media were statistically in the same group. The lowest seedling root dry weight value was obtained in M7 (0.42 g).

Changes in tomato seedling fresh weight in different growing media are shown in Fig. 3g. In the study, the differences between seedling fresh weights in different growing environments were found to be statistically significant ($P<0.0001$). The highest fresh

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weight value was determined in M8 (144.44 g) and the lowest fresh weight value was determined in M7 (37.14 g).

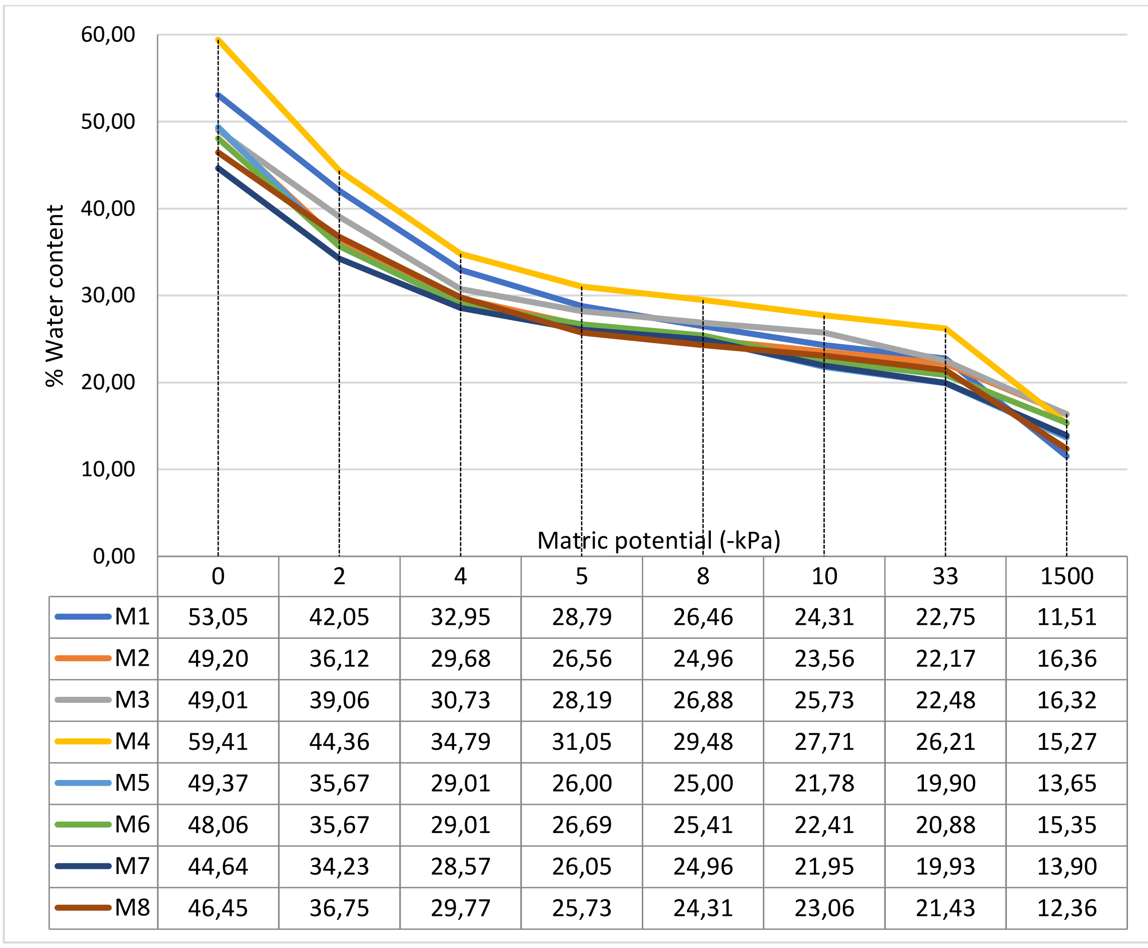
Changes in seedling dry weight of tomato seedlings in different growing media are shown in Fig. 3h. The differences between the dry weights of the seedlings in the growing media were statistically significant (P<0.0001). The highest seedling dry weight was obtained in M8 (15.24 g), while the lowest seedling dry weight was obtained in M7 (3.56 g), M2 (4.04 g), M3 (5.57 g) and M1 (6.09 g).

Changes in leaf chlorophyll content of tomato seedlings in different growing media are shown in Fig. 3i. The differences in leaf chlorophyll content of seedlings in different growing media were found statistically significant (P<0.0001). The highest chlorophyll content was measured in M6 (45.27spad) and the lowest chlorophyll content was measured in M1 (31.47spad).

4. Discussion

The highest amount of water retained at almost all matric potential (except for -1500 kPa) was found in the containing peat + vermicompost (M4). At -1500 kPa (15 atm) the water content stored in M1 media (%11.51) was lower than in the other media. This result implies that, under wilting point conditions, the M1 substrate contains a lower water content than the other media. This effect is due to the fact that the entire medium was composed of peat with a fibrous structure and had the lowest bulk density, thus releasing water easily under the low matric potential. Similar results to ourswere also reported by Fields et al (2004). According to Kutilek and Novak (1998), hydrological properties, such as water retention and water flow rate, are largely dependent on the total porosity and pore size distribution of the medium. However, according to the data obtained in our study, it is understood that chemical properties are as important as physical properties in the effect of growing medium on water properties. As a matter of fact, it was observed that the high EC values of the media with high proportions of vermicompost caused a decrease in their usefulness despite the high amount of water retention in these media. It is also thought that the increase in the osmotic pressure value of the medium with the increase in the EC value plays a role in this effect. In general, it is reported that the pH value for seedling growing media should be slightly acidic, and the EC should be lower than the value considered normal for many plants in the soil (De Boodt and Verdonck 1972). An ideal growth medium must have a pH range between 5.3 and 6.5; and the EC level must be less than 0.50 dS m^{-1} (Raviv et al. 1986; Abad et al. 2001).

On the other hand, under the highest matric potential, M1 had the lowest moisture content, but considering the moisture difference between -33 kPa and -1500 kPa, M1 had the highest moisture content. This is followed by M4 and M8. Physical (fiber content and pore size distribution) and some chemical (cation exchange capacity, electrical conductivity) properties of media



	0	2	4	5	8	10	33	1500
M1	53,05	42,05	32,95	28,79	26,46	24,31	22,75	11,51
M2	49,20	36,12	29,68	26,56	24,96	23,56	22,17	16,36
M3	49,01	39,06	30,73	28,19	26,88	25,73	22,48	16,32
M4	59,41	44,36	34,79	31,05	29,48	27,71	26,21	15,27
M5	49,37	35,67	29,01	26,00	25,00	21,78	19,90	13,65
M6	48,06	35,67	29,01	26,69	25,41	22,41	20,88	15,35
M7	44,64	34,23	28,57	26,05	24,96	21,95	19,93	13,90
M8	46,45	36,75	29,77	25,73	24,31	23,06	21,43	12,36

Figure 2. Water–release characteristic curves growing media.

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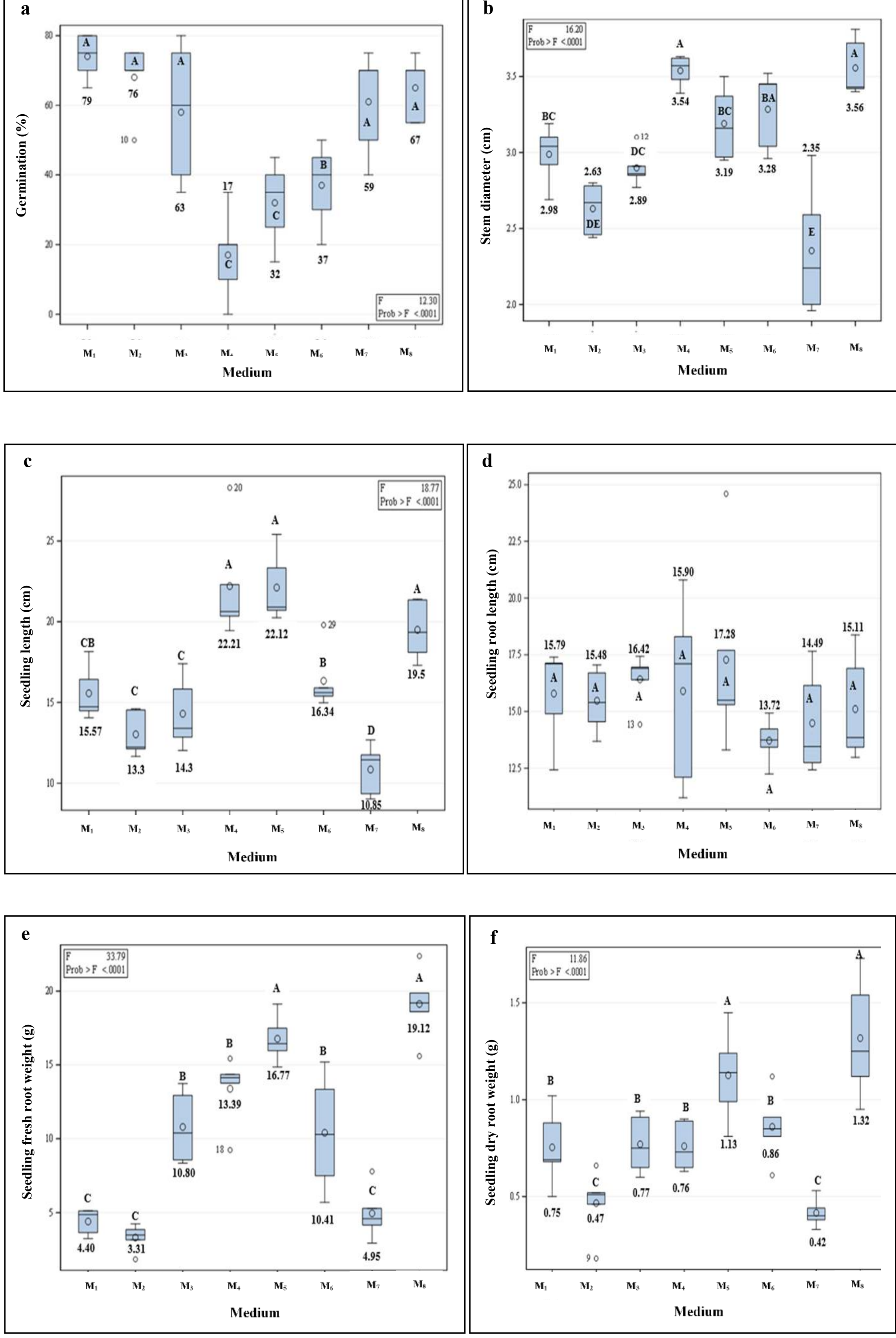

Figure 3. Some measurements of tomato seedlings grown in different growing media a) germination percentage b) stem diameter c) seedling length d) seedling root length e) seedling fresh root weight f) seedling dry root weight g) seedling fresh weight h) seedling dry weight and ı) chlorophyll content.

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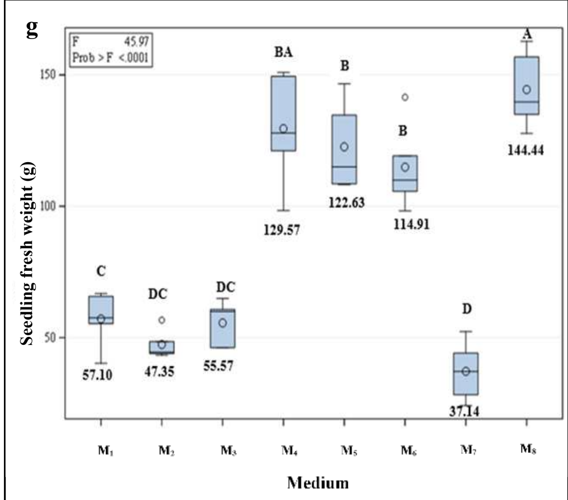

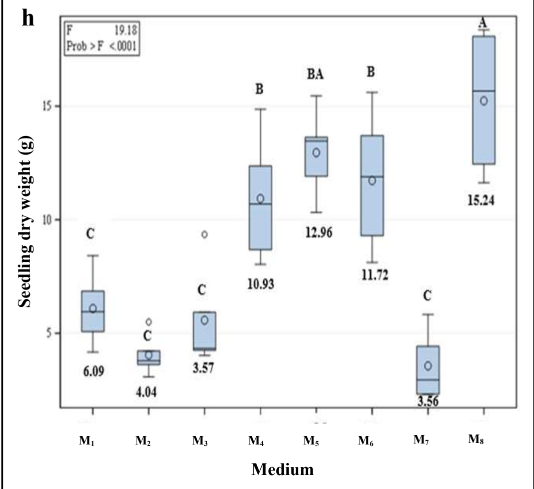

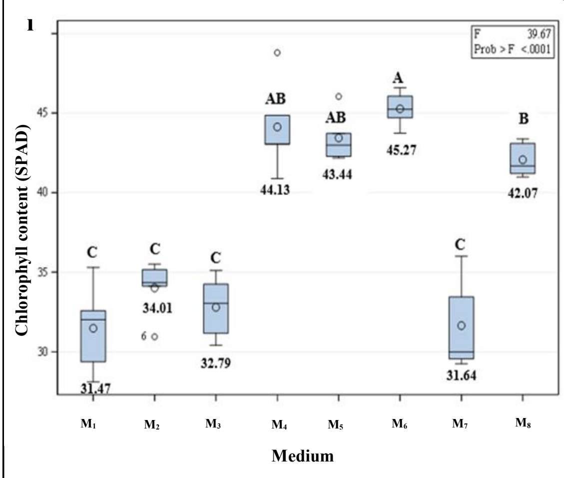

Figure 3. (continued)

may influence the amount of water. M1 and M4 have a more suitable physical structure than the other media due to their richness in fiber content and lowest bulk density values, which may lead to an increase in the amount of water available. Especially M4 has the highest moisture content between 0 kPa and -1500 kPa matric potential values. Therefore, M4 medium can be considered to be the most suitable medium with moisture supply continuity in seedling cultivation.

In almost all applications of soilless substrates, water retention is a decisive factor due to its impact on seed germination and plant growth. Seedling cultivation suffers from high drying rates due to free drainage in the media. The extreme importance of water supply in these situations makes the study of water-holding capacity and the potential increase in substrate formulations important issues. In this sense, the use of alternative substances to increase water retention capacity in soilless substrates is very important. Substances that can increase water-holding capacity have the potential to increase substrate water availability leading to greater plant growth and survival (Farrell et al. 2013). Aeration and water-holding capacity of the growing medium are important for seedling and plant growth. In addition, the water-holding capacity of the growing medium is one of the most effective and important factors in seedling and plant growth (Doneen and MacGillivray 1943; Bradford 1990). Balliu et al. (2017) stated that it is important that the water content of the substrate remains constant until seedling emergence. The physicochemical properties of peat, such as slow decomposition rate, low bulk density, high porosity, high water-holding capacity and high cation exchange capacity, make it suitable as a growing medium for many vegetables and ornamentals (Bohlin and Holmberg 2004).

Soilless growing systems generally use organic or mineral substrates that exhibit limited cation exchange and low buffer capacities, good water permeability, high water storage capacity and adequate aeration. In the absence of chemical and biological interactions, substrate performance depends predominantly on physical properties (Deepagoda et al. 2013). The addition of compounds with chemical activity to the medium may result in some, albeit small, changes in the water retention properties of the medium. This effect can be particularly noticeable in environments with materials that retain water with low strength. In addition to the suitability of the physical properties of the media, especially the high CEC value of M1 media may have had a positive effect on the amount of water retained. Indeed, among the media used in the study, M1 media has the lowest bulk density and the highest CEC value. Organic matter (OM) content and CEC play a vital role in water vapor sorption hysteresis, especially for clay minerals (Arthur et al. 2020). Based on the

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theory that water molecules form clusters on cations prior to full surface coverage of specific colloidal surfaces (Laird 1999; Prost et al. 1998), it has been confirmed that cation exchange capacity (CEC) and cation type play an important role in water vapor sorption magnitude and hysteresis (Arthur et al. 2019).

The amount of available water and EC level were significant in the germination rate. M4 and M1 medium had higher water retention capacity and water utilization capability than the other media, which led to an increase in the germination percentage in this medium. On the other hand, the lowest germination percentage obtained in M4 medium was attributed to the higher EC value of this medium compared to the other media. As a matter of fact, in some studies, the lowest values in germination percentages were obtained in the environments where vermicompost was used (Atiyeh et al. 2001; Ievinsh 2011; Tan 2014). The highest yield in vegetable cultivation depends on the balance between vegetative growth and generative development (Uzun 2001). Therefore, the relationship between stem diameter and plant height is important in determining the quality of the plant. The short length of the seedlings in the M7 medium is an indication that the seedlings are underdeveloped, and it is thought to be responsible for the thin stem diameters in this medium. The stem's small diameter may lead to variations in nutrient and water transport (Maltaş et al. 2017).

It is not desirable for quality seedlings to be too tall. Abnormalities in height are usually related to the growing medium temperature and the amount of water applied. The temperature regime of the seedling medium is very important for good and uniform germination. For this reason, temperature monitoring during germination is essential. For most crops, the temperature inside the germination room needs to be stable (Kubota et al. 2013). The optimum temperatures for tomato in seedling cultivation are 21-24°C and 18-21°C, respectively. (Balliu et al. 2017). Since the amount of sunlight decreases in winter, the light intensity also decreases. Unless this low light intensity is increased by additional lighting, the optimum temperatures specified for tomatoes should be reduced between 3-5°C according to the light intensity. If the temperature is not lowered, the seedlings will overgrow due to the high temperature (Ekşi 2012). The seedling heights obtained in our study were slightly above the average height (15-18 cm) stated by the seedling companies. This result may be due to the fact that the temperature values indicated in Table 2 in the first two weeks of the study were higher than the desired temperature values. In addition, the fact that no chemicals were used to inhibit the growth of the seedlings may have caused excessive growth of the seedlings. In some studies, on this subject, it was reported that seedling height was higher in environments where compost or vermicompost was used as a mixture (Atmaca 2012; Tan 2014; Sönmez 2017).

In the study, the highest fresh root weight was obtained in M8 medium. This result can be considered as an indication that M8 medium provides better root development. Yılmaz et al. (2017) examined the changes in seedling root weight parameters in different growing media and reported that the best seedling root wet weight results were obtained in the medium with the highest vermicompost ratio. In addition, vermicompost mixture was present in all environments where seedling fresh weight was high (M8, M6, M5 and M4). It was observed that the vegetative part increased in the environments with vermicompost, and it was thought that this effect may be due to the nutrient content of vermicompost and microbial activity in the environment (Atiyeh et al. 2001). On the other hand, Yılmaz et al. (2017) reported that the highest value (110.73 g) obtained in the fresh weight of tomato seedlings grown in different media was in the medium with 80% peat + 20% vermicompost proportional mixture.

High seedling dry weight indicates that the growth rate of the plants is high. In our study, the highest seedling dry weight and seedling height were obtained in M8 medium. This result was parallel with the findings of Uzun et al. (1998). On the other hand, vermicompost mixture was present in all environments (M8, M6, M5, M4) where seedling dry weight was high. Zaller (2007) reported that vermicompost added to the medium significantly affected seedling emergence and biomass distribution (root: shoot ratio) specifically for each tomato variety. The nutrient content and microbial enzyme content of vermicompost (Atiyeh et al. 2001) may be effective in achieving this result. In addition, since seedling fresh weight was low in M7 medium, it was considered to be a normal situation that root dry weight was also low.

Chlorophyll is one of the most important pigments that provide coloration in plants. The amount of chlorophyll in plants varies depending on many factors, such as plant species and growing conditions. It has been reported that it is important to know the average chlorophyll content in plants (Çetin 2017; Zeren et al. 2017), chlorophyll content in leaves affects dry matter production (Taiz and Zeiger 2008), and chlorophyll content is important in terms of quality and yield parameters (Kırbay and Özer 2015). In our study, although the best chlorophyll content was obtained in M6 medium, the chlorophyll content of seedlings in M4, M5 and M8 medium was also high. In conjunctionwith the results obtained in seedling dry weight, the presence of vermicompost in the medium also increased the chlorophyll content of the seedlings.

5. Conclusion

The physical properties of the growth media significantly affected the water content, influencing plant water uptake, yield, and seedling quality. Additionally, the electrical conductivity of the medium's water had a significant negative impact on plant water use. M4 medium retained the most water in its structure. In terms of the amount of available water, M1 and M4 media have approximately 1.5-fold the water content compared to the other media. The fact that both environments have the lowest bulk density played an important role in this effect. While high moisture content was obtained in the media with high organic content, it was determined that the moisture content decreased in the media with high mineral content. Tomato seedling yield and quality parameters (except germination percentage) decreased in the media containing only peat or mixing peat with other materials other than vermicompost. Although vermicompost increased both yield and quality in tomato seedlings, it is amounts greater than 15% in the medium which caused a negative effect on seedling germination. The M8 medium was the most favorable in terms of moisture capacity and other physicochemical properties and was the most suitable medium for tomato seedling growth and quality parameters. In summary, it was determined that the chemical capacities as well as the physical properties of the media to be used in soilless culture are important in terms of tomato seedling yield and quality parameters. For this reason, vermicompost should be included in the mixture in the preparation of growing media, but its proportion in the mixture should not exceed 15%.

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Acknowledgement

We thank Akdeniz University Scientific Research Projects Coordination Unit for their support (Project ID: FYL-2018-3114).

References

Abad M, Noguera, P, Bures S (2001) National inventory of organic wastes for use of growing media for ornamental potted plant production: case study in Spain. Bioresource Technology 77(2): 197-200. doi: 10.1016/S0960-8524(00)00152-8.

Arthur E, Tuller M, Norgaard T, Moldrup P, de Jonge LW (2019) Improved estimation of clay content from water content for soils rich in smectite and kaolinite. Geoderma 350: 40-45 doi: 10.1016/j.geoderma.2019.05.018.

Arthur E, Tuller M, Moldrup P, de Jonge LW (2020) Clay content and mineralogy, organic carbon and cation exchange capacity affect water vapour sorption hysteresis of soil. European Journal of Soil Science 71: 204-214. doi: 10.1111/ejss.12853.

Atiyeh RM, Edwards CA, Subler S, Metzger JD (2001) Pig manure vermicompost as a component of a horticultural bedding plant medium: effects on physicochemical properties and plant growth. Bioresource Technology 78: 11-20. doi: 10.1016/S0960-8524(00)00172-3.

Atmaca L (2012) Effects of using vermicompost as seedling growth medium. Ege University Graduate School of Natural and Applied Sciences. Master Thesis, İzmir, Turkey. (In Turkish)

Balliu A, Marsic NK, Gruda N (2017) Seedling production. Good agricultural practices for greenhouse vegetable production in the South East European countries. *Part II. Thematic approach 6. Seedling production.* FAO Plant Production and Protection Peper. ISSN 0259-2517, pp. 189-206.

Barral MT, Moldes AB, Cendon Y, Diaz-Fierros F (2007) Assessment of municipal solid waste compost quality using standardized methods before preparation of plant growth media. Waste Management & Research 25: 99-108. doi: 10.1177/0734242X07075514.

Bohlin C, Holmberg P (2004) Peat dominating growing medium in Swedish horticulture. Acta Horticulture 644: 177-181. doi: 10.17660/ActaHortic.2004.644.22.

Bradford KJ (1990) A water relations analysis of seed germination rates. Plant Physiology 94: 840-849. doi: 10.1104/pp.94.2.840.

Bures S, Landau DP, Ferrenberg AM, Pokorny FA (1993a) Monte Carlo computer simulation in horticulture: A model for container media characterization. Horticultural Science 28: 1074-1078. doi: 10.21273/HORTSCI.28.11.1074.

Bures S, Pokorny FA, Landau DP, Ferrenberg AM (1993b) Computer simulation of volume shrinkage after mixing container media components. Journal of the American Society of Horticultural Science 118: 757-761. doi: 10.21273JASHS.118.6.757.

Çetin M (2017) Change in amount of chlorophyll in some interior ornamental plants. Kastamonu University Journal of Engineering and Science 3(1): 11-19. (In Turkish)

De Boodt M, Verdonck O (1972) The physical properties of the substrates in horticulture. Acta Horticulture 26: 37-44. doi: 10.17660/ActaHortic.1973.26.5.

Deepagoda TKKC, Lopez JCC, Møldrup P, Wollesen de Jonge L, Tuller, M (2013) Integral parameters for characterizing water, energy, and aeration properties of soilless plant growth media. Journal of Hydrology 502: 120-127. doi: 10.1016/j.jhydrol.2013.08.031.

Demiralay İ (1993) Soil Physical Analysis. Atatürk University Faculty of Agriculture Publishing, No: 143, Erzurum, Turkey. (In Turkish)

Doğan D (2003) The effect of chicken manure added growing medium on growth and quality in seedling production of tomato and cucumber. Graduate School of Natural and Applied Sciences Department of Horticulture, Master Thesis, Ankara, Turkey. (In Turkish)

Doneen LD, MacGillivray JM (1943) Germination (emergence) of vegetable seed as affected by different soil moisture conditions. Plant Physiology 18: 524-529. doi: 10.1104/pp.18.3.524.

Ekşi C (2012) Seed Cultivation. General Directorate of Agricultural Research and Policies Alata Horticultural Research Station. Mersin, Turkey. https://www.alata.gov.tr. (In Turkish).

Farrell C, Ang XQ, Rayner JP (2013) Water-retention additives increase plant available water in green roof substrates. Ecological Engineering 52: 112-118. doi: 10.1016/j.ecoleng.2012.12.098.

Fields JS, William C, Fonteno BEJ, Owen JS (2004) Hydro physical properties, moisture retention, and drainage profiles of wood and traditional components for greenhouse substrates. Journal of the American Society of Horticultural Science 49(6): 827-832. doi: 10.21273/HORTSCI.49.6.827.

Gruda N, Qaryouti MM, Leonardi CH (2013) Growing media. In Good agricultural practices for greenhouse vegetable crops. Principles for Mediterranean climate areas. FAO, Plant Production and Protection Paper 217, Rome, pp. 271-302.

Gül A (2012) Soilless Agriculture, ISPN: 978-975-8377-83-1, Hasat Publishing, İzmir. (In Turkish)

Ievinsh G (2011) Vermicompost treatment differentially affects seed germination, seedling growth and physiological status of vegetable crop species. Plant Growth Regulation 65: 169-181. doi: 10.1007/s10725-011-9586-x.

Jackson ML (1967) Soil Chemical Analysis. Prentice Hall of India Pvt. Ltd., New Delhi.

Kacar B (1995) Chemical Analysis of Plant and Soil III. Soil Analysis, Ankara University Faculty of Agriculture Education Research and Development Foundation Publications, No: 3, Ankara, Turkey. (In Turkish)

Kırbay E, Özer H (2015) The Effects of Different Shading Applications on Yield and Quality of Organically Grown Cucumber (*Cucumis sativus* L.) in the Greenhouses. International Journal of Agriculture and Wildlife Science (IJAWS) 1(1): 7-14. (In Turkish)

Kubota CH, Balliu A, Nicola S (2013) Quality of planting material. In Good agricultural practices for greenhouse vegetable crops. Principles for Mediterranean climate areas. FAO Plant Production and Protection Paper Series, Chapter 13, Rome, pp. 355-378. doi: 10.13140/RG.2.1.1934.4808.

Kumar V, Guerreroa FM Tansel B, Savabi MR (2010) Hydro-physical characteristics of selected media used for containerized agriculture systems. Agricultural Water Management 98: 314-320. doi: 10.1016/j.agwat.2010.08.023.

Kutilek M, Novak V (1998) Exchange of water in the soil-plant-atmosphere system. International Agrophysics 12: 28-33.

Laird DA (1999) Layer charge influences on the hydration of expandable 2:1 phyllosilicates. Clays and Clay Minerals 47: 630-636. doi: 10.1346/CCMN.1999.0470509.

Lehman H, Clark EA, Weise SF (1993) Clarifying the definition of sustainable agriculture Journal of Agricultural Environmental Ethics 6: 127-143. doi: 10.1007/BF01965480.

Maltaş AŞ, Hız A, Kaplan M (2017) The Company and Cultivar Effects on Seedling Quality. Academia Journal of Engineering and Applied Sciences 2(3): 48-54. (In Turkish)

Mariyappillai A, Arumugam G (2021) Physico-chemical and hydrological properties of soilless substrates. Journal of Environmental Biology 42: 700-704. doi: 10.22438/jeb/42/3/MRN-1504.

Nelson PV (2012) Root Substrates. In: Greenhouse Operation and Management. 7th Edn., Prentice Hall, Upper Saddle River, NJ., pp. 161-194.

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Paradelo R, Moldes AB, González D, Barral MT (2012) Plant tests for determining the suitability of grape marc composts as components of plant growth media. Waste Management & Research 30: 1059-1065. doi: 10.1177/0734242X12451307.

Paradelo R, Vazquez-Nion D, Silva B, González A, Barral MT (2016) Acidification of mixtures of granite powder and compost to prepare nursery potting mixtures. Compost Science & Utilization 24: 1-10. doi: 10.1080/1065657X.2015.1020399.

Pokorny FA, Henny BK (1984) Construction of a milled pine bark and sand potting medium from component particles. I. Bulk density: A tool for predicting component volumes. Journal of the American Society of Horticultural Science 109: 770-773. doi: 10.21273/JASHS.109.6.770.

Pokorny FA, Gibson PG, Dunavent MG (1986) Prediction of bulk density of pine bark and/or sand potting media from laboratory analyses of individual components. Journal of the American Society of Horticultural Science 111: 8-11. doi: 10.21273/JASHS.111.1.8.

Prost R, Koutit T, Benchara A, Huard E (1998) State and location of water adsorbed on clay minerals: Consequences of the hydration and swelling-shrinkage phenomena. Clays and Clay Minerals 46: 117-131. doi: 10.1346/CCMN.1998.0460201.

Raviv M, Chen Y, Inbar Y (1986) Peat and peat substitutes as growth media for container-grown plants. In: Chen, Y., Avnimelech, Y. (Eds.), The Role of Organic Matter in Modern Agriculture. Martinus Nijhoff Publishers, Dordrecht, pp. 257-287.

Raviv M, Lieth JH (2008) Significance of soilless culture in agriculture. Soilless Culture 1-11. doi: 10.1016/B978-0-444-63696-6.00001-3.

Sevgican A (1999) Greenhouse vegetable cultivation (Soilless Agriculture- II) Ege University Publishing. No: 526, İzmir, Turkey. (In Turkish)

Sönmez İ (2017) Determination of the effects on growth and nutrient content of tomato seedlings of spent mushroom compost. Mediterranean Agricultural Sciences 30(1): 59-63.

Sonneveld C (1981) Items for application of macro-elements in soilless culture. Acta Horticulturae (ISHS) 126: 187-195. doi: 10.17660/ActaHortic.1982.126.23.

Taiz L, Zeiger E (2008) Plant Physiology. Sinauer Associates; 3 edition ISBN: 0878938230.

Tan E (2014) Determination of growing environments suitable for organic seedling production. Master Thesis, Ege University, İzmir, Turkey.

TÜİK (Turkey Statistical Institute) (2021) Plant production statistics. https://data.tuik.gov.tr/Kategori/GetKategori?p=tarim-111&dil=1. Accessed 26 December, 2021.

Tüzel Y, Gül A, Daşgan HY, Öztekin GB, Engindemiz S, Boyacı HF (2015) Changes and new searches in greenhouse cultivation. Turkey Agricultural Engineering VIII. Technical Congress, Ankara, Turkey, pp. 658-709. (In Turkish)

United Nations (UN) (2021) UN news: World population. https://www.un.org/en/global-issues/population. Accessed 26 December, 2021.

Uzun S, Demir Y, Özkaraman F (1998) Light interception and plant dry matter accumulation. Journal of Ondokuz Mayıs University Faculty of Agriculture 13(2): 133-154.

Uzun S (2001) Relationships between some growth and yield parameters of tomato and eggplant cultivation in greenhouse and temperature and light. 6. National Greenhouse Symposium. pp. 85-90. (In Turkish)

Wallach R, Da Silva F, Chen Y (1992) Hydraulic characteristics of tuff (scoria) used as a container medium. Journal of the American Society of Horticultural Science 117(3): 415-421. doi: 10.21273/JASHS.117.3.415.

Yelboğa K (2014) Growing power of agriculture: Seedling Industry. Garden News 3(2): 13-16. (In Turkish)

Yılmaz E, Özen N, Özen MÖ (2017) Determination of changes in yield and quality of tomato seedlings (*Solanum lycopersicon* cv. Sedef F1) in different soilless growing media. Mediterranean Agricultural Sciences 30(2): 163-168.

Zaller JG (2007) Vermicompost in seedling potting media can affect germination, biomass allocation, yields and fruit quality of three tomato varieties. European Journal of Soil Biology 43: 332-336. doi: 10.1016/j.ejsobi.2007.08.020.

Zeren İ, Cantürk U, Yaşar MO (2017) Change of chlorophyll quantity in some landscaping plants. Journal of Bartin Faculty of Forestry 19 (2): 174-182. (In Turkish)

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Acknowledgement of reviewers

MEDITERRANEAN AGRICULTURAL SCIENCES thanks to reviewers listed below for their enormous contribution to the articles published in Volume 37.

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e-ISSN 2528-9675

Journal web page: www.dergipark.org.tr/en/pub/mediterranean

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