MEDITERRANEAN AGRICULTURAL SCIENCES

Volume: 35
Number: 3
Year: December 2022

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

Prof. Dr. Mustafa ERKAN
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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
Fax: +90 242 310 2479

Subscription

Open access journal

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

Cover design
Assoc. Prof. Dr. Suleyman OZDERIN

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).

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: **35** Number: **3** Year: December **2022**

e-ISSN 2528-9675

MEDITERRANEAN AGRICULTURAL SCIENCES

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 115-120
DOI: 10.29136/mediterranean.1114313

Comparative characterization of the content and *in vitro* bioaccessibility of minerals in two Cornus species

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ARTICLE INFO

Received: May 9, 2022
Received in revised form: August 19, 2022
Accepted: August 23, 2022

***Keywords*:**

Cornus mas L.
Cornus sanguinea L.
Mineral content
Bioaccessibility

ABSTRACT

In this study, the content and bioaccessibility of minerals were investigated in four different cornelian cherry (*Cornus mas* L.) and one common dogwood (*Cornus sanguinea* L.) genotypes grown in Bursa, Turkey. Mineral content or bioaccessibility was determined using inductively-coupled plasma optical emission spectrometry or *in vitro* artificial gastrointestinal system, respectively. Results revealed that the common dogwood contained significantly greater amounts of minerals, particularly calcium and iron, compared with cornelian cherry genotypes. However, bioaccessibility of calcium or iron was greater in cornelian cherry genotypes (on average 90% or 25%, respectively) compared with that of common dogwood (13.72% or 4.48%, respectively). Bioaccessibility rates of potassium, magnesium and copper were over 50% in all genotypes. Among the cornelian cherry genotypes, G2 contained the highest amount of minerals, except for copper, and the highest amount of bioaccessible minerals. Although the mineral contents were different, amounts of bioaccessible minerals were comparable in both species due to the difference in bioaccessibility rates. In conclusion, the present study shows that fruits with rich mineral contents do not necessarily have high nutritional value due to lower bioaccessibility rates, and suggests that *in vitro* bioaccessibility studies are useful tools in the determination of the nutritional value of foods.

1. Introduction

The genus *Cornus*, classified under the Cornaceae family, is widely distributed in temperate and subtropical (rarely tropical) regions of the Northern Hemisphere, and it includes about 58 species, most of which are shrubs and small trees with hermaphrodite flower structure (Xiang et al. 2006). According to the chloroplast genome data and morphological characteristics, these species are divided into five groups: (1) alternate leaf, blue fruit, (2) opposite leaf, blue or white fruit, (3) Cornus cherries, (4) dwarf, and (5) large bractea, thorny (Truba et al. 2020). Most of the species in this genus are grown as ornamental plants and the most important species in terms of fruit cultivation is *Cornus mas* L. (cornelian cherry). The homeland of the cornelian cherry is Caucasus, Anatolia and Europe and it is found naturally in the forests of Northern Anatolia in Turkey. It grows in the mountains, forests and valleys of various provinces at up to 1400 m altitude in the Mediterranean, Aegean, Marmara and Black Sea regions of Turkey under suitable climatic conditions (Kökosmanlı and Keleş 2000, Demir et al. 2020). Slow-growing plants of this species are highly tolerant of cold, drought, disease and pests and their lifespan is up to 300 years (Bayram and Ozturkcan 2020). The elliptical or pear-shaped fruits, which ripen after midsummer, are 10-15 mm long, smooth-bright red-shelled, hard-core and sour tasting (Kökosmanlı and Keleş 2000). According to 2021 data, annual cornelian cherry production was 13745 tons in Turkey (TUIK 2022). Due to its sour taste, the fruits are used in various product groups after processing such as beverage, syrup, jelly, jam, yoghurt, ice cream, tarhana (a dried Turkish food product made with a fermented mixture of grain and yoghurt or fermented milk) and dried fruit pulp rather than fresh consumption (Kökosmanlı and Keleş 2000, Savaş et al. 2020). In addition to its successful growth in natural conditions without using any pesticides or synthetic fertilizers, the fact that the cornelian cherry is highly tolerant against different environmental conditions as well as pests and diseases makes this fruit suitable for organic production (Bijelić et al. 2011).

Fruits of species belonging to the genus *Cornus* have components (phenolic compounds, mineral content, vitamins, tannoid components and anthocyanins) that show quite strong biological activity. Therefore, they are used in both traditional and modern medicine and in the pharmaceutical industry (Stanković and Topuzović 2012). Studies with the genus *Cornus*, especially with the *C. mas* L. species, revealed anti-inflammatory effects and various beneficial effects (Sozański et al. 2014, Dadkhah et al. 2016). In addition, bioactive components and their

bioaccessibilities, as well as mineral contents of *C. mas* L., have been studied previously (Yilmaz et al. 2009, Ochmian et al. 2019, Anđela Martinović and Cavoski 2020, Olędzka et al. 2022). Although *C. mas* L. has been studied extensively, there are limited studies on other species of the *Cornus* genus. The majority of the species included in this genus has recently become the center of attention in the scientific community due to their biofunctional characteristics. For example, *Cornus alba* L., *C. sanguinea* L. (common dogwood), and *C. florida* L. species have been determined to contain rich nutritional sources such as phenolic acids and flavonoids (Truba et al. 2020). In addition, the fruits of *C. sanguinea* L. have been reported to be a promising candidate for a high-value natural antioxidant source (Stanković and Topuzović 2012). At the same time, there is no report on the bioaccessibility of the compounds identified in the fruits of *C. sanguinea*.

Fruits occupy a significant share in human nutrition all over the world and constitute one of the main sources of minerals needed by the organism to fulfill its vital functions. Minerals are essential for humans since they cannot be synthesized by the body and must be taken through diet (Rousseau et al. 2020). After foods are consumed, nutrients are converted into absorbable forms when traveling through the gastrointestinal system, and then they are transported to the relevant target tissues by entering the bloodstream (Boland et al. 2014). While bioaccessibility is defined as the portion of the nutrients released from the food matrix that is accessible for absorption from the stomach and intestine in relation to its total starting content, bioavailability refers to the part absorbed into the body and used in physiological functions or stored (Rousseau et al. 2020, Montiel-Sánchez et al. 2021). Bioaccessibility is examined *in vitro* by experiments that mimic the conditions at every stage of gastrointestinal digestion, while bioavailability is examined by *in vivo* animal and human studies (Rousseau et al. 2020). Since the *in vivo* methods are quite complex, expensive, time consuming and have ethical limitations, *in vitro* methods are preferred and widely used today because they are fast and safe. The *in vitro* method of gastrointestinal extraction has been shown to correlate well with *in vivo* human bioavailability studies (Miller et al. 1981).

The aim of this study was to determine and compare certain mineral contents as well as amounts of bioaccessible minerals by an *in vitro* gastrointestinal extraction method in fruits of four *C. mas* L. (cornelian cherry) and one *C. sanguinea* L. (common dogwood) genotypes grown in Cumalikizik village in the Bursa province in Turkey. To the best of our knowledge, no study has investigated the mineral content in *C. sanguinea* L. and bioaccessibility of minerals in species of *C. mas* L. and *C. sanguinea* L.

2. Materials and Methods

Fruits of four cornelian cherry (*C. mas* L.) (G1-G4) and one common dogwood (*C. sanguinea*) (G5) genotypes cultivated in Cumalikizik village in the Bursa province (Latitude: 40° 11' 25.1340" and Longitude: 29° 10' 20.1360") were used (Figure 1). About 3 kg healthy and mature fruits were randomly collected from four sides of trees in the second week of September for each genotype. Fruits, placed in paper bags, were brought to the laboratory within a short period of time and dried immediately at 65°C for 48 hours and then stored at -80°C until the analyses.

All solutions were of analytical purity and prepared using ultrapure water (18 MΩ cm resistant) with the TKA Ultra Pacific and Genpura water purification system. The 67% HNO_3 was obtained from Merck (Darmstadt, Germany). Argon (99.9995% purity, Linde, Turkey) was used as the carrier gas. Standard stock solutions (1000 mg L^{-1}) were used to prepare Merck (Darmstadt, Germany) calibration standards for each element. Standard solutions were prepared daily using 0.3% HNO_3. For botanical certificate reference materials for method validation: Certified Cabbage: IAEA - 359 Austria, Certified Tea NCSZC 73014- (GSB-7) China, Certified Strawberry LGC7162 UK, were utilized. As external standard solutions, 10 µg L^{-1} Cerium, Lithium, Yttrium, Thallium, and Cobalt were used. The Milestone Brand MLS 1200 Mega (Italy) microwave digestion system with a rotor with 12 sample chambers and polyethylene Teflon coated cups were used in the digestion process of the samples. Polyethylene Teflon containers were disinfected in 10% HNO_3 (67% v/v), then cleaned in ultra-pure water and dried in an oven at 40°C. The samples were homogenized and 0.5 g of samples were placed in Teflon cells and a mixture of 6 ml of HNO_3 (65%) and 1 ml of H_2O_2 (35%) was added. The samples were then digested using a Milestone Brand MLS 1200 Mega microwave burner according

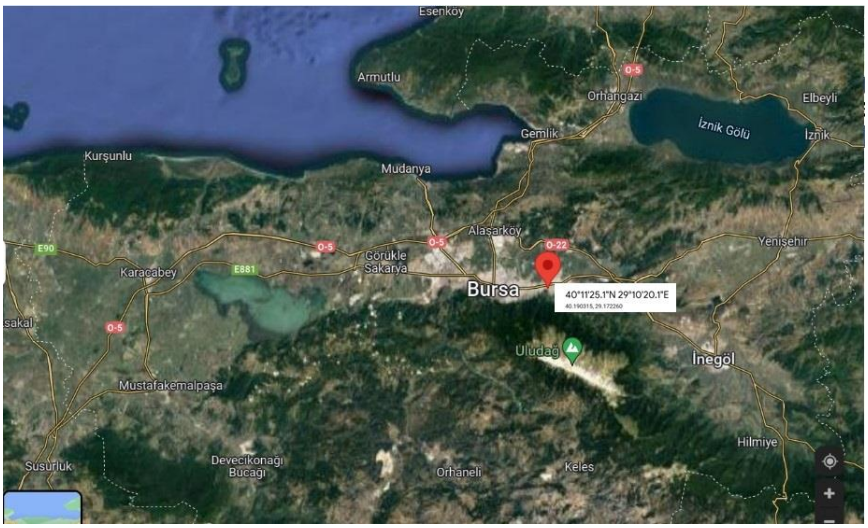


Figure 1. Cumalikizik village of Bursa province (Latitude: 40° 11' 25.1340" and Longitude: 29° 10' 20.1360").

to the following five-step program (250 W 2^{-1} min, 250 W 2^{-1} min, 250 W 6^{-1} min, 400 W 5^{-1} min, 600 W 5^{-1} min). Ultrapure water (Millipore Milli-Q 18.2 MΩ.cm) was added onto samples to reach a final volume of 25 ml which was followed by filtering through 0.45 µm filters (Hydropinilic PVDF Millipore Millex-HV). Filtered samples were then analyzed by ICP-OES (TS EN 13805). Operating conditions of the ICP-OES device have been presented in Table 1.

Table 1. Operating conditions of the ICP-OES

Parameter	Value
Instrument	Optima 2100 DV
Detector	CCD detector
Nebulizer	Concentric
RF generator	40 MHz
RF power	1300 W
Plasma gas flow rate	15.0 l·min^{-1}
Auxiliary gas flow rate	0.8 ml·min^{-1}
Nebulizer gas flow rate	0.5 l·min^{-1}
Pump speed	15 rpm
Auxiliary flow rate	1.0 l·min^{-1}
Integration mode	Field
Wavelengths	Ca 317.933. nm; Cu 324.754 nm; Fe 238.204 nm; Mg 285.213 nm; Mn 257.610 nm; K 766.490.nm; Zn 213.856 nm

Stock solutions were used at 1000 mg L^{-1} concentrations for ICP-OES analyses. Standard solutions were prepared by making dilutions from this stock solution. In addition, detection (LOD), quantification (LOQ) limits, and recovery studies were also carried out. Recovery studies were measured by spiking standard to the sample. The prepared samples were analyzed three times and the recovery values were determined from the results obtained (Table 2). K, Ca, Mg, Fe, Cu, Zn, and Mn contents of the samples were determined by ICP-OES (Perkin Elmer 2100 USA).

Table 2. Performance characteristic of the method

Element	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery %
K	2.2	7.4	105
Ca	2.7	9.1	91
Mg	2.1	6.9	75
Fe	0.3	1.0	77
Cu	0.2	0.7	100
Zn	0.3	0.8	84
Mn	0.1	0.4	90

Bioaccessibility studies with *in vitro* gastrointestinal extraction were conducted sequentially by creating an artificial stomach and intestinal system (Vitali et al. 2009). This method was carried out as follows: In the first stage, an artificial stomach solution (0.5 mg ml^{-1} pepsin solution adjusted to pH 2 with 5 M HCl) was added to the 0.5 g samples and then they were kept in a shaking water bath for two hours at 37°C. At the end of the shaking period, the samples were neutralized with 1 M $NaHCO_3$ and for the second stage, an artificial intestinal media (5 mg ml^{-1} pancreatine in phosphate buffer: pH 8.2) was added over the existing solution and incubated again for two hours in a shaking water bath at 37°C. Subsequently, samples were centrifuged at 4000 rpm for 20 minutes and 5 ml of the supernatant was taken and microwave combustion was applied, and then mineral analyses were performed with ICP-OES. The bioaccessibility ratio was calculated according to the equation given below:

Bioaccessibility %= [The value of the mineral content of the fruit after *in vitro* gastrointestinal extraction (mg kg^{-1}) / the value of the total mineral content of the fruit (mg kg^{-1})] x 100

In the experiment, at least three trees were used for each genotype and six parallel samples were prepared for each parameter. Each extracted sample was measured three times by ICP-OES. The data were evaluated statistically using the SPSS 23.0 software. LSD test at $P<0.05$ probability level was used to determine the statistically different groups among the average values obtained.

3. Results and Discussion

Functional nutrition has become an important part of our diet today. The increase in average life expectancy and the parallel increase in health costs led to the conduction of various studies in order to ensure a healthier life for people and to improve their quality of life (Odabaş-Serin and Bakır 2019). The importance of mineral substances for human health is indisputable. For example, potassium plays major roles in ion balance and cell functions, heart contraction, the proper functioning of the intestines and muscles. Calcium is responsible for the development of bones and teeth. It is also necessary for muscle contraction, nerve signal transfer, and secretion of hormones and enzymes. Magnesium plays an important role in the nervous and muscular systems. It is also important for the heart and kidneys to function properly (Mitic et al. 2019). Among the minerals found in very small amounts in the human body are iron (Fe), zinc (Zn) and copper (Cu) elements that are often lacking in human diets due to insufficient intake or inadequate absorption because of the food matrix. Insufficient mineral intake and especially iron, calcium and zinc deficiency in young children in industrialized countries are the main causes for diseases such as anemia, rickets, osteoporosis and immune diseases (Promchan and Shiowatana 2005). Daily intake of the minerals recommended by the United States Institute of Medicine for healthy men and women between the ages of 31-50 are as follows: K (4700 mg kg^{-1}), Ca (1000 mg kg^{-1}), Mg (320-420 mg kg^{-1}), Zn (11 mg kg^{-1}), Mn (2.3 mg kg^{-1}), Cu (0.9 mg kg^{-1}) (Koubová et al. 2018).

Mineral contents of cornelian cherry and common dogwood genotypes are presented in Table 3. K was the most abundant element quantified in both species. While the K contents of the cornelian cherry genotypes varied from 1486 mg kg^{-1} (G3) to 3762 mg kg^{-1} (G2), the amount of K in the common dogwood species was much higher (6114 mg kg^{-1}). Previous studies demonstrated that K content of cornelian cherry varied from 1400 to 5000 mg kg^{-1} and the K content of cornelian cherry genotypes fall into this range, in the present study. However, common dogwood was the richest genotype for the K mineral compared to all cornelian cherry genotypes analyzed in previous studies as well as in this study (Kalyoncu et al. 2009, Bijelić et al. 2011). The Ca content was high in both species. The highest Ca content was detected in common dogwood with the amount of 2441 mg kg^{-1} which is approximately 10 times higher than that in cornelian cherry genotypes. Among the cornelian cherry genotypes, the amount of Ca varied from 228 mg kg^{-1} in genotype G3 to 279 mg kg^{-1} in G2. Previous studies reported that the Ca content of cornelian cherry ranged from 27 mg kg^{-1} to 2000 mg kg^{-1} (Bijelić et al. 2011, Cetkovská et al. 2013) and our finding for the Ca content in cornelian cherry genotypes is compatible with these

Table 3. Mineral Contents of *Cornus mas* (G1-G4) and *Cornus sanguinea* (G5) genotypes

	Minerals (mg kg^{-1})						
Genotype	**K**	**Ca**	**Mg**	**Fe**	**Cu**	**Mn**	**Zn**
G1	2538±22.0^{c}	240±8.0^{c}	93±3.0^{d}	4.51±1.8^{bcd}	0.74±0.1^{c}	Nd	Nd
G2	3762±10.0^{b}	279±14.0^{b}	161±32.0^{b}	6.54±2.9^{bc}	0.76±0.1^{c}	Nd	Nd
G3	1486±10.0^{e}	228±20.0^{c}	90±3.0^{d}	3.08±0.8^{d}	0.78±0.1^{c}	Nd	Nd
G4	2472± 4.0^{d}	258±29.0^{bc}	103±16.0^{cd}	4.59±1.5^{bcd}	1.59±0.1^{b}	Nd	Nd
G5	6114±61.0^{a}	2441±205.0^{a}	491±15.0^{a}	41±19.0^{a}	3.33±0.1^{a}	2.37±0.1^{a}	Nd

Results are expressed as milligram dry weight per kilogram; ± standard deviation (n= 3); Nd: <LOQ; differences between averages in the same column bearing different letters are significant at P<0.05.

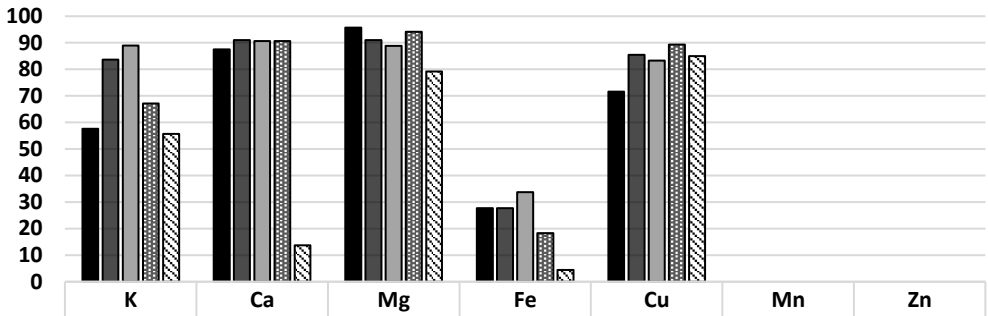
studies. On the other hand, the Ca content of common dogwood is higher compared to the highest Ca content reported in the literature for cornelian cherry. In terms of Mg content, the highest value was measured as 491 mg kg^{-1} in common dogwood. On the other hand, Mg contents in cornelian cherry genotypes varied from 90 mg kg^{-1} in G1 to 161 mg kg^{-1} in G2 genotype. The Mg level of the cornelian cherry detected in previous studies were remarkably variable (10-715 mg kg^{-1}) (Kalyoncu et al. 2009, Bijelić et al. 2011) and our results are in accordance with these studies. The Mg level of the common dogwood was in the range of the Mg level of the previously analyzed cornelian cherry genotypes. Iron mineral was found to be approximately 8-10 times lower in cornelian cherry genotypes than that in common dogwood species (41 mg kg^{-1}). Accordingly, the Fe content in cornelian cherry genotypes ranged from 3.08 mg kg^{-1} in G2 to 6.54 mg kg^{-1} in G3. The results obtained in the present study for both species agreed well with the Fe contents measured in other studies (0.4-48 mg kg^{-1}) (Cindrić et al. 2012, Cetkovská et al. 2013). As far as Cu is concerned, the highest amount among cornelian cherry genotypes was detected in the G4 genotype (1.59 mg kg^{-1}), while the other three genotypes were in the same statistical group. As with all other minerals, common dogwood species had the highest Cu content (3.33 mg kg^{-1}) among the analyzed samples. While Mn could not be determined in cornelian cherry fruits, it was detected in common dogwood with the amount of 2.37 mg kg^{-1}. Zn remained below the limit of detection (0.3 mg kg^{-1}) in all genotypes. Although we were not able to detect manganese and zinc in cornelian cherry genotypes, they were reported to be in the range of 0.2 to 29 mg kg^{-1} in the literature (Bijelić et al. 2011, Cetkovská et al. 2013, Ochmian et al. 2019). The reason for the variations in the content of minerals in different studies may be explained by genotypes as well as many other factors including geographic location, soil structure, agricultural practices, environmental factors, harvest method, storage conditions and analytical methods (Khoja et al. 2021). In the present study, all analyzed genotypes were grown in a garden with homogeneous cultivation practices. Therefore, the differences in minerals contents among the cornelian cherry genotypes and between two species could be due to the genotype.

While many studies have assessed the biological properties and chemical composition of *C. mas' fruits,* few phytochemical studies are present for common dogwood (*Cornus sanguinea* L.) fruits (Tenuta et al. 2022). In addition, there is no literature information regarding the mineral content of common dogwood fruits, therefore, a comparison could not be made with other genotypes of this species. On the other hand, many studies investigated the mineral content in other fruit species. Although the mineral content of the cornelian cherry species is similar or slightly higher than contents of plum, peach, blackberries, raspberries and strawberries except for Zn and Mn detected in other studies, the mineral content of the common dogwood is quite high compared to the commonly consumed fruit species (Baby et al. 2018). The fruits of common dogwood have a much richer mineral content than the fruits of *Corema album*, an edible wild species from the Ericaceae family (Brito et al. 2021). Table 4 shows the mineral content of cornelian cherry and common dogwood fruits measured after in vitro gastrointestinal extraction. When minerals are evaluated in terms of their bioaccessibility, the highest level of K content was determined in the common dogwood (3402 mg kg^{-1}), followed by G2 (3159 mg kg^{-1}), G4 (1661 mg kg^{-1}) G1 and G3 (1461 mg kg^{-1} and 1322 mg kg^{-1}) cornelian cherry genotypes. The results for Ca are quite surprising. Although the Ca content of the fruit is almost 10 times higher in the common dogwood genotype compared to the cornelian cherry genotypes, the bioaccessible Ca content of this species (335 mg kg^{-1}) is in the same statistical group with some cornelian cherry genotypes (G2: 254 mg kg^{-1} and G1: 218 mg kg^{-1}). While the bioaccessible amount of Mg was determined as 389 mg kg^{-1} in the common dogwood, it varied between 80-142 mg kg^{-1} among the cornelian cherry genotypes. Similar to calcium, although the Fe content in the common dogwood species is very high compared to that in cornelian cherry genotypes, the amount of iron measured by passing through the artificial gastrointestinal environment was found to be very close to each other in both species. The bioaccessible amount of Cu was determined as 2.83 mg kg^{-1} in common dogwood fruits and ranged between 0.53 and 1.42 mg kg^{-1} in cornelian cherry genotypes. Among all samples, Mn was determined only in the common dogwood species (Table 3) but its bioaccessibility was below detection limit. According to the proportional bioaccessibility of the mineral measured by in vitro gastrointestinal extraction method in the cornelian cherry and common dogwood species (Figure 2), the lowest bioaccessibility rate for K was detected in common dogwood (55.64%) and

Table 4. Bioaccessible mineral contents (mg kg^{-1}) of *Cornus mas* (G1-G4) *and Cornus sanguinea* (G5) genotypes

Genotype	**K**	**Ca**	**Mg**	**Fe**	**Cu**	**Mn**	**Zn**
G1	1461±42.0^{d}	218±62.0^{abc}	89± 4.0^{cd}	1.25±0.1^{a}	0.53±0.1^{cd}	Nd	Nd
G2	3159±92.0^{b}	254±10.0^{a}	142±16.0^{b}	1.81±0.8^{a}	0.65±0.1^{c}	Nd	Nd
G3	1322±30.0^{e}	209±11.0^{c}	80 ±11.0^{d}	1.04±0.1^{a}	0.65±0.1^{c}	Nd	Nd
G4	661±120.0^{c}	234±4.0^{b}	97±8.0^{c}	0.84±0.2^{ab}	1.42±0.1^{ab}	Nd	Nd
G5	3402± 54.0^{a}	335±79.0^{a}	389±50.0^{a}	1.84 ±0.9^{a}	2.83±1.3^{a}	Nd	Nd

Results are expressed as milligram dry weight per kilogram; ± standard deviation (n= 3); Nd: <LOQ; differences between averages in the same column bearing different letters are significant at P<0.05.



	K	Ca	Mg	Fe	Cu	Mn	Zn
G1	57.56	87.5	95.69	27.71	71.62	0	0
G2	83.67	91.03	91	27.67	85.52	0	0
G3	88.96	90.69	88.89	33.77	83.33	0	0
G4	67.19	90.69	94.17	18.3	89.31	0	0
G5	55.64	13.72	79.23	4.48	84.98	0	0

Figure 2. Bioaccessibility (%) of the mineral contents of *Cornus mas* (G1-G4) and *Cornus sanguinea* (G5) genotypes

the bioaccessibility of K in the cornelian cherry genotypes varied from 57.56 to 88.96%. In terms of Ca, the bioaccessibility of Ca in cornelian cherry genotypes was about 90%, while it was found to be at a very low level (13.72%) in common dogwood species. Mg has the highest bioaccessibility rate among the analyzed minerals varying from 79.23% in common dogwood to 95.69% in G1 genotypes. The bioaccessibility of Fe is the lowest among the minerals detected. Fe bioaccessibility in cornelian cherry genotypes ranged from 18.30% to 33.77%, while it was 4.5% in common dogwood species. The Cu bio-uptake rate was approximately 70-90% in all genotypes. While the bioaccessibility rates of the minerals for cornelian cherry fruits discussed in the present study were ranked as Mg>Ca>Cu>K>Fe, the most bioaccessible mineral in common dogwood fruits was Cu, followed by Mg, K, Ca, Fe and Mn (Figure 2).

The bioaccessibility of minerals is affected by many factors such as the chemical structure of the food, ligands in the food, redox activity of food components, chemical structure of the mineral of interest and mineral-mineral interaction (Lakshmi and Kaul 2011). According to the results, a significant amount of the analyzed mineral contents in the cornelian cherry fruits was found to be bioaccessible. Although the mineral contents of the common dogwood fruits were quite high, their bioaccessibility rates were found to be low. The reason for this discrepancy might be explained by the high proportion of compounds such as oxalic acid, carbonate and polyphenols which reduce absorption by forming insoluble complexes with minerals, and mineral-mineral interaction in the common dogwood species. At the same time, another reason might be low concentrations of proteins and amino acids (especially cysteine), because proteins and amino acids perform as reducing and chelating agents and increase the bioaccessibility of the element Fe in fruits and vegetables (Khouzam et al. 2011).

4. Conclusion

In conclusion, the amount of minerals that can be absorbed by the body is more important than that contained by the food. Therefore, the bioaccessibility of nutrients in foods has been important to demonstrate their nutritional value. Considering the mineral content in fruits of the two species in the study, *C. sanguinea* can be suggested as a very rich mineral resource for human nutrition. However, when *in vitro* bioaccessibility of the minerals is considered, both species have similar values. These results show that in investigating the potential of plant sources in terms of nutritional or functional properties, it may be misleading to evaluate the herbal matrix only by the amount of components it contains. The studies for evolution of herbal matrix should be supported by *in vitro* bioaccessibility determinations. Thus, more useful information can be obtained by determination of the nutritional potential of edible wild species. Today, in many areas of public opinion, recommendations are being made to consume wild species, especially dark fruits, due to their beneficial effects on people's health and rich nutritious content. However, as revealed in this study, the high content of a herbal food product may not mean that the food is very nutritious. In conclusion, in this study, the *in vitro* bioaccessibility of the mineral composition of *C. mas* and *C. sanguinea* fruits was examined for the first time and it was determined that the fruits of the *C. mas* species were a good mineral source for human nutrition when evaluated in terms of both total contents and bioaccessibility rates, and the G2 genotype was the most prominent among the studied genotypes.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 121-128
DOI: 10.29136/mediterranean.1082196

Potential of entomopathogenic fungi as biological control agents of *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera: Yponomeutidae)

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ARTICLE INFO

Received: March 3, 2022
Received in revised form: May 13, 2022
Accepted: July 1, 2022

Keywords:

Apple ermine moth
Entomopathogenic fungi
Molecular identification
Pathogenicity
Yponomeuta malinellus

ABSTRACT

The apple ermine moth, *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera Yponomeutidae), is a common pest of apple trees in Asia and Europe, and it has spread to North America. In apple growing regions of Turkey, the population of this pest may increase from time to time, requiring a separate control measure. In such cases, Turkish apple growers generally rely on synthetic insecticides to control this pest. The present study aimed to evaluate indigenous isolates of some entomopathogenic fungi (EPFs) against the pest as potential biological control agents. In the pathogenicity tests, 14 EPF isolates that belong to 4 fungal species [*Beauveria bassiana* (Bals.) Vuill. – 7, *Clonostachys rosea* (Link) Schroers – 3, *Isaria farinosa* (Holmsk.) Fr. – 2 and *Purpureocillium lilacinum* (formerly known as *Paecilomyces lilacinus* (Thom) Samson) (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson – 2] were assayed against the 4th instar larvae of *Y. malinellus* under laboratory conditions. All the EPF isolates were tested against the pest with three different conidial suspensions (1×10^7, 1×10^8 and 1×10^9 conidia ml^{-1}), using the spray method. The results of pathogenicity assays demonstrated that the effectiveness of the isolates increased with increasing concentration and elapsed time up to 9 days after treatment. Of the 14 isolates tested, 3 *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) were the most pathogenic, causing mortalities between 96.7% and 100% at the highest concentration 9 days post treatment. All the results suggest that the most pathogenic above-mentioned 3 isolates of *B. bassiana* have a significant biocontrol potential against *Y. malinellus*.

1. Introduction

The apple ermine moth, *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera Yponomeutidae), is a member of a European group of small ermine moths (Yponomeuta), consisting of nine species (McDonough et al. 1990). Adult *Yponomeuta* species are difficult to separate from one another, even by genitalia examination. Larval foodplant and some larval and pupal features are most reliable in their separation (Kimber 2021). *Y. malinellus* occurs in the Palaearctic region (in both Asia and Europe) (Kuhlmann et al. 1988; McDonough et al. 1990). However, there is a record that this species is also found in North America (Nearctic region) (Unruh et al. 1993).

The moths of this species have pure white forewings with black dots. First instars larvae overwinter under dense thick shield. In early spring, they crawl from under the shield, penetrate into swollen buds, and then mine the top part of leaflets of apple species. After the first molt, they leave the mines and live in the open. The later larval stages (2nd - 5th instars) feed all together in a silken web from May to early June. As larvae grow, their body varies from dark grey to yellowish grey in color, with dark spots along their sides (Iren 1960; Anonymous 2012). Different larval stages can be seen in the same web. A full- grown larva may reach 18 to 25 mm in length (Kimber 2021). During outbreak years, this species can negatively impact fruit production by defoliating apple trees (Anonymous 2012).

Until the last quarter of the last century, *Y. malinellus* was the second most important pest in apple orchards in Turkey after the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Iren 1960; Erturk 2016). Although it is still common in apple-growing regions of Turkey, no separate control measures are required for this univoltine pest species in apple orchards in most growing seasons, due to insecticide applications against other pests, especially *C. pomonella*. However, in some years, the population of this pest may increase enough to require a separate control measure. In such cases, chemical control remains a dominant management tactic against this pest. Although the presence of some effective parasitoids of this species has been reported in Turkey, their effectiveness has been greatly diminished due to excessive use of synthetic pesticides in apple orchards in many parts of the country (Gencer 2003; Narmanlioglu and Coruh 2018). Due to the many undesirable results of chemical applications, biological control methods have become a trend in recent years (Sönmez and Mamay 2018; Mamay and Mutlu 2019). Microbial agents have an important place among biological control agents including entomopathogens such as fungi (Alramadan and

Mamay 2019a), bacteria (Alramadan and Mamay 2019b), viruses (Alramadan and Mamay 2019c) and nematodes (Alramadan and Mamay 2019d). Microbial control agents may be viable alternatives to synthetic insecticides in the control of this pest, with no hazardous effects to human health or the environment. Recently, some *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) isolates were tested against *Y. malinellus* as microbial agents and some good results were obtained (Erturk 2016). Entomopathogenic fungi (EPFs) are common in terrestrial environments and play an important role in the regulation of insect populations (Alramadan and Mamay 2019a). EPFs are the most common microbial agents, and they are used in many parts of the world with great success and advantage due to the large number of virulent strains known, easy mass production and improving formulation (Butt et al. 2001; Wraight et al. 2001; Butt 2002; Goettel et al. 2010). Hence, this study had the objective of evaluating the biocontrol potential of indigenous isolates of some EPFs against this pest.

2. Materials and methods

2.1. Insect material

Insects used in the experiments were collected from the infested apple orchards in Cavdir (Burdur, Turkey) in June 2020. Silken nests containing 3rd and 4th instars larvae were carefully pruned from branchlets of trees and transported in 5-liter containers to the Entomology Laboratory in the Plant Protection Department, Akdeniz University (Antalya) for testing. The larvae were supplied with fresh apple (*Malus domestica* Borkh. cv. 'Starking') foliage under controlled conditions (25 ± 2°C, 60 ± 5 RH, and 16:8 h L: D photoperiod) in a climate room. They were used in the experiments when all of them reached the 4th instar larval stage (Baki et al. 2021).

2.2. Indigenous EPF isolates

Fourteen indigenous isolates belonging to three soil-borne EPF species, which had been previously isolated from soil samples collected from the selected agricultural habitats and their natural surroundings in Antalya province (South-western part of Turkey), and have already been maintained at the EPF Collection of Plant Protection Department of Akdeniz University, were tested in this study. The species and code names, habitats, sampling sites and geographic coordinates of the isolates tested are presented in Table 1.

2.3. Preparation of conidial suspensions

The EPF isolates were grown on PDA (Potato Dextrose Agar) in Petri dishes (9 cm diameter) and maintained in darkness at 26 ± 2°C and 65 ± 5 RH for 14 days for the completion of sporulation. Then, conidia were collected by scraping the surface of the culture of each fungal isolate gently with an inoculation needle and put into vials containing 10 ml of sterile distilled water + 0.03% Tween-80 (Sigma Chemical, St. Louis, Mo, USA). Prepared stock suspensions were filtered using a sieve (60-mesh) to remove hyphae and growing substrate and then homogenized for 3 minutes using a vortex (Yuyao Haiju Laboratory Equipment Co., Ltd., Zhejiang, China). The conidial concentration of stock suspensions was determined by direct count using a Neubauer hemocytometer (Fancelli et al. 2013). Serial dilutions (10^7–10^9 conidia ml^{-1}) were prepared in sterile distilled water containing Tween-80 and preserved at 4°C until used in the assays. Three concentrations (1×10^7, 1×10^8 and 1×10^9 conidia ml^{-1}) of each EPF isolate were tested, using the spray method. For each isolate, conidial viability was determined using the method of Goettel and Inglis (1997) before being used in the assays. The isolates with a viability of >95% were used in the assays.

2.4. Pathogenicity assays against the larvae of Y. malinellus

The assays were carried out under controlled conditions (25 ± 2°C, 60 ± 5 RH, 16:8 h L: D photoperiod) in the Entomology Laboratory of Plant Protection Department of Akdeniz University. For each treatment, randomly selected ten 4th instar larvae of *Y. malinellus* were introduced to each Petri dish (9 cm in diameter) lined with 3-layer filter paper. The insects were then sprayed through a handheld sprayer from a distance of 30 cm, using 2 ml of any conidial concentration of any EPF isolate. A control treatment (distilled water containing 0.03% Tween-80) was also included in the assays. All the treatments were replicated 3 times, and each treatment contained 10 larvae. After air-drying, treated larvae were transferred to new dishes containing clean fresh apple foliage using a fine camel-hair brush. The lids of the Petri dishes were closed and then perforated with a hot needle for ventilation (20 times per each). All the dishes were kept in the laboratory under the above-mentioned conditions of temperature, humidity and photoperiod. Surviving larvae in each of the dishes were fed on clean fresh apple foliage until the end of the experimental period.

Table 1. Indigenous entomopathogenic fungal isolates used in this study.

Isolate code	Fungal species	Sub-region	Vegetation	Latitude and longitude
BbKm-3	*Beauveria bassiana*	Kumluca	Orange	N 36°22'39.6" E 30°17'40.0"
BbKr-1	*B. bassiana*	Kemer	Forest	N 36°35'51.0" E 30°33'22.7"
BbMp-1	*B. bassiana*	Muratpaşa	Fig	N 36°53'07.2" E 30°44'30.4"
BbAl-1	*B. bassiana*	Alanya	Banana	N 36°33'40.8" E 31°56'43.7"
BbDs-2	*B. bassiana*	Döşemaltı	Pomegranate	N 37°00'02.4" E 30°38'16.1"
BbKs-1	*B. bassiana*	Kaş	Olive	N 36°12'08.8" E 29°38'46.3"
BbDm-1	*B. bassiana*	Demre	Orange	N 36°14'39.7" E 29°58'45.0"
CrFn-1	*Clonostachys rosea*	Finike	Orange	N 36°19'11.2" E 30°09'12.1"
CrFn-2	*C. rosea*	Finike	Orange	N 36°21'17.2" E 30°07'59.6"
CrKm-1	*C. rosea*	Kumluca	Orange	N 36°21'07.6" E 30°14'36.9"
IfKm-1	*Isaria farinosa*	Kumluca	Wheat	N 36°20'41.5" E 30°15'25.3"
IfDs-1	*I. farinosa*	Döşemaltı	Pomegranate	N 37°01'39.2" E 30°36'46.9"
PlKa-1	*Purpureocillium lilacinum*	Konyaaltı	Apple	N 36°53'53.5" E 30°37'51.8"
PlMp-1	*P. lilacinum*	Muratpaşa	Sassafras tree	N 36°53'42.6" E 30°39'56.7"

The dishes were observed daily under a stereomicroscope, and treatment mortalities were recorded at 3, 5, 7 and 9-days post infection. At each count, all dead larvae were removed individually from the dishes, and they were transferred to new Petri dishes containing damp filter paper individually. The dishes were incubated at 25 ± 2°C and 65 ± 5 RH in complete darkness for up to 14 days to monitor signs of mycosis in order to confirm fungal infestation as a probable cause of death. Larvae that did not display fungal outgrowths with identical characteristics to those of the applied fungus as treatment were not included in the count because their mortality was attributed to another factor or factors.

2.5. Molecular identification and phylogenetic analysis of EPF isolates

Molecular phylogenetic analyses were executed only for the three most virulent isolates of *B. bassiana* (BbKm-3, BbDs-2 and BbDm-1). For DNA isolation of the BbKm-3, BbDs-2 and BbDm-1 isolates, firstly pure cultures were developed in SDA (Sabouraud dextrose agar) medium at 25°C for 7-10 days incubation. Fungal genomic DNA was extracted from monosporic cultures of these three *B. bassiana* isolates through CTAB method described by Doyle and Doyle (1990). In this study, ITS 1/ITS 4 PCR primers of the ITS gene region, which are used in the molecular identification of many organisms White et al. (1990), and 983/2218 primers used in the amplification of the mRNA translation elongation factor 1-alpha (EF1alpha) (TEF) gene region Rehner (2001) were used in the diagnosis of high virulence entomopathogenic fungus isolates.

The classic PCR was conducted in a Peqlab Thermocycler Primus 96 device using two different primer sets, sequence and PCR conditions as shown in Table 2.

The phylogenetic analyzes were performed using the MEGA7 software (Biodesign Institute, Tempe, Arizona) using the Maximum Likelihood (ML) method based on the Tamura 3-parameter model (Tamura et al. 2011). Phylogenetic analysis was performed relative to the ITS and mRNA translation elongation factor 1-alpha (EF1 alpha) region sequence of the fungal isolates and the nucleotide sequence of other fungi from GenBank.

2.6. Data analysis

During the pathogenicity assays, no control mortality was detected; therefore, no adjustment was made for the mortality values. Prior to analysis, mortalities were arcsine-transformed and analyzed using the general linear model of the SPSS 23.0 Windows by one-way ANOVA (IBM Corp. 2015, USA). Tukey's honest significant difference test ($P<0.05$) was used to define significant differences among the treatment means. For all EPF isolates, lethal time (LT_{50} and LT_{95}) values with 95% confidence limits were also calculated using Probit analysis and the Log-probit method (SPSS® 23.0).

3. Results

3.1. Effectiveness of EPF isolates on Y. malinellus larvae

The results from the pathogenicity assays with the 4th instar larvae showed that all the EPF isolates tested had different efficacy rates against *Y. malinellus* (Table 3). Mortality was generally time- and concentration-dependent. Mortality rates caused by the isolates varied over time, and differences in mortality at each count date were generally significant among the different fungal isolates ($P<0.05$). Of all the EPF isolates tested, 4 isolates of *B. bassiana* (BbDm-1, BbDs-2, BbKm-3 and BbKs-1) and 1 isolate of *C. rosae* (CrFn-2) were most pathogenic and caused mortalities between 80% and 100% at all the concentrations tested 9 days post treatment. Even, at the shortest incubation time (3 days after application), these isolates exhibited ≥60% mortalities, except for the isolate BbKs-1 (Table 3).

For the highest concentration (1×10^9 conidia ml^{-1}) of EPF isolates tested, the time required for 50% and 95% mortality (LT_{50} and LT_{95}) of the 4th instar larvae of *Y. malinellus* varied between 0.79-4.57 days and 4.87-34.82 days, respectively (Table 4). The lowest LT_{50} and LT_{95} values were calculated for isolates BbDm-1, BbDs-2, BbKm-3, BbKs-1 and CrFn-2 (LT_{50}: 0.79, 1.83, 2.59, 2.66 and 2.36; LT_{95}: 4.87, 7.93, 9.06, 13.93 and 20.22 respectively), implying their high virulence and their biocontrol potential against *Y. malinellus*.

3.2. Phylogenetic placement of the EPF isolates tested

The accession numbers of the three most virulent EPF isolates found in this study, which belong to *B. bassiana*, and those of other isolates of the related species in GenBank are given in Table 5. After alignment analysis, the ITS and TEF region sequences of these three *B. bassiana* isolates data set consisted of 460 aligned positions. All these three *B. bassiana* isolates had 99%-100% homology with other isolates of the respective species in the GenBank (Figures 1 and 2).

Table 2. PCR primers and programs used in the identification of EPF isolates in this study.

Primers and References	Sequence	PCR conditions		
		Temperature (°C)	Time (Seconds)	Cycles
ITS1/ ITS4 White et al. (1990)	5′-TCCGTAGGTGAACCTGCGG-3' 5′-TCCTCCGCTTATTGATATGC-3'	94	300	1
		94	30	30
		56	60	
		72	90	
		72	300	1
983/ 2218 Rehner (2001)	5'-GCYCCYGGHCAYCGTGAYTTYAT-3' 5'-ATGACACCRACRGCRACRGTYTG -3'	95	300	1
		95	60	35
		58	60	
		72	60	
		72	300	1

Table 3. Mortality in the 4th instar larvae of *Yponomeuta malinellus* assayed with different conidial concentrations of EPF isolates at different time intervals after treatment

Fungal species and isolate name*	Dose (spores ml^{-1})**	Percent mortality (± SE)			
		3rd day***	5th day	7th day	9th day
Beauveria bassiana					
	1×10^7	40.0±0.0 BCDEFbIII	63.3±3.3 ABCaII	76.7±3.3 ABCaI II	90.0±5.8 AaI
BbKm-3	1×10^8	46.7±3.3 CDEabIII	73.3±3.3 ABaII	86.7±3.3 ABaI II	93.3±6.7 ABCaI
	1×10^9	60.0±5.8 BCDaII	76.7±3.3 ABCaI II	90.0±5.8 ABaI	96.7±3.3 ABaI
	1×10^7	36.7±3.3 CDEFbIII	50.0±0.0 CDEbII	63.3±3.3 ABCbI	70.0±0.0 ABbI
BbKr-1	1×10^8	50.0±5.8 BCDEabII	63.3±3.3 BCDab I II	70.0±0.0 ABCabI	76.7±3.3 CDabI
	1×10^9	63.0±6.7 BCaI	73.3±6.7 ABCDaI	76.7±3.3 ABaI	83.3±3.3 ABaI
	1×10^7	33.3±3.3 CDEFbII	40.0±5.8 EFGbII	63.3±3.3 ABCaI	76.7±6.7 ABaI
BbMp-1	1×10^8	33.3±3.3 DEFbIII	56.7±3.3 BCDabII	66.7±6.7 BCaI II	76.7±3.3 CDaI
	1×10^9	53.3±3.3 BCDaII	60.0±0.0 CDEFaII	66.7±3.3 BaI II	83.3±6.7 ABaI
	1×10^7	20.0±5.8 EFaII	33.3±3.3 FGaII	56.7±6.7 CDaI	73.3±3.3 ABaI
BbAl-1	1×10^8	23.3±3.3 FaII	36.7±6.7 EaII	60.0±5.8 CaI	80.0±0.0 BCDaI
	1×10^9	36.7±3.3 DaIII	46.7±6.7 FaII III	66.7±6.7 BaI II	80.0±5.8 BaI
	1×10^7	63.3±3.3 ABaI	70.0±0.0 ABbI	80.0±5.8 ABaI	86.7±8.8 AaI
BbDs-2	1×10^8	70.0±3.3 ABaII	73.3±3.3 ABabII	86.7±3.3 ABaI	96.7±3.3 ABaI
	1×10^9	73.3±3.3 ABaIII	83.3±3.3 ABaII III	90.0±0.0 ABaI II	100.0±0.0 AaI
	1×10^7	50.0±5.8 ABCDaIII	60.0±0.0 BCDaII III	76.7±3.3 ABCaI II	80.0±5.8 ABaI
BbKs-1	1×10^8	53.3±8.8 ABCDaII	63.3±3.3 BCDaI II	80.0±5.8 ABCaI	86.7±3.3 ABCDaI
	1×10^9	56.7±8.8 BCDaII	70.0±5.8 BCDEaI II	83.3±6.7 ABaI II	90.0±0.0 ABaI
	1×10^7	70.0±0.0 AbII	76.7±3.3 AbI II	83.3±3.3 AaI	86.7±3.3 AbI
BbDm-1	1×10^8	73.3±3.3 AbIII	86.7±3.3 AabII	93.3±3.3 AaI II	100.0±0.0 AaI
	1×10^9	90.0±0.0 AaI	93.3±3.3 AaI	96.7±3.3 AaI	100.0±0.0 AaI
Clonostachys rosea					
	1×10^7	43.3±6.7 BCDEaII	60.0±0.0 BCDaI II	63.3±3.3 ABCaI	70.0±0.0 ABbI
CrFn-1	1×10^8	50.0±0.0 BCDEaIII	63.3±3.3 BCDaII	73.3±3.3 ABCaI II	83.3±3.3 ABCDaI
	1×10^9	53.3±3.3 BCDaIII	66.7±3.3 BCDEFaII III	76.7±3.3 ABaI II	86.7±3.3 ABaI
	1×10^7	56.7±3.3 ABCaII	60.0±0.0 BCDaII	70.0±5.8 ABCaI II	80.0±0.0 ABaI
CrFn-2	1×10^8	60.0±5.8 ABCaII	66.7±3.3 BCaI II	73.3±3.3 ABCaI II	83.3±3.3 ABCDaI
	1×10^9	60.0±0.0 BCDaII	66.7±3.3 BCDEFaII	80.0±0.0 ABaI	86.7±3.3 ABaI
	1×10^7	36.7±6.7 CDEFaIII	46.7±3.3 DEFaII III	60.0±5.8 BCaI II	70.0±0.0 ABbI
CrKm-1	1×10^8	40.0±5.8 CDEFaIII	50.0±0.0 CDEaII III	63.3±6.7 BCaI II	73.3±3.3 DabI
	1×10^9	43.3±3.3 CDaIII	53.3±3.3 DEFaIII	66.7±3.3 BaII	80.0±0.0 BaI
Isaria farinosa					
	1×10^7	16.7±3.3 FcIII	30.0±3.3 GbII	36.7±3.3 DbII	56.7±3.3 BbI
IfKm-1	1×10^8	30.0±0.0 EFbIII	46.7±3.3 DEabII III	63.3±3.3 BCaI II	73.3±6.7 DabI
	1×10^9	46.7±3.3 CDaIII	53.3±6.7 DEFaII III	70.0±5.8 BaI II	80.0±0.0 BaI
	1×10^7	26.7±3.3 DEFaII	43.3±6.7 EFGaI II	56.7±3.3 CDaI	60.0±0.0 BbI
IfDs-10	1×10^8	30.0±0.0 EFaIII	46.7±3.3 DEaII III	60.0±5.8 CaI II	73.3±3.3 DabI
	1×10^9	43.3±6.7 CDaIII	50.0±0.0 EFaII III	66.7±3.3 BaI II	80.0±5.8 BaI
Purpureocillium lilacinum					
	1×10^7	43.3±3.3 BCDEaII	53.3±3.3 CDEaI II	60.0±0.0 BCaI II	70.0±5.8 ABaI
PlKa-1	1×10^8	46.7±3.3 CDEaII	60.0±0.0 BCDaI II	70.0±5.8 ABCaI	76.7±3.3 CDaI
	1×10^9	50.0±0.0 BCDaIII	60.0±5.8 CDEFaII III	73.3±6.7 ABaI II	83.3±3.3 ABaI
	1×10^7	36.7±8.8 CDEFaIII	43.3±3.3 EFGbII III	70.0±5.8 ABCaI II	73.3±6.7 ABaI
PlMp-1	1×10^8	46.7±3.3 CDEaIII	56.7±3.3 BCDaII III	70.0±5.8 ABCaI II	73.7±3.3 CDaI
	1×10^9	50.0±5.8 BCDaIII	60.0±0.0 CDEFaII III	73.3±6.7 ABaI II	83.3±3.3 ABaI
Control	dH_2O	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

*The differences between the means with different capital letters in different isolates on the same day are statistically significant (P<0.05; Tukey test).
**The differences between the means with different lowercase letters on the same day and in the same isolate are statistically significant (P<0.05; Tukey test).
***The differences between the means with different roman numerals on different days in the same isolate are statistically significant (P<0.05; Tukey test).

Table 4. LT_{50} and LT_{95} (days) values of indigenous EPF isolates tested at 1×10^9 conidia ml^{-1} to the 4th instar larvae of *Yponomeuta malinellus*.

Isolate name	Fungal species	LT_{50} (LCL-UCL)*	LT_{95} (LCL-UCL)	Chi-Square (df)	Regression equation (y=ax+b)
BbKm-3	*Beauveria bassiana*	2.59 (1.52-3.29)	9.06 (7.07-15.81)	54.942 (10)	y= -1.253+3.027x
BbKr-1	*B. bassiana*	1.59 (0.05-2.80)	34.82 (15.18-6.75)	34.330 (10)	y= -0.251+1.229x
BbMp-1	*B. bassiana*	3.00 (1.36-3.97)	32.37 (16.63-64.7)	34.569 (10)	y= -0.761+1.593x
BbAl-1	*B. bassiana*	4.57 (3.36-5.43)	21.60 (13.95-61.73)	47.433 (10)	y= -1.609+2.439x
BbDs-2	*B. bassiana*	1.83 (0.96-2.54)	7.93 (6.42-12.14)	30.332 (10)	y= 0.724+2.633x
BbKs-1	*B. bassiana*	2.66 (1.29-3.51)	13.93 (9.56-39.93)	50.951 (10)	y= -0.972+2.287x
BbDm-1	*B. bassiana*	0.79 (0.0-1.87)	4.87 (2.83-16.32)	51.006 (10)	y= 0.209+2.086x
CrFn-1	*Clonostachys rosea*	2.86 (2.07-3.45)	18.46 (13.32-34.09)	17.580 (10)	y= -0.927+2.031x
CrFn-2	*C. rosea*	2.36 (1.77-2.83)	20.22 (15.25-31.93)	14.011 (10)	y= -0.659+1.764x
CrKm-1	*C. rosea*	3.97 (3.29-4.52)	26.10 (18.01-50.73)	15.468 (10)	y= -1.205+2.011x
IfKm-1	*Isaria farinosa*	3.69 (2.53-4.50)	26.79 (16.03-96.84)	31.836 (10)	y= -1.086+1.913x
IfDs-1	*I. farinosa*	4.08 (2.96-4.92)	26.28 (15.79-95.05)	36.717 (10)	y= -1.245+2.035x
PlKa-1	*Purpureocillium lilacinum*	3.24 (2.07-4.02)	22.48 (14.14-70.46)	31.779 (10)	y= -0.999+1.956x
PlMp-1	*P. lilacinum*	3.26 (2.09-4.04)	24.88 (15.22-83.87)	29.116 (10)	y= -0.957+1.864x

*95% confidence limits (CL); LCL, lower limit; UCL, upper limit.

Table 5. GenBank accession numbers and gene regions used in phylogenetic analysis of indigenous three most virulent *Beauveria bassiana* isolates and their relatives.

Isolate name	Species	Gene	Accession no.	Isolate name	Species	Species	Accession no.
BbDm-1	***B. bassiana***	**TEF**	**OM489219**	**BbDm-1**	***B. bassiana***	**ITS**	**MT441872**
BbDs-2	***B. bassiana***	**TEF**	**OM489220**	**BbDs-2**	***B. bassiana***	**ITS**	**MT441879**
BbKm-3	***B. bassiana***	**TEF**	**OM489221**	**BbKm-3**	***B. bassiana***	**ITS**	**MT441870**
792	*B. bassiana*	TEF	AY531957	F19-N	*B. bassiana*	ITS	MG640376
3097	*B. bassiana*	TEF	AY531925	MG562497	*B. bassiana*	ITS	MG562497
ArgB33	*B. bassiana*	TEF	KT748548	SHU.M.161	*B. bassiana*	ITS	KU158472
TM1613	*B. bassiana*	TEF	LT220758	SHU.M.131	*B. bassiana*	ITS	KU158461
TMCR05	*B. bassiana*	TEF	LT220759	EABb04	*B. bassiana*	ITS	KC753382
GMCR51	*B. bassiana*	TEF	LT220745	SASRI BB444	*B. bassiana*	ITS	JX110368
CHE-CNRCB 84	*B. bassiana*	TEF	MH203473	2718	*B. bassiana*	ITS	KU364353
TMSL142	*B. bassiana*	TEF	LT220761	EABb 04/01	*B. bassiana*	ITS	DQ364698
2579	*B. bassiana*	TEF	AY531916	HHWG1	*B. brongniartii*	ITS	JX110385
GMGJ75A	*B. bassiana*	TEF	LT220749	SASRI	*B. brongniartii*	ITS	JX110388
CHE-CNRCB 414	*B. bassiana*	TEF	MH203489	FUM03	*B. varroae*	ITS	MF667767
LPSc1213	*B. bassiana*	TEF	MK047585	B5	*B. varroae*	ITS	MH374536
B47	*B. caledonica*	TEF	MK040132	ARSEF 2641	*B. amorpha*	ITS	HQ880808
BUB421	*B. caledonica*	TEF	MG642903	B518a	*B. amorpha*	ITS	HQ880806
YFCC 7025	*B. vermiconia*	TEF	MN576997	BYYC-05	*B. asiatica*	ITS	MG345071
BCC14510	*B. asiatica*	TEF	MN401502	BUB824	*B. asiatica*	ITS	MG642836
BCC12907	*B. asiatica*	TEF	MN401481	ARSEF 4622	*B. australis*	ITS	HQ880790
YFCC 5600	*B. asiatica*	TEF	MN576996	ARSEF 4598	*B. australis*	ITS	HQ880789
BCC75846	*B. asiatica*	TEF	MN401462	F585	*B. caledonica*	ITS	DQ529233
BCC2120	*B. asiatica*	TEF	MN401465	BG47	*B. caledonica*	ITS	MT180427
C18-2_b	*B. brongniartii*	TEF	KJ908277	1717	*B. vermiconia*	ITS	FJ973063
RUG50-1_b	*B. brongniartii*	TEF	KJ908276	ARSEF 7281	*B. sungii*	ITS	HQ880815
RUB11-2_b	*B. brongniartii*	TEF	KJ908275	EFCC 5657	*B. sungii*	ITS	JX463219

4. Discussion

The results of the present study showed that all indigenous EPF isolates tested had a pathogenic activity against the 4th instar larvae of *Y. malinellus* under laboratory conditions; however, three isolates of *B. bassiana* (BbDm-1, BbDs-2 and BbKm-3) were more pathogenic than others. A review of the relevant literature revealed that there is no study on the biological control of *Y. malinellus* using EPFs. So, we could not compare our results with others. In a previous study with entomopathogenic bacteria, Erturk (2016) evaluated the insecticidal effects of some *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) isolates as biological control agents against *Y. malinellus*. He reported that two *B. thuringiensis* isolates (HD-1 and BTS-1) were the most pathogenic and caused 97% and 83% mortalities of 4th larvae of *Y. malinellus* at the concentration of 1.8 x 10^9 72 h post treatment under laboratory conditions. The results obtained from the Erturk's study confirmed that the biological control of the pest may be possible by using entomopathogenic agents. However, the efficiency of entomopathogenic fungi as well as bacteria is greatly influenced by many abiotic factors, such as temperature, relative humidity, solar radiation, etc. (Vidal and Fargues 2007). That is why it is necessary that EPF applications be made at a

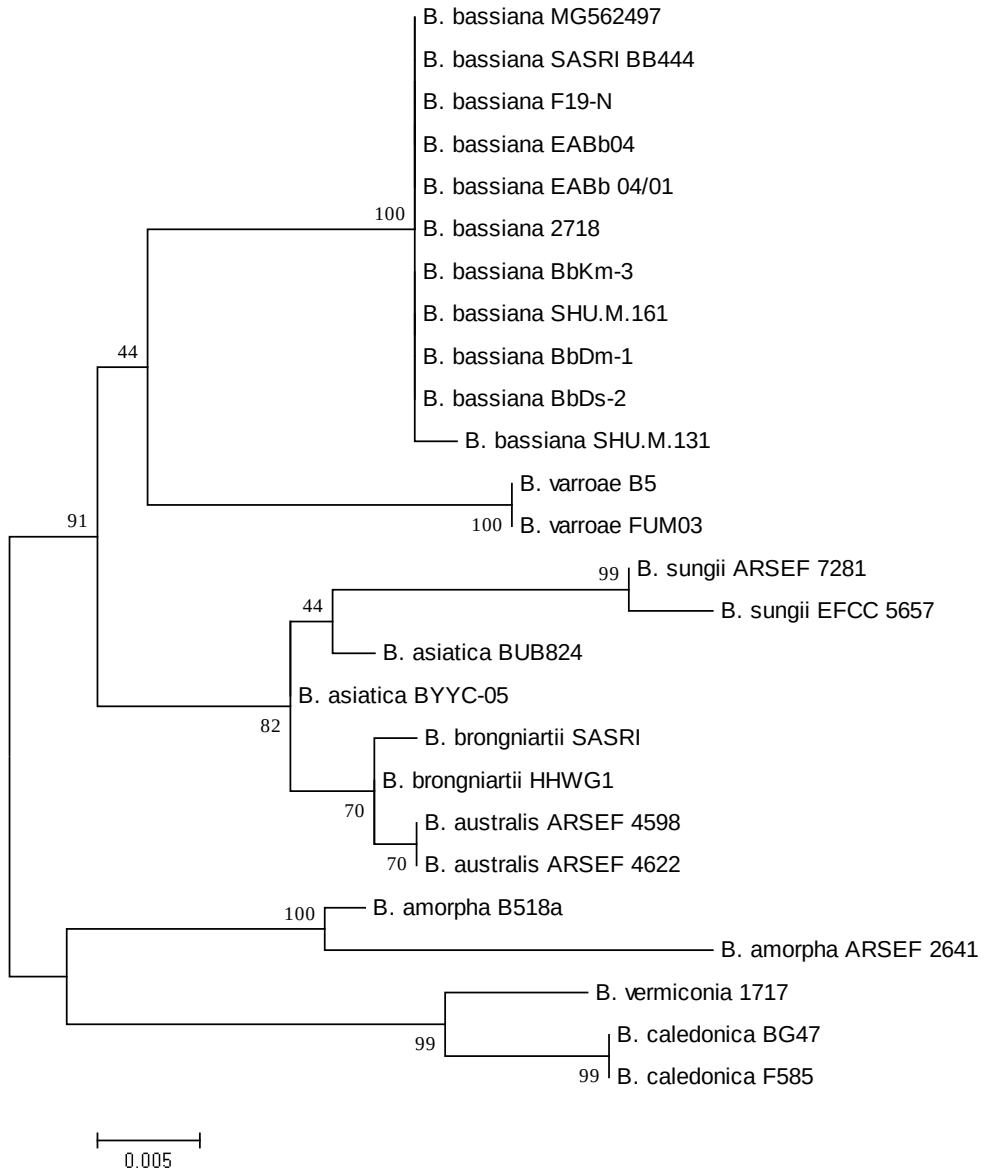

Figure 1. The Maximum Likelihood tree based on the Tamura 3-parameter model showing the phylogenetic relationship between the three *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) found to have high virulence in the present study and other *B. bassiana* isolates from GenBank based on ITS region sequence.

time when ambient temperature and humidity is suitable for optimal entomopathogenic activity. Likewise, it is more appropriate to do applications in the evening or early in the morning when the solar radiation is low or nonexistent.

As for the phylogenetic placement of the three most virulent EPF isolates, which are 3 isolates of *B. bassiana* (BbKm-3, BbDs-2 and BbDm-1), the results demonstrated that these three isolates had 99% evolutionary homology with other *B. bassiana* isolates from the NCBI database. In the present study, two different gene regions (ITS and TEF regions) were used for identifying and comparing the above-mentioned EPF isolates. Rehner and Buckley (2005) reported that molecularly based sequences based on a single region can be misleading in determining *B. bassiana* isolates. Also, many researchers used the multiple gene sequencing approach for identifying and comparing *B. bassiana* isolates (Glare and Inwood 1998; Glare 2004; Rehner and Buckley 2005; Glare et al. 2008). Serna-Domínguez et al. (2019) identified 44 *B. bassiana* isolates from different pests in the west-central Mexico (The state of Colima) using a translation elongation factor 1-α (TEF) and Bayesian phylogenetic analysis of the nuclear intergenic Block region. They did not detect any significant genetic associations between any substrate, insect-host, or geographic origin combination. Their results also indicated that the TEF region was effective in identifying *B. bassiana* isolates, similar to those of the present study. Likewise, Castro-Vásquez et al. (2021) molecularly identified 32 *B. bassiana* isolates, 26 from Costa Rica, 5 from Puerto Rico and one from Honduras, using the Bloc, TEF-1α and RPB2 regions. Their results showed that the TEF region can be used effectively in the identification of *B. bassiana* isolates in molecular characterization and there is a low correlation between geographic origin and variation between isolates.

Based on the results of this study, it was concluded that three indigenous isolates (BbDm-1, BbDs-2 and BbKm-3) of

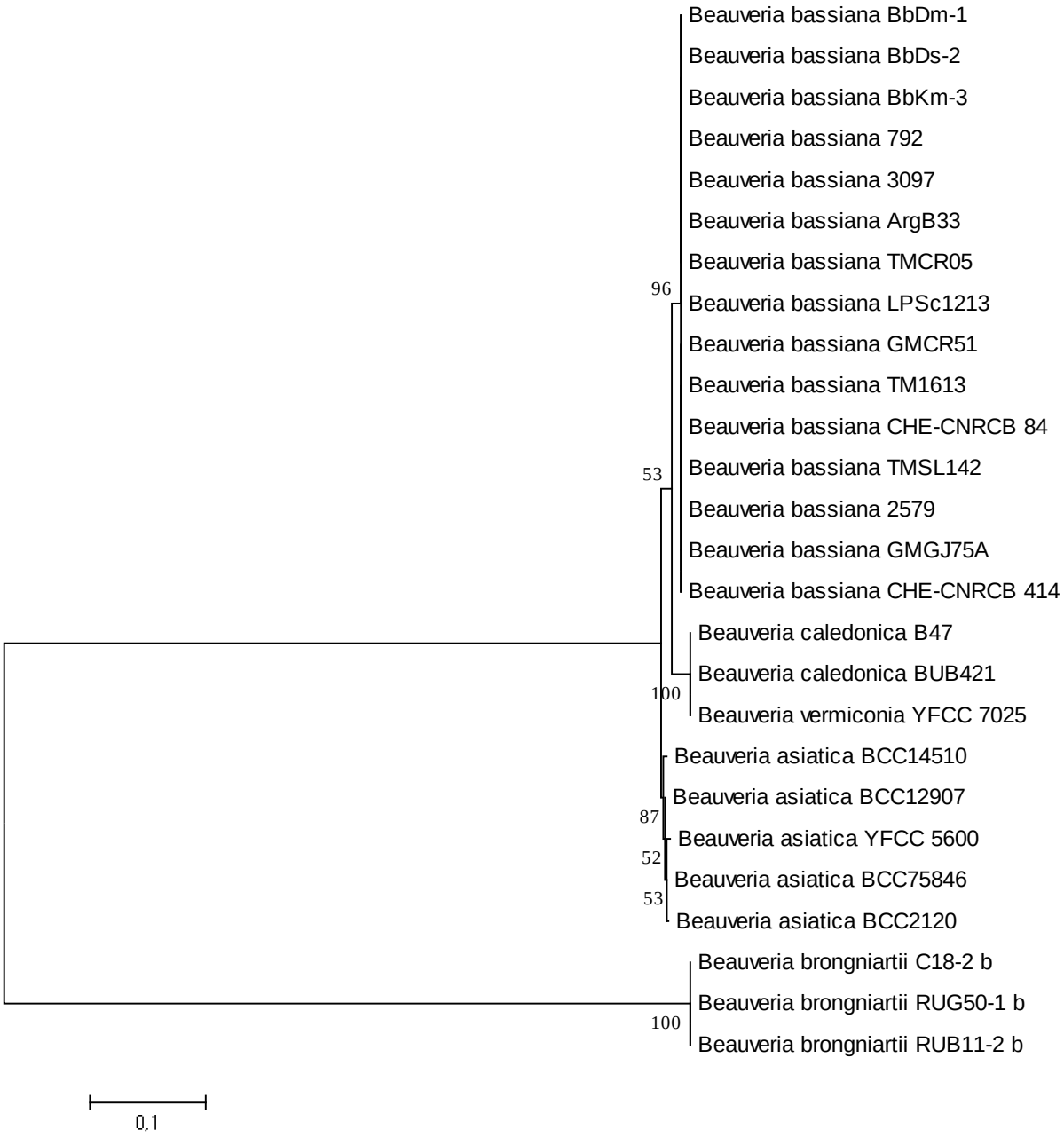

Figure 2. The Maximum Likelihood tree based on the Tamura 3-parameter model showing the phylogenetic relationship between the three *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) found to have high virulence in the present study and other *B. bassiana* isolates from GenBank based on TEF region sequence.

B. bassiana can be used as potential alternatives for the management of *Y. malinellus*. However, further studies should be conducted under field conditions to better understand the efficacy of these three *B. bassiana* isolates and their potential as effective biocontrol agents within the framework of an integrated pest management (IPM) program in apple orchards.

Acknowledgements

The authors thank to the Scientific Projects Coordination Unit of Akdeniz University for financial support and to the growers for their helps during the collection of insect material.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 129-134
DOI: 10.29136/mediterranean.1101666

Molecular analysis of resistance gene locus to bacterial canker and wilting disease in tomato mutants

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ARTICLE INFO

Received: April 11, 2022
Received in revised form: May 13, 2022
Accepted: July 1, 2022

***Keywords*:**

Polymerase Chain Reaction
SSR markers
Maping
Clavibacter michiganensis subsp. *michiganensis*

ABSTRACT

The tomato plant is one of the most widely produced vegetables in the world. However, there are several disease factors which limit tomato production. The *Clavibacter michiganensis* subsp. *michiganensis (Cmm)* pathogen causes wilting and canker symptoms on the leaves of plants and bird's eye symptoms on the tomato and then the plants completely die. Development of resistant tomato varieties is prerequisite due to absence of an effective control methods against the bacterial disease. The resistant M3-9 and M3-15 tomato plants have been developed because of mutation from susceptible NCEBR3 tomato seeds with ethyl methanosulfonate (EMS). For each chromosome of the tomato genome, 24 SSR markers were selected from each end of the haploid, 12 tomato chromosomes, and polymorphic differences between susceptible and resistant tomato plants were studied. Polymorphisms were found with SSR13 and SSRB18031 markers located on chromosome 5 with resistant mutants, M3-9, M3-15 and susceptible original NCEBR3 plants. It is envisaged that a resistance gene is located on the 5th chromosome of resistant M3-9 and M3-15 plants. Further fine mapping studies will reveal the location of the resistance gene(s) for controlling bacterial canker and wilting pathogen.

1. Introduction

Tomatoes are among the most important vegetables for agricultural production worldwide. According to data from the United Nations Food and Agriculture Organization, 180766329 tons of tomatoes were produced on 5030545 hectares worldwide in 2019. Turkey ranks third in the world with a tomato production of 12841990 tons in an area of 181488 ha (FAO 2021), whereas 13204015 tons of tomato production were recorded in 2020 according to the Turkish Statistical Institute (TÜİK 2021).

Among plant pathogenic bacteria, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) causes complete death of tomato plants by showing severe symptoms of canker and wilting in plants. On young plants, when an infection is caused by an inoculum directly placed on the vascular bundle tissue, systemic infection manifests itself with wilting symptoms. In older plants, wilting symptoms appear more slowly and gradually than in young plants. Even though this disease agent has such a high importance, there is no effective cultural, biological or chemical control management. In a fight against the disease, chemical applications protect the plants, but cause negative effects on environment and human health. Considering all these factors, the most important issue would be a permanent and effective controlling of the bacterial pathogen, this is possible with the development of resistant plant varieties (Agrios 2005; Çalış et al. 2013).

The bacterial pathogen *Cmm*, likely other plant pathogens must test for their host's suitability to multiply in the host; if a receptor protein encoded by *resistance* (*R*) gene recognizes elicitor protein encoded from *avirulence* gene of pathogen during interactions between the host and the pathogen are resulting resistance. When the resistance gene recognizes pathogens, it activates defense mechanisms and controls the pathogen (Rathjen and Mofefett 2003; Meyers et al. 2005). In these relationships, called gene-for-gene theory, resistance arises when the *R* genes of the host plant against the *avirulence* genes of the pathogen are in harmony with each other because the plant understands the presence of the pathogen (Lahaye 2004). This resistance mechanism in plants is called systemic immunity or systemic acquired resistance (Wiermer et al. 2005; Grant and Lamb 2006).

There is no reliable resistance gene to bacterial wilt and canker pathogen. Therefore, the study aims to map resistant gene using promising resistant M3-9 and M3-15 plants which were obtained from chemical treated susceptible NCEBR3 tomato plants and to determine polymorphic markers on these plants.

2. Material and methods

2.1. Supply of material

In the study, NCEBR3 tomato line seeds were obtained from Prof Dr. Randy GARDNER at Mountain Horticultural Crops Research and Extension Center, North Caroline State University, USA. The NCEBR3 line is known as North Caroline Early Blight Resistant 3 and is a semi-dwarf table tomato genotype (Nash and Gardner 1988). In previous studies, mutation of NCEBR3 pure line tomato seeds with a single-base mutagen ethyl

metanosulfonate (EMS, Sigma, Germany) and mutation in the nucleotide sequences on the plant genome resulted in the conversion of cytosine (C) / guanine (G) to thymine (T) / adenine (A). By transforming into (A) nucleotides, 15 M2 mutant plants out of 450 M2 tomato mutant plants were found to be resistant to the disease agent *Cmm2*. Seeds of M3 populations were produced from these plants and plants in the M3-9 and M3-15 mutant families did not show any susceptibility against the *Cmm2* pathogen by pathogenicity tests (Çalış et al. 2013). The seeds of promising resistant mutants M3-9 and M3-15, which were obtained by mutating with EMS chemical mutagen, were obtained from the seed collection in Tokat Gaziosmanpasa University Faculty of Agriculture Plant Protection Department Phytopathology Laboratory.

2.2. Cultivation of plants

The tomato seeds used in the study were sown in vials containing sterile peat in the Tokat Gaziosmanpasa University Faculty of Agriculture Biotechnology greenhouse at 16 hours daytime and 8 hours night length, 23±5ºC temperature and 60% relative humidity conditions. Tomato seeds planted in vials were irrigated at regular intervals to ensure their germination. When the plants reached the stage with 2-3 true leaves, they were planted in 20 cm diameter and 30 cm deep pots containing a mixture prepared by autoclaving 1:1:1:1 ratio of peat: perlite: soil and animal manure at 121ºC for 15 minutes and left to develop in a greenhouse environment.

2.3. Growth of the pathogen in GYCA media and inoculation into test plants

Clavibacter michiganensis subsp. *michiganensis* isolate *2* (*Cmm2*) disease agent with high virulence, was obtained from Professor Hüseyin BASIM (Faculty of Agriculture, Akdeniz University). The pathogen was subcultured on Glucose Yeast Carbonate Agar (GYCA) distilled water was added to the mixture, formed with 2 grams (g) glucose, 5 g peptone, 5 g yeast extract, 40 g calcium carbonate, until it reached 1 liter (L) and the pH of the mixture was brought to 7.2 with either KOH or HCl. Then, 15 g of Agar (Merck, Germany) was added to the mixture and the medium was prepared by autoclaving at 121ºC at 1 atmosphere (atm) pressure for 15 minutes (Oxoid 2013). *Cmm2* bacterial isolates were taken from the stock solutions with the help of a loop, and drawings were made on glass Petri dishes containing GYCA, prepared in sterile conditions. The Petri dishes were wrapped with cling film and kept in an incubator at 28ºC for 3 days, to ensure the growth of bacteria. Inoculation was performed on the plant stems using sterile toothpicks.

2.4. Molecular markers used

In the study, the chromosome number of the tomato plant was considered in the selection of SSR markers in order to reveal the polymorphism. The tomato plants are diploid species but a haploid set of 12 chromosomes were considered with 24 SSR markers, one for each chromosome see Table 1, which are specialized for the beginning and end of each chromosome. The SSR markers were selected from the markers and maps in the Solanacea Genomics Network and used in our study (Solgenomics 2013).

2.5. DNA isolation from plants

A total of 27 leaf samples were taken from 9 plants each from the promising resistant M3-9 and M3-15 mutants and the original susceptible EBR3 tomato plants at 4-5 true leaf stage. The pellet obtained using the Fermentas DNA isolation kit was dissolved in 100 µL of 1× Tris-EDTA buffer (TE) and stored at -80°C (Fermentas 2014). 1×Tris-EDTA prepared with Tris-HCl (pH: 8) 12.1 g L^{-1}, $Na_2EDTA2H_2O$ (pH: 8) 3.7 g L^{-1} (Sambrook et al. 1989).

2.6. Classical PCR analyzes

PCR was performed with the modification of the protocol specified by Sambrook et al. (1989) dsH_2O 11.4 µL, $MgCl_2$ 4 µL, 10X Taq Buffer 10 µL, dNTP Mixture 4 µL, Primer Forward 4 µL, Primer Reverse 4 µL, Taq Polimerase 0.6 µL and by adding 2 µL of DNA, total of 40 µL of the PCR mixture was prepared. A Peqlab Pirimus96 (Germany) thermal cycler device was used in the study. Denaturation at 94ºC for 5 minute, 35 cycles at 94ºC, Denaturation at 94ºC for 1 minute, annealing variable with primer for 1 minute, Extension at 72ºC for 1.5 minute, Final Extension at 72ºC for 10 minute and Store 8ºC program was used (Çalış and Topkaya 2011).

2.7. Agarose gel preparation and Gel Imaging

As a result of PCR amplifications, 1% or 2% agarose gels were prepared according to the product size. For preparation 30 mL gel: Agarose 0.37 g, 5XTBE 7.4 mL, dsH_2O 29.6 mL, Buffer (1%): 5XTBE 60 mL, dsH_2O 240 mL and Ethidium Bromide 4 µL were used. 5X Tris/Borate/EDTA (TBE) buffer was prepared by adding 20 mL L^{-1} of Tris-Base (pH:8) 54 g L^{-1}, Boric Acid 27.5 g L^{-1} and 0.5 M EDTA (pH:8) used in gel preparation (Sambrook et al. 1989). The PCR products prepared with 5 µL of DNA, 3 µL of loading dye and 7 µL of dsH_2O were loaded into each well on the prepared gel. Promega brand 100 bp ladder (molecular weight marker) was loaded into the first well in the gel. The samples were connected to a direct current source from negative pole through to positive pole and run at 70-160 Volt cm^{-1} direct current which varies according to the size of the gel tank. The samples, which were run in horizontal electrophoresis gel tanks, were analyzed in the Vilber Lourmat brand gel imaging system, and printed out on a thermal printer.

3. Results

In pathogenicity tests of M3-9, M3-15 mutant plants are revealing resistance phenotype but their original NCEBR3 plant is susceptible to the *Cmm2* (Figure 1). The resistance status of mutant genotypes was determined by molecular SSR markers and their possible resistance locus were searched on chromosomes. A total of 24 SSR markers were used from each chromosomes and lower parts at haploid 12 chromosomes (Figure 2). The 24 SSR markers were examined for polymorphisms among the NCEBR3 parent, M3-9 and M3-15 mutant plants. There were no band formation or polymorphism found with SSR335 and SSR34 markers (Figure 3) located on chromosome 8 and 10 respectively. The PCR analyses revealed that differences in genetic material of resistant mutants M3-9 and M3-15 and susceptible NCEBR3 original plants identified with SSRB18031 molecular marker produced different amplicons between M3-9 and M3-15 mutants

Table 1. The 24 SSR markers used in the study for revealing polymorphism between resistant and susceptible plants.

Chromosome	Marker	Nucleotide Sequence	Annealing Temperature	Position
1	SSR92	F5'-AAGAAGAAGGATCGATCGAAGA-3' R5'-TCATGACCACGATACTACATGTTTC-3'	50ºC	0.00 cM
1	SSR65	F5'-GGCAGGAGATTGGTTGCTTA-3' R5'-TTCCTCCTGTTTCATGCATTC-3'	50ºC	159.00 cM
2	SSR96	F5'-GGGTTATCAATGATGCAATGG-3' R5'-CCTTTATGTCAGCCGGTGTT-3'	50°C	36.50 cM
2	SSR22	F5'-GATCGGCAGTAGGTGCTCTC-3' R5'-CAAGAAACACCCATATCCGC-3'	60ºC	99.00 cM
3	SSR86	F5'-AGGGCAACAAATCCCTCTTT-3' R5'-GGAGACGAG GCTGCTTACAC-3'	50°C	54.00 cM
3	SSR27	F5'-CCCAAATCA AGGTTTGTGGT-3' R5'-TCAGATGCCACCACTCTCAG-3'	50°C	169.00 cM
4	SSR638	F5'–TGTTGGTTGGAGAAACTCCC-3' R5'-AGGCATTTAAACCAATAGGTAGC-3'	50°C -60°C	58.00 cM
4	SSR214	F5'-AAATTCCCAACACTTGCCAC-3' R5'-CCCACCACTATCCAAACCC-3'	50°C	95.00 cM
5	SSR13	F5'-GGGTCACATACACTCATACTAAGGA-3' R5'-CAAATCGCGACATGTGTAAGA-3'	50°C	27.00 cM
5	SSRB18031	F5'AGACTCAGTCCCGAACAAGTTGAAG-3' R5'ACATTACACTAAACCCCCAATTGCC-3'	55°C	119.00 cM
6	SSR47	F5'-TCCTCAAGAAATGAAGCTCTGA-3' R5'-CCTTGGAGATAACAACCACAA-3'	50°C	6.50 cM
6	SSR350	F5'-GGAATAACCTCTAACTGCGGG-3' R5'-CGATGCCTTCATTTGGACTT-3'	55°C	101.00 cM
7	SSR241	F5'-TCAACAGCATAGTGGAGGAGG-3' R5'-TCCTCGGTAATTGATCCACC-3'	55°C	0.00 cM
7	SSR45	F 5'-TGTATCCTGGTGGACCAATG-3' R5'-TCCAAGTATCAGGCACACCA-3'	50°C	60.00 cM
8	SSR335	F5'-CCTCTCCATTCTGTGGTGGT-3' R5'-AACCGTCCTCGATTTCACAC-3'	55°C	49.70 cM
8	SSRB105694	F5'-AAGCCAAAGTGGAAGAACTCAAGG-3' R5'-CTCGTAAAACGTTCATCAATCTCGC-3'	53°C	87.00 cM
9	SSR69	F5'-TTGGCTGGATTATTCCTGTTG-3' R5'-GCATTTGATAGAAGGCCAGC-3'	50°C	24.90 cM
9	SSR599	F5'-GATTTCTCATGGAGAATCAGTC–3' R5'-TCCCTTGATCTTGATGATGTTG-3	55°C	109.0 cM
10	SSR34	F5'-TTCGGATAAAGCAATCCACC–3' R5'-TCGATTGTGTACCAACGTCC–3'	50°C – 45°C	25.30 cM
10	SSR479	F5'-TGTAAGAGTGTCTGCCTGCAC–3' R5'-ATGGGTTCGGGTTAGCTCTT –3'	52°C	86.00 cM
11	SSR136	F5'-GAAACCGCCTCTTTCACTTG–3' R5'-CAGCAATGATTCCAGCGATA –3'	50°C	11.00 cM
11	SSR67	F5'-GCACGAGACCAAGCAGAT TA–3' R5'-GGGCCTTTCCTCCAGTAGAC–3'	50°C	24.00 cM
12	SSR44	F5'-TCATCTGCAATTCATGGCTC–3' R5'-AGGTCAAGGATGTGCTTCCC–3'	45°C	60.00 cM
12	SSR345	F5'-AAGCCAAGCTCGAACCTGTA-3' R5'-ATCCATGCTGTCGCTTTCAT-3'	60°C	72.50 cM

Figure 1. Stem sections ofM3-15 and M3-9 resistant mutants and susceptible NCEBR3 tomato plants. The plants are inoculated with *Clavibacter michihganensis* subsp. *michiganensis* isolate 2 at 15 days post inoculations.

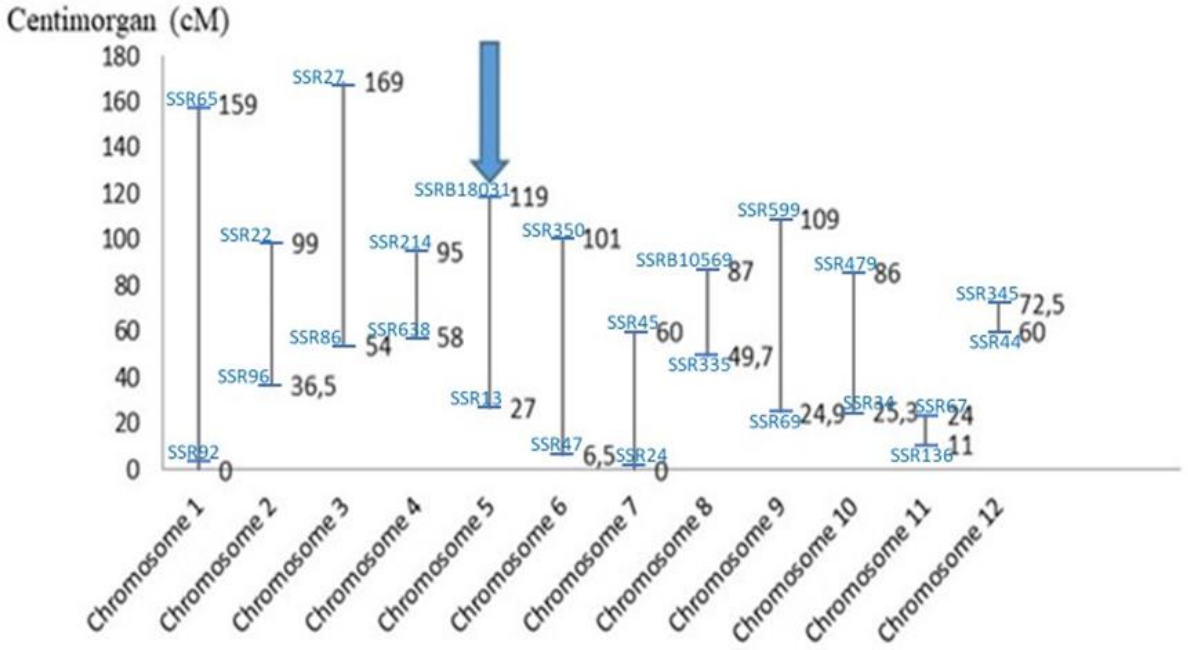

Figure 2. Chromosome distribution of *Solanum lycopersici* specific simple sequence repeat (SSR) markers.

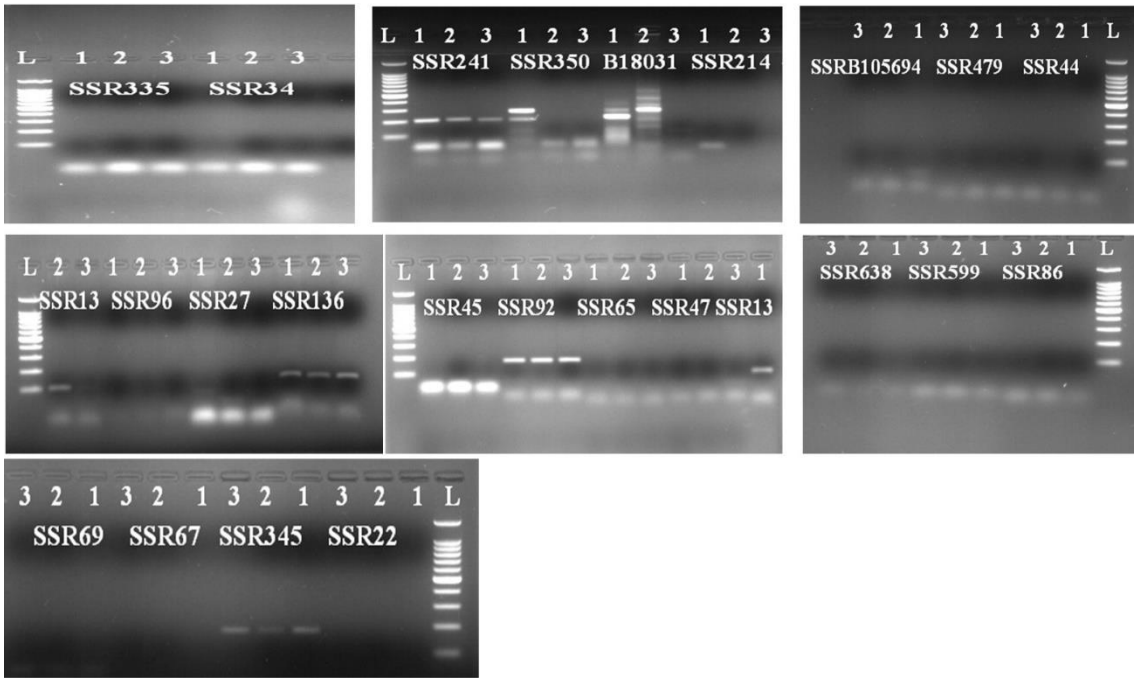

Figure 3. The PCR amplifications performed using 24 Simple Sequence Repeats (SSR) molecular markers and their amplicons were run on 2% agarose gel.1: M3-9, 2: M3-15, 3: NCEBR3, L: 100 bp Ladder.

but no amplification product was formed in susceptible NCEBR3 plant samples (Figure 3). The SSRB18031 molecular marker is located on chromosome 5 and the marker has been distinguished in mutants and original tomato plants. In the use of SSR214 and SSR27 molecular markers, they consisted of different size bands with M3-9 and M3-15 resistant mutant plants, no band formation was observed in the susceptible NCEBR3 plant (Figure 3). Another SSR13 marker located on chromosome 5 produced a band M3-9 resistant mutant but it did not form a distinct band, neither M3-15 resistant mutant nor in susceptible NCEBR3 plant (Figure 3). The SSR136, SSR92 and SSR345 markers amplified same size band on resistant M3-9, M3-15 and susceptible NCEBR3 plants. Apart from these, SSRB105694, SSR479, SSR44, SSR96, SSR27, SSR65, SSR47, SSR638, SSR599, SSR86, SSR69, SSR67 and SSR22 molecular markers did not amplify any bands on resistant M3-9, M3-15 and susceptible NCEBR3 original plants (Figure 3).

4. Discussion

For the genetic control of tomato bacterial canker and wilt disease, M3-9 and M3-15 resistant mutants found as a result of chemical mutation, the two resistant mutants were amplified and their genotypes were searched for polymorphisms with their original susceptible parent. In previous studies, *Cmm2* inoculated M3-9 and M3-15 resistant plants' extracts were analyzed in High Pressure Liquid Chromatography (HPLC) system. In HPLC analysis, chlorgenic acid and rutin hydrate were increased 6.7 and 13 times higher in resistant plants than in susceptible NCEBR3 plants respectively (Bayan 2011). Additional inoculation tests of M3-9 and M3-15 plants were carried out with 3 different *Cmm* isolates obtained from Tokat tomato production areas in the Black sea region, Turkey (Çalış et al. 2015). This phenotypic knowledge led us to search polymorphism to locate possible resistance gene(s) on genotypes of mutants compared with susceptible NCEBR3 plants. Molecular analyses with 24 SSR markers were randomly chosen from each terminal part of chromosomes and their molecular analyses were associated with SSR13 and SSRB18031 markers at chromosome 5 (Figure 2 arrow indicates). The results reveal that a mutation occurred on M3-15 and M3-9 resistant plants but the susceptible NCEBR3 plant did not have an amplified band. The chemical mutation might create a mutation on M3-9 and M3-15 plants and control resistance to the *Cmm2* pathogen. These results should be verified with constructing individual mapping populations with the M3-9 and M3-15 plants.

SSR markers provide high efficiency and accuracy in detecting DNA difference in any plant. SSR markers are preferred in population genetics and gene mapping studies because they require a low level of DNA, have high levels of polymorphism and codominant features (Powell et al. 1996). All these attributed to use the codominant SSR markers where SSRB18031 and SSR13 markers have polymorphism between resistant mutant and susceptible NCEBR3 plants indicating phenotypes of the plants are matched in genotypes with these markers.

5. Conclusions

In this study, genetic polymorphisms were investigated in resistant M3-9 with M3-15 mutants and susceptible NCEBR3 plants. Altogether 24 SSR markers selected from each end of haploid 12 tomato chromosomes, were used to determine a possible resistance locus using PCR based molecular analyses. The PCR amplification method revealed polymorphisms with SSRB18031 and SSR13 molecular markers in M3-9, M3-15 mutants and susceptible NCEBR3 plants. As a result of the polymorphisms the resistance locus is on chromosome 5 because the two SSR markers are located on the chromosome. Further fine mapping studies should uncover possible resistance locus, which controls resistance to the bacterial canker and wilting pathogen.

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Research Article

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MEDITERRANEAN
AGRICULTURAL SCIENCES
(2022) 35(3): 135-139
DOI: 10.29136/mediterranean.1097816

Metal tolerance of *Spirulina platensis*

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ARTICLE INFO

Received: April 3, 2022
Received in revised form: May 29, 2022
Accepted: June 22, 2022

***Keywords*:**

Bioremediation
Environment-friendly
Heavy metals
Primary sewage treatment
Spirulina platensis

ABSTRACT

Microorganism-based bioremediation is a well-sought after method for industrial wastewater treatment and forms the primary stage. The current research suggests using *Spirulina platensis* as an organism of choice for bioremediation. This study provides an insight into the potential use of primary-treated wastewater as the growth media for *Spirulina platensis.* The tolerance of *S. platensis* was confirmed for metals such as mercury (Hg), cadmium (Cd), manganese (Mn), zinc (Zn), and copper (Cu) by using media enriched with these metals. *S. platensis* was most tolerant to Hg followed by Cd and Cu. Further, it is suggested that the biomass and bioactive compounds extracted from *S. platensis* be tested for their application in animal and aquaculture feed, supplements, and pharmaceuticals.

1. Introduction

Improper discharge of waste causes heavy metal contamination that causes detrimental and long-term effects on the ecology and human population (Wang and Chen 2009; Priyadarshini et al. 2019). Heavy metals cannot be removed entirely from the system but can be biotransformed (Juwarkar et al. 2010). Bioremediation, identified as the biological deterioration of contaminants, intensifies this process (Gouma et al. 2014).

There has been a shift in the focus to organisms capable of biosorbing toxic compounds from their environment (Dhankhar and Hooda 2011) - to overcome the drawbacks of existing technologies. Thus, bioremediation is environment-friendly and cost-effective. *Spirulina* (*Arthrospira*) *platensis* (Gomont 1892), a filamentous cyanobacterium, is recognized as a nutritious food supplement, alongside being a source for commercially valuable bioactive compounds, phycocyanin (Güroy et al. 2017; Paramanya et al. 2019). This cyanobacterium thrives in a high-salt environment, tolerant of osmotic stresses (Usharani et al. 2012).

Biosorption is a physio-chemical process involving the passive uptake and accumulation of toxicants (heavy metals) by biological materials (usually dead or inactive) from their surroundings (Yan and Viraraghavan 2003). Microorganisms have evolved many bioprocesses to exploit the chemical properties of selectively acquired metals for catalyzing reactions and maintaining protein structure (Murali and Mehar 2014).

In wastewater management, the dry biomass of *S. platensis* is used for precipitation (Fariduddin et al. 2018) and biosorption of heavy metals (Ahmad et al. 2010; Michalak et al. 2020) at low cost (Rangsayatorn et al. 2002). *Spirulina* sp. is effective in bioremediating water polluted with petroleum hydrocarbons (Ciferri 1983), pesticides (Khan et al. 2005), some estrogens (Shi et al. 2010), radioactive elements (Fukuda et al. 2014), and fluoride ions (Tabagari et al. 2019). Dead biomass is preferred for several reasons: high tolerance to toxic heavy metal ions, no necessary nutrient supply, and no limiting culture conditions (Kőnig-Péter et al. 2014). However, little is known about the use of its live biomass for biosorption.

Culturing *S. platensis* in low metal concentrations can potentially be used for tertiary treatment for metal-contaminated effluent (Murugesan et al. 2008). Commercially grown and consumed *Spirulina* supplements have traces of inorganic elements and heavy metals at concentrations that do not exceed the present regulation levels; if appropriate measures are taken, it can be considered safe food (Al-Dhabi 2013). This application has the potential to combat the issues of contaminated biomass. In addition, the efficiency of *S. platensis* in the biosorption of heavy metals makes it a potential organism for cost-effective and environment-friendly wastewater treatment.

Lu et al. (2000) suggested that mercury inhibits the quantitative photosynthetic yield of cyanobacteria. Cu and Zn directly affect photosynthetic pathways, leading to a decrease in cell growth (Lone et al. 2008). Therefore, changes and variations in biomass yield and chemical composition of *Spirulina* sp. are considered when bioactive compounds are extracted or used as dietary supplements or fertilizers.

This pilot study aims to verify the growth of *Spirulina platensis* and its tolerance to heavy metals in wastewater. Preliminary experiments were conducted to standardize and optimize the growth conditions of *S. platensis*, in turn establishing a growth curve.

2. Materials and Methods

2.1. Procurement and cultivation of Spirulina platensis PCC 7345

The strain of *S. platensis* PCC 7345 was procured from BITS Pilani, India. The pure culture was sub-cultured in the Blue-Green 11 (BG11) medium (Dineshkumar et al. 2016) and grown under photoautotrophic conditions - 28°C in constant white light (pH 7.2) - on a shaker.

2.2. Determination of Standard Growth Curve

To standardize growth, *S. platensis* was grown in triplicate in 250-mL flasks containing 200 mL BG11 culture medium (Table 1) for four weeks. 500-µL inoculum was added to the medium at an initial cell count of $2.5x10^4$ cells mL^{-1}.

Table 1. Composition of Blue-Green 11 (BG11) medium (Adapted from Moghazy 2019)

Macroelement nutrients	Concentration (g L^{-1})
$NaNO_3$	1500.00
K_2HPO_4	40.00
$MgSO_4.7H_2O$	75.00
$CaCl_2.7H_2O$	36.00
Citric Acid	6.00
Na_2CO_2	20.00
Na_2EDTA	1.00
Ferric ammonium citrate	6.00
Microelement nutrients	**Concentration (mg L^{-1})**
H_3BO_3	2.860
$MnCl_2.4H_2O$	1.810
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.7H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

The Direct Cell Counting method was used to estimate the growth density of *S. platensis*. This method involves using a calibrated grid placed over the culture chamber (Neubauer hemocytometer), followed by a cell count per grid square under a microscope (Liu 2017). Samples were collected aseptically every third day from the day of inoculation and observed at 45x magnification. Counts were performed until cell density was constant. To obtain reproducible results, the seeded material had a constant dilution factor from count to count. In addition, the number of cells per millilitre was calculated (Selvakumaran and Jell 2005).

Number of viable cells mL^{-1}= The average number of viable cells per 0.1 cm^2 area × 10^4 (correction factor for the volume of shaded area) × Dilution factor

2.3. Metal Tolerance of Spirulina platensis PCC 7345

S. platensis was grown in the presence of metal ions to test its potential to tolerate metals detected in wastewater. The metal ions and their final concentrations (Table 2) in the medium were selected based on the standards for permissible heavy metal concentrations in inlet wastewater laid by the Ministry of Environment and Forests, Government of India (2010) for Common Effluent Treatment Plants (CETP) as per The Environment (Protection) Rules (1986). The inoculum was added at an initial cell count of $1x10^4$ cells mL^{-1}. The potential of *S. platensis* to grow in metal-enriched media was visually observed and later direct cell counts were done on the twenty-eighth day, from the day of inoculation.

Table 2. Final concentration of heavy metals in the medium

Sr. No.	Heavy Metal	Concentration (ppm)
1.	Cadmium (Cd)	1
2.	Cobalt (Co)	49
3.	Copper (Cu)	3
4.	Manganese (Mn)	1812
5.	Mercury (Hg)	0.01
6.	Zinc (Zn)	222

3. Results

3.1. Growth curve of Spirulina platensis PCC 7345

The Growth Curve was used to determine Exponential Growth Time. As seen in Figure 1, for the first five days there was a gradual increase in the number of *S. platensis* cells followed by a steep increase for the next three days (exponential phase). It remained nearly constant between 12-28 days (stationary phase) and decreased beyond 28 days (death phase).

3.2. Tolerance of Spirulina platensis to Metals – Qualitative

S. platensis grew in media enriched with cadmium, copper, and mercury; no observed growth in media with cobalt, manganese, and zinc (Table 3). Quantitative methods confirmed these results.

Table 3. Growth of *S. platensis* in BG11 medium with various heavy metals

Heavy Metal	Growth of *S. platensis*
Cadmium (Cd)	+
Cobalt (Co)	-
Copper (Cu)	+
Manganese (Mn)	-
Mercury (Hg)	++
Zinc (Zn)	-

+ represents growth; - represents no growth

3.3. Tolerance of Spirulina platensis to Metals – Quantitative

To quantitatively investigate *Spirulina*'s metal tolerance, the living cells cultivated in media were counted. *S. platensis* was most tolerant of Hg ($11x10^4$ cells mL^{-1}), followed by Cd and Cu, respectively (Figure 2).

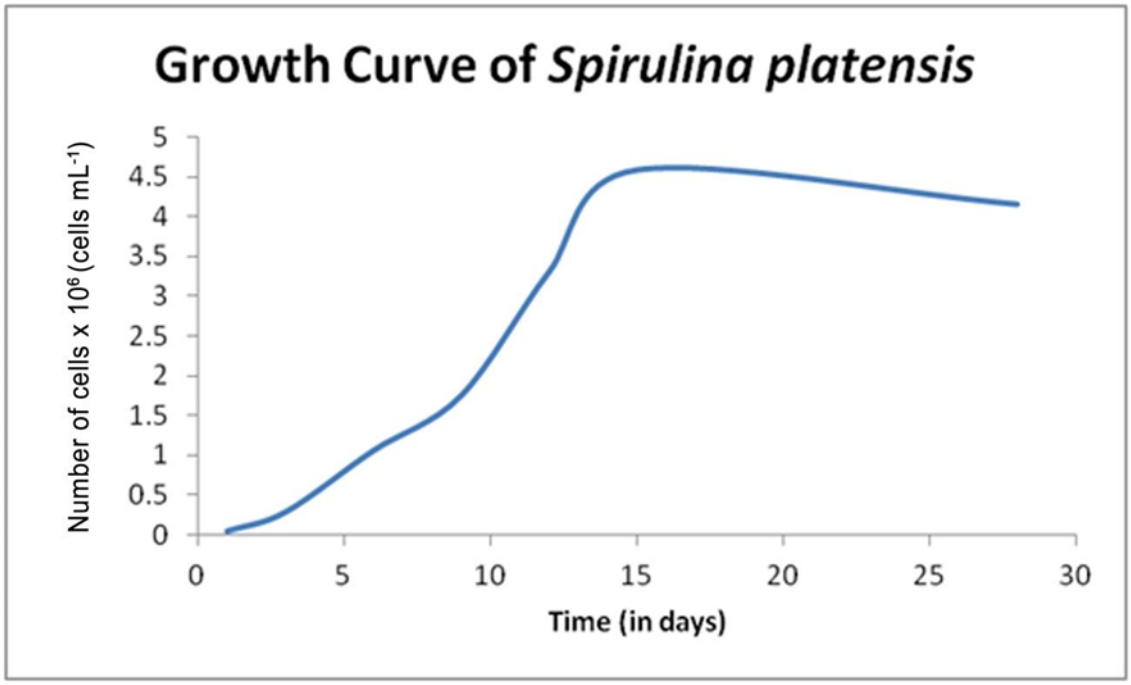

Figure 1. Growth curve of *S. platensis* in BG11 medium.

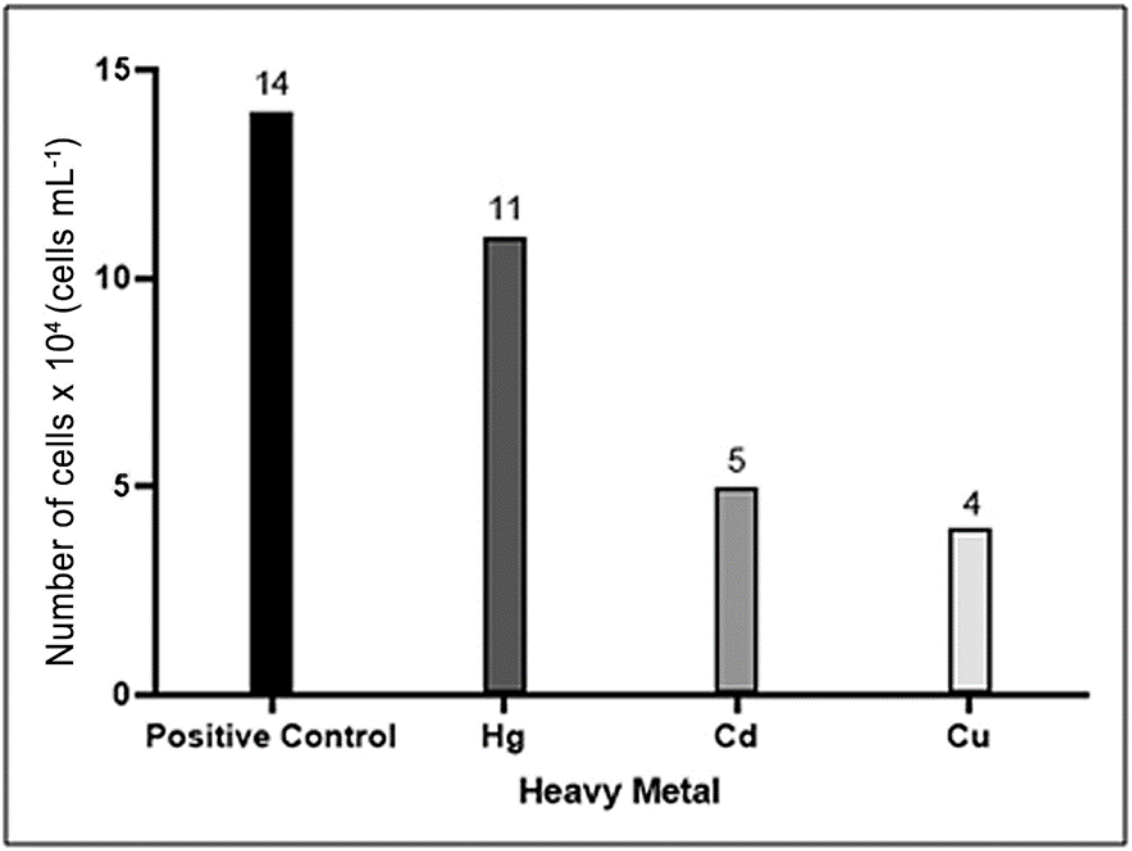

Figure 2. Number of cells counted of *S. platensis* in medium with various heavy metals.

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4. Discussion

Spirulina platensis contributes 30% of the total global algae biomass production, majorly cultivated using chemical-based culture media (Lim et al. 2021). Moreover, the excessive use of chemicals and nutrients has adverse effects on the environment. This highlights the need for an alternative culture medium such as wastewater, rich in nitrogen, phosphorus, and indigenous bacteria (Jia and Yuan 2016).

Cobalt, manganese, and zinc probably disrupted the metabolic pathways of *S. platensis*, so no growth was recorded for these media. Quantitative analysis of S. platensis tolerance to mercury was contrary to the order of toxicity proposed by Thomson and Kurup (2010); clear reasons for this were not identified. In view of these results, *S. platensis* can be used as an effective organism for the uptake of metal ions in wastewater treatment, as also suggested by Murali and Mehar (2014), Michael et al. (2019), and Lim et al. (2021).

This further paves the way for value-added products of *S. platensis*, such as food supplements, wastewater treatment (bioremediation), biofuels, animal feeds, and fertilizers (Padgaonkar et al. 2021). *S. platensis* tolerance for heavy metals could find applications in wastewater management and address the environmental issues of heavy metals in the natural ecosystem. High tolerance for mercury can imply the cyanobacteria's suitability for mercury treatment. Through further investigation, it is necessary to understand the tolerance range of *S. platensis* to Hg (II) and the subsequent effect on the organism's metabolic pathways.

The current studies support the use of sewage as the growth media (Lim et al. 2021), but studies are needed to establish proper evidence on the bioremedial potential of *S. platensis*. Since it exclusively uses light as an energy source (phototrophic organism), its use in biological reactors for bioremediation of hazardous substances is proposed as a cost-effective approach. Major cities of India generate Around 72368 million litres per day of wastewater (Central Pollution Control Board 2021), which can ensure better productivity of *S. platensis*.

5. Conclusion

The results of this study confirm the tolerance of *Spirulina platensis* to mercury (0.01 ppm), copper (3 ppm) and cadmium (1 ppm). However, the organism could not tolerate other metals such as cobalt, manganese, and zinc at the amounts found in primary wastewater. One solution to this problem could be diluting the wastewater before inoculation with the organism.

The study also shows that *Spirulina platensis* has a high tolerance to mercury (at 0.01 ppm). Its tolerance towards Cu and Cd was considerably less, but noteworthy. However, it is necessary to further determine the suitability of *S. platensis* as a bioremediator for heavy metals. The biomass can be studied for the extraction of bioactive compounds or value-added products.

Acknowledgements

We acknowledge the financial support received from the University of Mumbai as Minor Research Project**.**

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Research Article

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MEDITERRANEAN
AGRICULTURAL SCIENCES
(2022) 35(3): 141-145
DOI: 10.29136/mediterranean.1109948

Chlorine and ozone applications used for fruit and vegetable disinfection in tourism accommodation facilities

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ARTICLE INFO

Received: April 28, 2022
Received in revised form: August 2, 2022
Accepted: August 31, 2022

***Keywords*:**

Food
Chlorine
Disinfection
Ozone

ABSTRACT

Samples of vegetable and fruit were taken from the kitchens of tourism accommodation facilities and microbiological analyses were made. The samples were analysed after being disinfected with 100 ppm chlorine for 2-10 minutes and with 2 ppm ozone for 2-10 minutes. In the examined vegetables and fruit samples, *Escherichia coli*, *E. coli* O157, *Salmonella* spp., *Listeria monocytogenes* were examined in terms of microorganisms. Before disinfection, *E. coli* was detected in all 30 samples: *E. coli* O157 in 8 samples, *Salmonella* spp. in 4 samples, and *L. monocytogenes* in 3 samples. As a result of disinfection applications with 100 ppm chlorine for 2 minutes, *E. coli* decreased by more than 45%. *Salmonella* spp. was detected in 1 sample and *E. coli* O157 was detected in 2 samples. After disinfection applications with 2 ppm ozone for 2 minutes, *E. coli* decreased by more than 60%. A decrease of more than 70% was observed in *E. coli.* after disinfection applications with 100 ppm chlorine for 5 minutes. As a result of disinfection applications with 2 ppm ozone for 5 minutes, *E. coli* decreased by more than 85%. No bacteria were found in the samples after disinfection with 100 ppm chlorine and 2 ppm ozone for 10 minutes. It was observed that there was a decrease in the microorganism loads as a result of the chlorination and ozonation processes of the vegetable and fruit samples.

1. Introduction

A healthy life and nutrition are among peoples most basic needs. The main problem in food products in developing countries is the inability to disinfect foods properly and effectively. In order to protect food and ensure its hygiene and sanitation, it is necessary to protect raw materials and water, to prevent cross-contamination of cooked and raw foods, to hold the storage conditions at the appropriate temperature, to cook the food at the appropriate time and temperature, to protect the food from the pollution of humans, animals and parasites (Gökçe 2011).

In recent years, in line with the increasing demands for raw fruit and vegetable products, consumers' orientation towards quality, healthy and nutritious products has increased. Therefore, disinfection processes for these food products are of great importance. Chlorine, ozone, organic acid, hydrogen peroxide, calcium oxide, and thyme water are disinfectant agents used in places of mass consumption.

Today, chlorinated compounds continue to be widely used in the food industry for the disinfection processes of fruit and vegetable in tourism accommodation facilities, especially because they are economical.

Ozone is accepted as a usable antimicrobial substance in waters by the US Food and Drug Administration (FDA) and as a substance that has generally been accepted as safe a (GRAS) status in foods. As a result of the research, it has been revealed that ozone, which can be used in gas or liquid form, and reduces the presence of disease-causing microorganisms, can quickly decompose and leave the environment when applied to food products (Gücükoğlu and Küplülü 2005).

Vegetable and fruit samples were analyzed after being subjected to chlorination and ozonation processes. Vegetable and fruit samples were examined in terms of microorganisms corresponding to washed, chopped and packaged raw vegetables and frozen or dried vegetables separately or mixed in the Turkish Food Codex Microbiological Criteria Regulation Annex-1 section and the ready-to-eat chopped fruits and vegetables groups in Annex-2 (Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği 2011).

In the light of the information obtained from the findings in the study, it was necessary to reach the source of the problems. The disinfection processes of fruits and vegetables in tourism accommodation facilities were examined and the advantages and disadvantages of disinfection agents were revealed. It is thought that the research will benefit ozonation and chlorination applications in enterprises such as places of mass consumption. The findings obtained from the study will be a prediction for the disinfection activities in vegetables and fruits to continue more effectively and well, and to eliminate the threats posed by the product groups that can be consumed raw and present them to the consumer in a healthy way.

Disinfection processes have an important place in order to prevent physical, chemical and microbiological deterioration that may occur in the chain from the production to consumption of vegetables and fruits (Aytemiş 2021).

According to studies (Erkoç 2019), diseases caused by pathogenic microorganisms, due to the consumption of vegetables and fruits, have increased in recent years. It has been observed that the increase in foodborne diseases are caused by conditions such as cross-contaminations in food products and food contact materials, sewage water used in vegetable and fruit irrigation, the land where the products are planted, and lack of personnel training.

Chlorine is one of the most preferred disinfectant agents due to its low cost and ease of use. Hypochlorite and liquid chlorine are the most commonly used chlorine forms to extend the shelf life of vegetables and fruits (Gölgeçen 2014). However, the disadvantages, such as the fact that bacteria are not sufficient to destroy spore forms of bacteria and viruses, that they can produce harmful products and that they have a high risk of harming the environment have led to a tendency towards different types of disinfection (Sevilgen 2009).

In a study conducted with chopped lettuce, which was disinfected with chlorinated water containing 100 mg l^{-1} concentration at 4°C and 47°C it was reported that lettuce washing at 47°C was more effective in the inhibition of microorganisms. As a result, the effect of chlorine activity on microorganism inactivation, depending on temperature, was observed (Delaquis et al. 1999).

In another study, iceberg and fresh broccoli vegetables were kept in a solution containing *Escherichia coli* for 1 minute and then for 2-5 minutes in a chlorine solution (50 ppm-100 ppm chlorinated water studies). As a result, it has been reported that chlorinated water causes a decrease in *E. coli* (Behrsing et al. 2000).

One of the areas where ozone applications are used the most is food product groups (Tümay 2019). Fresh vegetables and fruits are foods that are offered to the consumer without any processing. The risk of contamination is high due to mechanical damage during and after harvest. Therefore, it is important to increase the shelf life of chopped vegetables and fruits and to protect their nutritional values and sensory properties (Savaş et al. 2014).

Ozone is used as a disinfectant that can be easily adapted to processes, eliminating disease-causing microorganisms (pathogens) in food products, preventing quality losses, prolonging the shelf life of products, removing pesticide residues and mycotoxins (Tetik et al. 2006; Karaca 2010).

It is stated that very low application concentrations of ozone give very positive results in microorganism inactivation during cold chain storage in vegetables and fruits. Ozone is applied as a gas during transport or storage in the food chain. At the same time, it acts as a strong fumigation and sanitizer, protecting food products against pests, reducing the number of microorganisms on the surfaces of the products (Xu 1999).

This research was carried out with the aim of microbiological examination of disinfection processes with chlorine and ozone of vegetables and fruits offered for the consumption of the guests in the kitchens of tourism accommodation facilities.

2. Material and Method

2.1. Materials

In this study, samples of cress, lettuce, parsley, mint, arugula, dill, tomato, apple, plum, and carrot, which were offered to the consumption of guests in June, July and August of 2020 from some tourism accommodation facilities in Antalya, were used. Random selections were made for the analysis of food samples.

2.2. Methods

2.2.1. Preparation of the samples

The products were purified from coarse dust, dirt and insects by using only tap water in the kitchen. At least 700 g of these samples were taken and at least 100 grams of them were analyzed without any disinfection process.

2.2.2. Chlorination and ozonization applications

Afterwards, 300 grams of the samples were divided into 3 parts, and disinfection was carried out with 2 minutes-5 minutes-10 minutes of chlorination at 100 ppm chlorine concentration with chlorination (Diversey, South Carolina, USA). Rinsing was performed again in order to prevent chlorine from leaving residue. The remaining average of 300 grams of the samples was divided into 3 and disinfection was carried out with 2 minutes-5 minutes-10 minutes ozonation at a concentration of 2 ppm with ozonizer (Prozon, Antalya, Türkiye). No rinsing was done as ozonation does not leave any residue.

The samples were delivered to the laboratory under aseptic conditions, in the cold chain, for analysis. They were analyzed by classical methods in terms of *E. coli*, *E. coli* O157, *Salmonella* spp., and *Listeria monocytogenes.* According to the product groups of "washed, chopped and packaged raw vegetables and frozen or dried vegetables separately or mixed" in the Turkish Food Codex Microbiological Criteria Regulation Annex-1 section and "the ready-to-eat chopped fruits and vegetables" groups in Annex-2, and the validation stages were carried out (Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği 2011).

2.2.3. Escherichia coli Analysis

Ten g of the sample was homogenized with 90 ml of Maximum Recovery Diluent (Biolife 4016912). It was inoculated into 1 ml empty sterile petri dish. Approximately 15 ml of Tryptone Bile Glucuronide Agar (Himedia M1591) previously cooled in a water bath at 44-47°C was poured into each petri dish and mixed. After incubation at 44°C for 18-24 hours, blue-green colonies were counted as *E. coli* (TSE 2012).

2.2.4. Escherichia coli O157 analysis

A 25g sample was homogenized with 225 ml of Modified Tryptone-Soy *Broth* (MTSB) with Novobiocin (Merck 1092050500) under aseptic conditions. As a result of immunomagnetic separation, streaking was performed on Cefixime Tellurite Sorbitol Macconkey Agar (Biolife 4016692) and Harlequin SMAC-BCIG (Labm HAL 006) medium. It was incubated for 18-24 hours at 37°C. Colonies with clear and usually colorless yellowish-brown zones were taken for indole verification and incubated at 37°C for 18-24 hours. Petri dishes with positive indole confirmation were confirmed with the latex confirmation test (TSE 2003).

2.2.5. Analysis of Listeria monocytogenes

A 25g sample was incubated in 225 ml Half Fraser Broth (Biolife 4014952) medium at 30°C for 25±1 hour. The culture was taken with a loop from the pre-enrichment medium incubated for 25±1 hours at 30±1°C, streaked on Listeria According to Ottaviani and Agosti Agar (Biolife 4016052) and Oxford Agar (Biolife 4016002) media.

The 0.1 ml taken from the pre-enrichment medium into 10 ml second enrichment medium was transferred to a tube containing Fraser Broth (Biolife 4014952). Fraser Broth was incubated at 37°C for 24±2 hours. Streaks on ALOA and Oxford Agar media were incubated for 24±2 hours at 37°C. The culture was taken with a loop from Fraser Broth, the second pre-enrichment medium, which was incubated for 24±2 hours at 37°C, and streaked again on ALOA and Oxford Agar mediums. It was incubated in ALOA medium for 48±2 hours at 37°C and in Oxford Agar at 37°C for 24 hours. Colonies of *L. monocytogenes* on the ALOA medium were colonies with a blue-green opaque zone. On the Oxford Agar, black-brown colonies with 2-3 mm diameter blackish sunken center were *L. monocytogenes* colonies (TSE 2017a). Commercially available Microgen Listeria-ID kits were used for the confirmation test (Kemiteks Kimya 2021a).

2.2.6. Salmonella spp. analysis

A 25g sample was homogenized with 225 ml Buffered Peptone Water (Biolife 4012782) under aseptic conditions. Enrichment was done by incubating at 34-38°C for 18 hours. 0.1 ml of the pre-enriched sample was inoculated into 10 ml of Rapaport Vassiliadis Medium (Biolife 4019812) and 1 ml into tubes containing 10 ml of Muller-Kauffman Tetrathionate Novobiocin Broth (Biolife 4017452). A second enrichment was made by incubating RVS broth at 41.5°C for 24 hours and MKTTn broth at 37°C for 24 hours. At the end of the incubation, streaks were made from RVS and MKTTn onto solid media Brillant Green Phenol Red Agar (Biolife 4012552) and XLD agar (Biolife 4022082) with loops. It was incubated for 24±3 hours at 37±1°C. Typical colonies, pink-red, rarely colorless, on the BGA medium after incubation formed a red zone around them. In the XLD medium, on the other hand, they formed pink colonies with black centers (TSE 2017b). A commercially available biochemical Microgen GN A-ID panel was used for validation (Kemiteks Chemistry 2021b).

3. Result and Discussion

From the tourism accommodation facilities in Antalya province, in June, July and August of 2020, cress, lettuce, parsley, mint, arugula, dill, tomato, apple, plum, carrot, vegetable and fruit samples, including the appropriate microorganisms according to the Turkish Food Codex Microbiological Criteria Regulation Annex-1 and Annex-2, were taken into analysis before and after the disinfection process and examined (Tables 1 and 2).

Table 1 shows detected microorganisms according to samples before and after the disinfection process and Table 2 shows the number of samples detected before and after the disinfection process.

In the analyzes performed before the disinfection applications of vegetable and fruit samples, *E. coli* bacteria were detected in all 30 samples, *E. coli* O157 in 8 samples, *Salmonella* spp. in 4 samples, and *L. monocytogenes* in 3 samples. *E. coli* is known as a fecal-derived bacterium that lives in the large intestine of mammals. *E. coli* O157 is the serotype of *E. coli*, and it is a toxin-forming bacterium that lives in the human intestines like *E. coli*.

The reason why it is found in fruit and vegetable samples is that these samples, which are consumed raw, are cross-contaminated by washing with unclean washing water before reaching the consumer. Before these foods are washed, they provide a suitable environment for the reproduction of microorganisms by contacting different unhygienic products in

Table 1. Detected microorganisms according to samples before and after the disinfection process

Vegetable and fruit	**Without disinfection**	**Chlorine application (100 ppm)**			**Ozone application (2 ppm)**		
Time	-	2 min	5 min	10 min	2 min	5 min	10 min
Cress	1	0	0	0	0	0	0
Lettuce	1, 2, 4	1	0	0	0	0	0
Parsley	1, 2	0	0	0	0	0	0
Mint	1	1	0	0	0	0	0
Rocket	1, 2, 3	1, 2	0	0	1	0	0
Dill	1, 2	1	0	0	1	0	0
Tomatoes	1	0	0	0	0	0	0
Apple	1	0	0	0	0	0	0
Plum	1	0	0	0	0	0	0
Carrot	1	0	0	0	0	0	0

0: not detected, 1: *E. coli*, 2: *E. coli* O157, 3: *Salmonella* spp., 4: *L. monocytogenes.*

Table 2. Number of samples detected microorganisms according to before and after the disinfection process

Vegetable and fruit	**Without disinfection**	**Chlorine application (100 ppm)**			**Ozone application (2 ppm)**		
Time	-	2 min	5 min	10 min	2 min	5 min	10 min
E. coli	30	16	7	0	11	4	0
E. coli O157	8	2	0	0	0	0	0
Salmonella spp.	4	2	0	0	0	0	0
L. monocytogenes	3	0	0	0	0	0	0

the storage and storage areas. Waste water, soil, air, animal feed, insects, birds, mice, rodents are known as factors that are effective in the spread of *Salmonella* spp.. *L. monocytogenes* can be found in many areas such as slaughterhouse waste, water, sewage water, animal-human feces, mastitis or healthy milk. *L. monocytogenes* contaminates green fodder and soil from infected animals, and it is known that the bacteria re-infects milk and meat animals fed with them. This causes the bacteria to remain alive in nature and form a contamination cycle. The viability of bacteria also varies according to the type of food, storage conditions and storage areas.

As a result of disinfection applications with 100 ppm chlorine (2 minutes), *E. coli* in 16 of 30 samples, *Salmonella spp*.in 1, *E. coli* O157 in 2 microorganisms were detected. *L. monocytogenes* were not found in any of the samples. As a result of disinfection applications with 2 ppm ozone (2 minutes), *E. coli* were detected in 11 of 30 samples. *E. coli* O157, *Salmonella* spp., and *L. monocytogenes* were not detected in any sample disinfected with ozone. Due to the low effect of chlorine on pathogenic bacteria, no pathogenic bacteria were observed in ozone-treated samples. In the study conducted on 30 samples, it was observed that as a result of chlorination disinfection applications, *E. coli* remained in contact with food more than through the ozone application. This is thought to be due to the fact that ozone disinfection processes are more effective than chlorination applications. The detection of *E. coli* O157 and *Salmonella* spp. microorganisms after chlorine application for 2 minutes is due to the lack of time for inactivation of these bacteria. In addition, it shows how important it is that the product groups and the microorganism load they contain are more or less in disinfection.

In the study, *E. coli* bacteria were detected in 7 of 30 samples as a result of disinfection applications with 100 ppm chlorine (5 minutes). *E. coli* O157, *Salmonella* spp., *L. monocytogenes* were not found in any of the samples. As a result of disinfection applications with 2 ppm ozone (5 minutes), *E. coli* were detected in 4 of 30 samples. *E. coli* O157, *Salmonella* spp., *L. monocytogenes* were not detected in any of the products. As a result of disinfection applications with 100 ppm chlorine (10 minutes) and 2 ppm ozone (10 minutes), *E. coli, E. coli* O157, *Salmonella* spp., *L. monocytogenes* were not found in any of the 30 samples. In disinfection processes with chlorine and ozone, when the concentration is constant and the washing times are increased, it has been determined that the vital activities of pathogenic microorganisms are stopped by preventing the proliferation of bacteria. This shows that as the disinfection processes take longer, microorganisms that are harmful to human health are destroyed from the environment.

The high oxidation effect of ozone has a greater effect on the destruction of bacteria than chlorine. It has been observed that ozone is more effective than chlorine in the inhibition of microorganisms in the same period.

In the application of 2 minutes chlorine disinfection of lettuce containing *E. coli* bacteria, a decrease of more than 45% of the bacteria, a decrease of more than 70% in the application of 5 minutes, and 100% elimination in the application of 10 minutes were detected. Aruscavage et al. (2006) in their study, it was determined that there was a 2.5 log cfu g^{-1} decrease in the microorganism as a result of the treatment of lettuce samples containing 10^5 cfu g^{-1} *E. coli* with 200 ppm chlorine. Since *E. coli* is a fecal-derived bacterium, its incidence is high in fruits and vegetables. When the studies are compared, it is seen that chlorine reduces the *E. coli* microorganism by creating an antimicrobial effect.

In the study, the *E. coli* O157 microorganism was detected in 8 of 30 fruit and vegetable samples before the disinfection process. It was detected in 2 of the samples after 2 minutes of chlorination was applied to the products containing this bacterium. The *E. coli* O157 microorganism was not found in 5-10 minute chlorine applications. Nou and Luo (2010) disinfected the lettuces in chlorinated water at a concentration of 70 ppm in their research. They reduced the *E. coli* O157 load, which was 6.3 log cfu g^{-1} at the beginning, to 1 log cfu g^{-1} in the first wash for 60 seconds. In the second wash for 30 seconds, they reported a decrease of 0.6 log cfu g^{-1} in the load. As a result of disinfection processes in food products, it has been determined that the amount of time and concentration of chlorination are important in the gradual decrease of microorganism loads.

In the study, after 2 ppm ozonation treatment, *E. coli* decreased by over 45% after 2 minutes of treatment, over 85% after 5 minutes of treatment, and 100% after 10 minutes of treatment. *E. coli* O157, *Salmonella* spp., *L. monocytogenes* could not be detected in the environment with ozone applications for 2-5-10 minutes. In his study on green leafy vegetables in which he applied disinfection with ozone for 5-10-15 minutes at 2-5-10 ppm concentrations, Tümay (2019) reported the highest reductions of *E. coli, S. aureus, L. monocytogenes, B. cereus* and *S. typhimurium* after ozonation as 0.65 log cfu g^{-1}, 0.32 log cfu g^{-1}, 0.46 log cfu g^{-1}, 0.47 log cfu g^{-1}, 0.15 log cfu g^{-1}, respectively. In his study, Karaca (2010) reported that *E coli* showed a decrease of 1.25-2.09 log cfu g^{-1} and *L. innocua* showed a decrease of 1.54-2.17 log cfu g^{-1} in 5 minutes ozone application in lettuce, spinach and parsley. In the same study, lettuce, spinach and parsley samples were washed with water and chlorine to eliminate *E. coli* and *L. innocua*. He reported that ozone with chlorine achieved better results than washing with pure water. When the studies were compared, it was seen that the microorganism loads decreased depending on the time and concentration after the ozonation process in all of the fruit and vegetable samples.

4. Conclusion

Disinfection processes with chlorination are economical and have low investment costs. However, it causes corrosion as it creates the risk of leaving residues. Disinfection with ozonation is known as an environmentally friendly disinfectant that does not leave any residue, but with a high cost (Karaca 2010).

Features such as eliminating bacteria and viruses, oxidation, environmental sensitivity, color removal, investment cost are more advantageous in ozonation than chlorine. Chlorination can cause more damage to the respiratory and skin in humans than ozonation. However, it is known that ozone is more difficult to apply than chlorine. After the chlorination process, the food must be re-watered so that the chlorine does not leave any residue. This leads to quality loss (Sevilgen 2009). In ozone application, there is no need for such a process. In tourism accommodation facilities, disinfection with chlorine is generally preferred in fruit and vegetable samples due to its costs. As a result, in this study conducted in the kitchens of tourism accommodation facilities, it was concluded that ozonation eliminates microorganisms more effectively than chlorine applications, and that ozonation is a healthier disinfection process in terms of human health. Disinfection processes of fruits and vegetables consumed raw in places of mass consumption such as tourism accommodation facilities should be carried out in an appropriate and reliable manner in a way that does not pose a risk to human health and does not cause food poisoning.

This study is a contribution to other research done on the effectiveness of microorganism inactivation by comparing ozone and chlorine applications to be used for disinfection in the kitchens of tourism accommodation facilities.

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Research Article

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

MEDITERRANEAN
AGRICULTURAL SCIENCES
(2022) 35(3): 147-154
DOI: 10.29136/mediterranean.1086107

Organic maize farming practices in Nigeria: Drivers and barriers

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ARTICLE INFO

Received: March 11, 2022
Received in revised form: June 16, 2022
Accepted: June 17, 2022

***Keywords*:**

Organic farming
Maize production
Challenges
Determinants
Food safety

ABSTRACT

Organic farming is gaining global recognition in terms of the role it plays in providing safe and healthy food, income, and maintaining a sustainable environment. Despite these aspects , it faces constraints that, if identified, will play a vital role in its development and formulating policy for its sustainability. Creating an effective policy to improve organic farming necessitates identifying the influencing factors in organic farming method selection as well as barriers encountered. This study, therefore, examined the common types of organic maize farming, their determinants, and their challenges in Nigeria. Primary data collected from 480 respondents were analysed with descriptive statistics and multivariate logistic regression. The result revealed that organic manure, compost manure, biocontrol, and cover cropping were used by the farmers to enrich the soil. Farming experience, membership in a farm-based organization, farm distance, education, income, extension contacts, farm size, and cultural compatibility were the influencing factors of different organic farming systems used in maize production. Inadequate organic food accrediting agencies, inability to meet export demand, high cost and scarcity of organic seeds, lack of financial support, poor marketing system, inadequate supporting infrastructure, poor technical know-how, and pest infestation were the most common problems encountered in organic farming. To enhance participation in organic maize farming practices, there is a need to support organic farmers with financial support, accessible organic accreditation centres, training, educational support, and inputs.

1. Introduction

The agricultural sector is no doubt a major and important aspect of most developing countries' economies. In Nigeria, agriculture plays a significant role in food production, employment opportunities, and the national economy (Mukaila et al. 2022). It employs over seventy percent of the nation's workforce, contributes significantly to non-oil exports, and contributes 22 percent to Nigeria's GDP (Mukaila 2021; National Bureau of Statistics 2020). Thus, agriculture plays a vital role in ensuring food security and economic development. Meanwhile, Nigeria's agricultural sector has faced challenges as the farmers recorded low productivity due to poor agricultural finance, low level of education among farmers and the use of crude implements by the farmers. The country's subsistence agriculture and low level of technology adoption has also contributed significantly to farmer productivity (Fawole and Rahji 2016).

One of the major food crops widely grown and consumed in Nigeria is maize. It is a major cereal and is ranked first among the cereal crops grown in Nigeria. Maize (Zea mays) belongs to the family Gramineae. Maize gives a fast return on investment compared to other food crops such as cassava, yam, and rice. Maize gives a return on investment as short as sixty days in the case of fresh maize and less than six months for dry maize. It serves as food for many households and feed for livestock, especially in poultry farming. Maize constitutes the largest proportion of feeds given to poultry in Nigeria, which indirectly contributes to protein intake. Maize is, therefore, an important crop for attaining food security, poverty alleviation, and enhancing the economic growth of a nation. Nigeria's maize production in 2020 was 10 million tonnes (Knoema 2020). Nigeria's low maize productivity, compared to other countries with higher productivity (e.g. the US, Mexico, India, Ukraine, Argentina, China, Brazil, Canada, and Indonesia), is attributed to soil nutrient depletion, pests and diseases (such as Striga and fall armyworm), drought, and low research funding. The use of inorganic farming inputs, such as fertilizer, has long been emphasised to enhance soil nutrients. Agrochemical inputs, such as inorganic fertilizers, herbicides, fungicides, and pesticides are used in agriculture to increase crop productivity (Digal and Placencia 2019).

Although the use of agrochemicals increases crop yield, they pose a serious threat to human health, natural resources, the environment and increased soil acidity, which, in turn, affects farm productivity in the long run (Bui and Nguyen 2020). They are also very expensive. Continuous use of agrochemicals in conventional agriculture results in the deterioration of soil health and nutrient imbalance. The use of inorganic inputs in conventional agriculture results in the poisoning of about 30 million people, leading to the death of 220000 people yearly (Muhammad et al. 2016). The side effects of inorganic farming are now of great concern to people (consumers), policymakers, and researchers. Organic farming can solve the problems of food poisoning and deterioration of soil health as well as enhance

productivity among farmers in the long run. Organic farming is, therefore, suitable for farmers and more environmentally friendly than conventional agriculture.

Organic farming is gaining global recognition in terms of the role it plays in providing safe food and income. Organic agriculture avoids the use of chemical fertilizers, herbicides, synthetic pesticides, gene manipulation, or growth hormones; it instead uses techniques that reduce pollution and sustain the ecosystem (Oyawole et al. 2016). It emphasizes the use of mechanical, biological, and agronomic methods rather than the use of synthesis materials (Atoma et al. 2020). It has a low risk of contamination, which gives organic products a positive image (Łuczka and Kalinowski 2020). Thus, organic farming is needed to sustain agriculture, provide healthy foods for human consumption, safeguard animals and protect the environment (Bui and Nguyen 2020).

Despite its importance, organic farming encounters serious constraints which, if identified, will play a vital role in its development and formulating policy for its sustainability (Łuczka and Kalinowski 2020). Developing an effective policy to improve organic farming necessitates identifying the factors influencing the choice of organic production methods as well as the barriers encountered (Läpple and Kelley 2013). Previous studies on the use of organic farming focused on farmers' decisions to engage in organic farming (Läpple 2010; Sodjinou et al. 2015; Ullah et al. 2015; Ashari et al. 2017; Digal and Placencia 2019; Bui and Nguyen 2020; Yazdanpanah et al. 2022). They investigated the probability of using organic practices without examining the factors responsible for different types of organic farming practices. In addition, none of the studies focused on organic maize farming. The few studies on organic maize farming did not examine the factors that determine the use of types of organic maize practices (Adamteya et al. 2016; Choudhary and Kumar 2013; Liverpool-Tasie et al. 2017; Mucheru-Muna et al. 2014). Thus, the need for this study is to fill the research gap and add to the existing research.

Because of the above mentioned factors, the objective of this study is to investigate organic farming practices in maize production. Specifically, the study identified the common organic practices for maize farming among farmers, examined the factors influencing the types of common organic farming used, and identified the problems associated with organic maize farming in Nigeria. Understanding the driving factors of using different organic farming practices and the barriers faced in organic maize production would help in formulating policies to enhance participation in organic farming. This would, in turn, ensure food safety and security both in the short and long run.

2. Materials and Methods

2.1. Sampling procedure and data collection

This study employed a four-stage sampling technique to select the respondents. In the first stage, a purposive selection of two states (Kaduna and Niger) with the highest maize production was made. This was done to have a good representation of organic maize producers. In the second stage, four local government areas (LGAs) were selected, randomly, from the two states. In the third stage, three communities were randomly selected from each LGA. The last stage involved the selection of twenty organic maize farmers from each rural community using the snowball technique, which involved identified farmers referring to other organic maize farmers in the study area. This resulted in a total of 480 respondents.

This study used primary data. The data were collected through the administration of a structured questionnaire to the organic maize farmers. The organic maize farmers considered in this study were those who practice monocropping, that is, growing only organic maize on the farmland. The data were collected on socioeconomic and production characteristics such as the common organic practices for maize farming and the problems associated with organic maize farming.

2.2. Method of data analysis

Descriptive statistics and multivariate logistic regression were the means of data analysis. Descriptive statistics were used to describe the socio-economic features of the organic farmers, and common organic practices, and identify the problems associated with organic maize farming. The multivariate logistic regression model was used to analyse the determinants of the types of common organic farming used by maize farmers.

Multivariate logistic regression is an extension of logistic regression with more than one binary outcome. It tries to find out how the values in the dependent variables respond simultaneously to changes in independent variables. Below is the generalized equation for the multivariate regression model:

$$Y = \beta_0 + \beta_1 A + \beta_2 ED + \beta_3 INC + \beta_4 FS + \beta_5 FD + \beta_6 FE + \beta_7 FO + \beta_8 EX + \beta_9 CC + e$$

Where:

Y_i= Organic practices that were considered in this study are as follows:

i. Use of compost manure (1= yes, 0= no)
ii. Use of cover crop (1= yes, 0= no)
iii. Use of biocontrol (1= yes, 0= no)
iv. Use of organic manure (1= yes, 0= no)

The independent variables that were considered are as follows: A is the age of the farmers (years), ED is educational level, INC is income, FS is farm size (hectares), FD is farm distance from home (km), FE is farming experience (years), FO is farm-based organization membership (yes= 1, 0= otherwise), EXT is number of extensions contact,

CC is cultural compatibility (yes= 1, 0 otherwise), and e is the error term.

3. Results and discussion

3.1. Socio-economic characteristics of the organic maize farmers

This section described the socio-economic characteristics of the respondents and how they related to organic maize farming. The results presented in Table 1 show that the majority of organic maize farmers were male. This could be because males adopt new agricultural practices more quickly than their female counterparts in most African countries. Atoma et al. (2020) and Digal and Placencia (2019) reported a similar result, that more males adopted and practised organic farming than females. The larger proportion (55.83%) of the farmers were below 40 years of age. Their mean age was 40.5 years. This suggests that organic

Table 1. Distribution of the organic farmers by socioeconomic characteristics

Variables	Categories	Frequency	Percentage	Mean
Gender	Male	468	97.50	
	Female	12	2.50	
Age	21 – 30	68	14.17	
	31 – 40	200	41.66	40.5
	41 – 50	116	24.17	
	51 – 60	16	20.00	
Level of education	No Formal	16	3.33	
	Primary	72	15.00	
	Secondary	256	53.33	
	Tertiary	136	28.33	
Marital status	Married	376	78.33	
	Single	104	21.67	
Household size	1 – 5	88	18.33	
	6 – 10	348	72.50	7.54
	11 – 15	44	9.17	
Major occupation	Farming	422	87.92	
	Trading	12	2.50	
	Civil Servant	24	5.00	
	Artisanship	22	4.58	
Cooperative society membership	Yes	144	30.00	
	No	336	70.00	
Access to extension services	Yes	316	57.57	
	No	204	42.53	
Monthly income (₦)	1000 – 50000	260	54.17	
	50001 – 100000	164	34.17	75270.83
	100001 – 150000	24	5.00	
	>150000	32	6.67	
Organic maize farm size in hectares	1 – 2	292	60.83	
	3 – 4	156	32.50	2.41
	5 – 6	32	6.67	
Farming experience in years	1 – 10	76	15.80	
	11 – 20	308	64.20	15.91
	21 – 30	96	20.00	
Organic farming duration in years	1 – 5	164	34.17	
	6 – 10	180	37.50	7.71
	11 – 15	136	28.33	

Source: Field Survey (2021)

farmers can be said to be at their most youthful and economically active age when they can practice organic farming economically. Farmers at their active age can effectively and economically make use of scarce production resources to achieve their goals (Mukaila et al. 2021). Their active age may be considered an advantage with lots of potential considering the fact that this age group could be able to adopt simple and effective technologies to increase their productivity. Only 3.33% of the farmers had no formal education. The majority (53.33%) were secondary school certificate holders. About 28% had tertiary education with either a degree certificate, a national diploma certificate, a national certificate in education or their equivalents. Fifteen percent of the respondents were primary school certificate holders. These results imply that the organic farmers had some level of education that could enhance their productivity and decision-making process, unlike other farmers who engaged in conventional farming and had a low level of education. Mukaila et al. (2020) reported a low educational level among conventional farmers.

The majority of the organic farmers were married, which suggests that the farmers had some family responsibilities to cater to. The majority had between six and ten people in their households. They, however, had a mean household size of eight people per household. This suggests a large household size, which could serve as family labour. The majority of the organic farmers had farming as their major occupation. This implies that farming served as a major means of livelihood for them. The average organic maize farm size was 2.41 hectares, implying that organic farming is on a small scale. The farmers had a mean farming experience of 15.91 years, implying that the farmers had a high level of farming experience. They, however, had 7.71 years of organic farming experience. This suggests that organic farming has been practised for a couple of years in the study area, although relatively new. The majority were not members of cooperative societies, while only 30% belonged to cooperative societies. About 58% had access to extension services. This could enhance their knowledge of organic farming as the extensionist provides relevant information about best agricultural practices and innovation. Their mean monthly income was ₦75270.83 (USD 182.92). This implies that organic farming serves as a source of income for farmers and enhances their economic status.

3.2. Common organic practices for maize farming

The results presented in Table 2 show that the majority of the farmers used organic manure to enrich the soil. This could be widely used to supply nutrients to the soil due to its availability at a relatively cheaper rate. Most farmers were able to access it very close to their farms. About half of the respondents made use of cover crops to enrich the soil, while 49.2% did not make use of such practices. About 23% of the farmers used biocontrol to control pests on their farms, while 76.67% of the respondents did not use it. A higher percentage of the farmers (86.67%) used compost manure, while a smaller percentage of the respondents (13.33%) did not use compost manure to increase soil nutrients. From the results, it can be inferred that organic manure and compost manure were majorly used by the farmers since they had a higher percentage. While very few farmers made use of cover crops as an organic practice in the study area.

Table 2. Common organic practices for maize farming

Organic practices	Yes	No
Organic manure	456 (95.00)	24 (5.00)
Cover crop	244 (50.83)	236 (49.17)
Biocontrol	112 (23.33)	368 (76.67)
Compost manure	416 (86.67)	64 (13.33)

Source: Field survey (2021)

3.3. Drivers of the types of organic farming used by the farmers

Table 3 shows the factors that influence different types of organic farming practices used by farmers. As shown in Table 3, farmers' educational level (P<0.05), income (P<0.05), farm distance (P<0.01), farming experience (P<0.01), farming organization (P<0.01) and extension contacts (P<0.01) significantly influenced farmers' use of organic manure. The educational level of the farmers had a negative influence on the use of organic manure in maize production. This implies that a high level of education did not necessarily enhance organic manure usage. Thus, less educated farmers used organic manure to enrich the soil. This could be because most farmers are aware that organic manure is an important means of supplying nutrients to the soil. The coefficient of farmers' income was positive in relation to organic manure usage. This implies that the higher the farmers' income, the higher the likelihood of using organic manure in maize production. Due to the capital involved in getting organic manure, high-income farmers used more organic manure than their counterparts with low incomes. This is because farmers' income determines the level of agricultural investment (Falola et al. 2022a). Farm distance also had a positive influence on using organic manure. This implies that the longer the farm distance, the higher the probability of using organic manure. Thus, farmers who travel a long distance to reach their farms prefer to use organic manure due to the proximity of organic manure sellers (livestock farmers) to the maize farm.

Farming experience had a positive effect in relation to the use of organic manure practice. An increase in the farming experience of organic farmers leads to an increase in the probability of organic manure usage. Therefore, farming experience is an enhancing factor in the use of organic manure to boost soil nutrients and enhance maize growth and productivity. This is because the more farmers invested in organic farming, the better their understanding of organic manure usage and its significance. The coefficient of membership in a farm-based organization also had a positive effect on the use of organic manure practices. Thus, an increase in the likelihood of being a member of a farm-based organization by the farmers increases the probability of organic manure usage. This could be because of the dissemination of relevant information about the application of organic manure in farm-based societies to their members. Thus, membership in farm-based organizations is an enhancing factor in the use of organic manure for organic maize farming. The coefficient of extension contacts had a negative influence on organic manure usage. This shows that an increase in extension contact did not increase the likelihood of organic manure usage among the farmers. This could be because most farmers have knowledge of organic manure usage and its importance.

Farmers' use of cover cropping was significantly influenced by their educational level (P<0.01), farming experience (P<0.01), farm distance (P<0.01), farming organisation (P<0.01) and cultural compatibility (P<0.01). The educational level had a positive influence on using cover cropping as a means of supplying nutrients organically to the soil. Thus, the higher the educational level of farmers, the greater the likelihood of using cover cropping to enrich the soil with nutrients. This is because educated farmers know the type of cover crop to be grown alongside maize to supply the needed nutrients. Farm distance was positive and significant in relation to the use of cover cropping. Thus, an increase in farm distance increases the probability of using cover cropping practices by farmers. This is because a longer-distance farm requires a practice that will limit farmers' visitation to their farms. Thus, cover cropping can play a significant role in this regard as the cover crops supply nutrients to the soil, reduce soil erosion, avoid washing away soil nutrients, protect the soil from adverse environmental conditions, and suppress the growth of weeds on the farm. It also serves as crop diversification or polyculture organic farming, which can provide additional income to organic farmers.

Farming experience had a negative influence on the use of cover cropping. This suggests that a high level of experience did not necessarily increase the likelihood of cover cropping usage by farmers. Thus, the likelihood of its usage increases among farmers with little experience, which could be due to experienced farmers concentrating on other methods of organic farming. Farming organizations positively and significantly influence farmers' use of cover crops in their organic farms. This shows that the higher the likelihood of farmers being members of a farm-based organisation, the higher the probability of using cover crops to supply nutrients to the soil in order to enhance maize growth and yield. This could be due to the dissemination of organic farming knowledge in the society. Being a member of a cooperative society influenced farmers' usage of innovation as they learn from each other (Yokouchi and Kazuki 2016). Cultural compatibility also, positively, influenced cover cropping usage among organic maize farmers. Thus, the likelihood of its usage increases due to its acceptability by the farmers' culture.

Regarding the use of biocontrol as organic practice, educational level (P<0.01), income (P<0.01), farm size (P<0.05), farming experience (P<0.01) and extension contacts (P<0.01) were the significant factors. The level of education was positive and significant under the use of biocontrol. This implies that the probability of biocontrol (the use of natural pesticides) usage increases as the level of education of the farmers increases. This could be because well-educated farmers might have information about the importance and usage of employing natural pesticides to control pests in an organic way, which might increase the use of natural pesticides among them. Less educated farmers, on the other hand, are less likely to use natural pesticides to control pests

Table 3. Factors influencing the types of organic farming used by the farmers

Variables	Organic manure			Cover cropping			Biocontrol			Compost manure		
	Coef	Std Err	P>|z|	Coef	Std Err	P>|z|	Coef	Std Err	P>|z|	Coef	Std Err	P>|z|
Age	0.001	0.005	0.820	0.002	0.006	0.790	0.005	0.007	0.437	0.000	0.003	0.879
Education	-0.170**	0.072	0.028	0.241***	0.079	0.006	0.265***	0.091	0.008	0.005	0.037	0.903
Income	2.65e-06**	1.28e-06	0.049	5.39e-08	7.02e-08	0.450	-2.65e-07***	8.13e-08	0.003	-1.91e-09	3.28e-08	0.954
Farm size	-0.022	0.040	0.597	-0.006	0.044	0.901	0.021**	0.010	0.045	0.003	0.021	0.876
Farm distance	0.069***	0.014	0.000	0.058***	0.015	0.001	0.017	0.018	0.350	0.015**	0.007	0.042
Farming experience	0.031***	0.006	0.000	0.027***	0.006	0.001	0.030***	0.008	0.001	0.124***	0.253	0.000
Farming organization	0.782***	0.248	0.004	1.264***	0.272	0.000	-0.436	0.315	0.179	0.271**	0.127	0.044
Extension contacts	-0.219***	0.043	0.000	-0.064	0.047	0.183	0.177***	0.054	0.003	0.056**	0.023	0.019
Cultural compatibility	-0.684	0.494	0.179	0.173***	0.541	0.004	0.736	0.627	0.252	0.002	0.003	0. 610
Constant	1.963***	0.326	0.000	2.403***	0.358	0.000	-1.431***	0.414	0.002	1.056***	0.167	0.000
LR chi^2	25.06			25.91			23.73			22.25		
Prob > chi^2	0.002			0.002			0.004			0.008		
Pseudo R^2	0.579			0.567			0.528			0.489		
Log-likelihood	-9.101			-9.903			-10.628			-11.613		

***, **, * represent 1%, 5% and 10% levels of significance, respectively.
Source: Field survey (2021)

in their maize farms. Farmers' income had a negative influence on using biocontrol on organic farms. This implies that high income did not increase the likelihood of using biocontrol on the farm. Thus, farmers with lower incomes used natural pesticides such as wood ash and other biocontrol to control pests on their farms, which could be due to their low capital involvement.

Farm size was positive and significant under the use of biocontrol. This implies that an increase in organic farm size will increase the probability of biocontrol usage. The introduction of biocontrol on a large farm could reduce the cost of production on the farm. Farming experience also positively influenced biocontrol usage. This implies that the likelihood of using biocontrol increases as the farming experience increases. Thus, the farming experience was an enhancing factor for the use of biocontrol in organic farms. Extension contacts had a positive influence on the use of biocontrol among organic maize farmers. This implies that the likelihood of using biocontrol increases as the number of extension contacts increases. This could be due to the dissemination of useful information on biocontrol and teaching farmers how to use it on their farms. Extension agents inform farmers about innovation and its benefits (Mwangi and Kariuki 2015).

Under the use of compost manure, farm distance (P<0.05), farming experience (P<0.01), farming organisation membership (P<0.05) and extension contacts (P<0.05) were statistically significant. Farm distance had a positive influence on compost manure usage. This implies that farm distance enhances the probability of using compost manure to enrich the soil nutrients among organic maize farmers. This is due to the fact that preparing and using compost on the farm reduces the transportation cost of moving other means of enriching the soil; thus, farmers who travel a long distance prefer to use compost manure to reduce production costs. Farming experience had a positive influence on the use of compost manure. This implies that an increment in organic farming experience leads to an increment in the likelihood of using compost manure. This shows that the well-experienced farmers used compost manure to add nutrients to the soil more than the less experienced ones. The coefficient of membership in a farm-based organization was also positive and significant concerning the use of compost manure by the farmers. This suggests that an increase in the likelihood of being a member of a farm-based organization will increase the probability of using compost manure by farmers. This could be a result of accumulated labour available in the organisation, which could be used to prepare compost manure from which all members can benefit. The farmers' extension contacts also had a positive influence on the use of compost manure by the farmers. This shows that extension contacts increase the likelihood of using compost manure among farmers. This could be because of the knowledge required to make compost manure, which extension agents supplied to the farmers.

3.4. Problems associated with organic maize farming

The result presented in Table 4 shows that inadequate accrediting agencies was perceived as a severe constraint to organic farming in the study area by the majority (81.7%) of the farmers. Most of the farmers found it difficult to get their organic farms accredited. There was a lack of quality standards for bio manures by government agencies. Most of the organic farmers (67.50%) encountered the inability to meet the export demand as one of the problems associated with organic maize farming. This could be a result of low production or inadequate accreditation agencies. Low yield (59.17%) was also seen as a problem for organic farming. This could be due to the inability of the farmers to effectively control pests and diseases in organic farming in the study area, as the farmers mostly used physical methods and biological control, which have low efficacy. Most of the organic farmers (63.56%) reported that a lack of financial support was one of the problems associated with organic maize farming. Inadequate finance affects rural farmers' productivity and the rural economy, thus limiting farmers' potential to produce more food to feed the growing populace (Falola et al. 2022b). A shortage of organic seeds was also a challenge in organic maize farming. Some noted that organic seeds were not readily available within their locality. Half of the respondents perceived the shortage of organic matter as one of the problems associated with organic maize farming.

Table 4. Problems associated with organic maize farming in the study area

Problems	Yes	No
Inadequate accrediting agencies	392 (81.70)	88 (18.30)
Inability to meet the export demand	324 (67.50)	156 (32.50)
Low yield	284 (59.17)	196 (40.83)
Lack of financial support	300 (63.56)	180 (37.50)
Shortage of organic seeds	308 (64.17)	172 (35.83)
Shortage of organic matter	240 (50.00)	240 (50.00)
Inadequate supporting infrastructure	308 (64.17)	172 (35.83)
High input costs	288 (60.00)	192 (40.00)
The poor market for output	176 (36.67)	304 (63.33)
Lack of awareness	228 (47.50)	252 (52.50)
Lack of technical know-how	308 (64.17)	172 (35.83)
Pest control	292 (60.83)	188 (39.17)

Source: Field survey (2021)

Inadequate supporting infrastructure (64.17%) such as irrigation systems and motorable roads were also major problems associated with organic maize farming. The majority of the farmers practised rainfed agriculture, which limits their production to the availability of rain. High input costs (60%) were also a problem associated with organic maize farming. The price of organic maize seeds was expensive compared to the conventional maize seeds used in production. About 37% of the organic maize farmers perceived poor market output as a challenge to organic maize farming. This could be due to the absence of organic produce markets in the study area. Bello (2008) also reported that inadequate market information was a problem for organic farming. About 48% of the farmers encountered a lack of awareness as one of the problems associated with organic maize farming. Some maize consumers in the study area, especially in rural areas, were not aware of the health risk of conventional agriculture and the benefits of organic agriculture. Most of the farmers (64.17%) reported a lack of expert knowledge as one of the problems associated with organic maize farming. This supports the opinion of Bello (2008) that organic farming is faced with a lack of technical knowledge. Pest and disease control (60.83%) was a constraint to organic maize farming. Investigations revealed that, unlike the conventional farming system where chemical pesticides with high efficacy were used to control pests on maize farmers, the organic farmers in the area used natural pesticides and wood ash to control pests that had low efficacy.

Conclusion

This study investigated the determinants of different organic maize farming practices and the challenges encountered by farmers in Nigeria. The common organic practices in the study area were organic manure, compost manure, biocontrol, and cover cropping. The use of organic manure was influenced by income, farming experience, farm distance, educational level, farming organization, and extension contacts. Farming experience, farm distance, educational level, farming organisation, and cultural compatibility influenced the use of cover crops in organic maize farming. The use of biocontrol in organic maize farming was influenced by income, farming experience, farm size, educational level, and extension contacts. The use of compost manure was influenced by farm distance, farming experience, farming organisation membership, and extension contacts. Inadequate accrediting agencies, inability to meet export demand, high cost and scarcity of organic seeds, lack of financial support, high cost of inputs, poor marketing system, inadequate supporting infrastructure, poor technical know-how, and pest control were the most common problems faced by organic farmers.

Given these findings, this study calls for support from government and non-governmental organisations to encourage farmers in order to promote organic farming. This could be in the form of an effective marketing system for organic products to appreciate the benefits of organic farming. Adequate funding is also a prerequisite to promote the continuity of organic farming and to enhance judicious research in developing sustainable and feasible organic agricultural practices. Programmes should be set up by the government to create public awareness regarding how safe the consumption of organic food products is. This will, in turn, promote the sale of organic food products, thereby enhancing the interest of farmers to go more into organic food production. Also, adequate enlightenment regarding organic maize farming practices programmes should be frequently established for farmers so that more farmers can adopt such practices. Moreover, the government, non-governmental organizations, and research institutes should facilitate the dissemination of organic farming innovations among farmers.

Acknowledgement

We appreciate the organic maize farmers in the study area for providing the required information for this study.

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 155-165
DOI: 10.29136/mediterranean.1163714

Ethnic crop consumption and marketing in the Eastern United States: Trends and prospects

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ARTICLE INFO

Received: September 2, 2022
Accepted: September 19, 2022

***Keywords*:**

Locally grown
Ethnic greens and herbs
Hispanic populations
Asian populations
Eastern United States

ABSTRACT

The population demographics of the eastern U.S has changed in recent years with an increase in immigrants, particularly of Asian and Hispanic origins. This trend motivated the identification of foods preferred by these ethnic communities in 16 states in the region and Washington, D.C., focusing on greens and herbs. Over 100 ethnic greens and herbs were identified as being preferred food choices, from which 40 were selected for further study, representing 10 crops important to four ethnicities: Asian Indian, Chinese, Mexican, and Puerto Rico. Bulletin board focus group and telephone survey participants responded to questions regarding their consumption in 2010. The relevant information was collected to assess retail sales for each crop for each of the four ethnic groups. Results demonstrated that the ethnic crop demand in the eastern U.S is significant, and the prospects for future growth are promising as the population of ethnic consumers in the region is projected to continue to grow.

1. Introduction

Since 1980, growers in the eastern U.S. have been profoundly challenged with profitability and subsequent farm viability due to highly volatile markets (Govindasamy et al. 2010). Eastern growers tend to operate on relatively small farms and face higher production costs. The commercial production prospects for specialty crops and catering to the ethnically diverse consumers in the eastern U.S. have progressed in the last decade (Bhugra et al. 1999; Tubene 2001; Mendonca et al.2006; Arumugam et al. 2016). Population demographics are significantly changing with the increase of an ethnic population, which has been more pronounced in the East Coast region. As per the 2020 Census Bureau Reports, Hispanics and Asians remain the rapidly increasing minorities in the U.S. Between 2016 and 2060, the Hispanic population is projected to grow from 58 to 111 million, whereas the Asian population will increase from 18 to 37 million (Vespa et al. 2020). Beginning in 2030, net international migration is expected to be the main factor in population growth in the U.S. For instance, while the natural increase in population is projected to add one million people by 2030, net international migration will add 1.1 million people (Vespa et al. 2020).

According to a study of the multicultural economy by the University of Georgia Selig Center, the combined buying power of Asian Americans and Hispanic populations has dramatically increased since 2000, becoming the fastest-growing minority market in the country. In 2018, Asian-Americans contributed 6.2 percent, roughly $1 trillion (Humphreys 2018), to the economy, an increase of 267 percent since the beginning of the millennium (Humphreys 2018), while Hispanics contributed about $1,5 trillion, an increase of 212 percent, since 2000. The purchasing power of Hispanics increased to $494 billion in 2000 and is projected to increase by more than $1,924 trillion by 2023. The Asian buying power was estimated to be about $276 billion in 2000 and is expected to increase to $1,34 trillion by 2023 (Humphreys 2018). The fast growth of the ethnic population and their increasing purchasing power translates to substantial opportunities for the ethnic produce sector. Greens and herb growers in the region can take advantage of their proximity to densely populated areas where these groups often reside. However, ethnic consumers are often looking for produce with

specific attributes and flavors (Govindasamy et al. 2007; Park et al. 2007; Govindasamy et al. 2015).

Consumer food choices that result in nutritional patterns are considered important for achieving sustainability targets (Sciarappa et al. 2016). Food choice behaviors are linked to the social and economic appearance of identities, preferences, and cultural meanings and are an essential determinant of nutritional status and health. Food can be a sign of individual identity, group affiliation (Lindgren et.al 2018), and cultural identity (Bisogni et al. 2002). Consumers make food decisions based on psychological and cultural factors, as well as lifestyle and food trends (Fischler 1988; Asp 1999; Gilbert 2000). Recent food trends in the U.S. reflected more on "home-cooking" eating habits, with more whole/plain foods such as fruit, vegetables, cooking fat, grains, unsweetened milk, juice, and others; whereas, the trends also showed a negative preference for processed food groups such as fast-food meals and snacks (Monterrosa et al. 2020).

The food system is important in understanding the many factors that influence food choice at the individual level and the role of culture in driving those choices. For instance, there is an established linkage between food and culture (Piernas et al. 2014). This correlation drives an immigrant's integration into a new culture, like that of the U.S., while still maintaining their identity with their home country (Peñaloza 1994; Piernas et al. 2014; Arumugam et al. 2016). Evidence for the importance of these groups is further demonstrated by the fact that ethnic foods are categorized as specialty items, which have increased in value by 9.8 percent between 2016 and 2018, reaching $148,7 billion in sales (Bojanic and Xu 2006). Thus, it would only be prudent for growers to assess their ability to meet the demand for ethnic food. Against this background, this paper has documented Hispanic and Asian consumers' consumption patterns and identified the most preferred ethnic greens and herbs in the eastern U.S.

2. Materials and Methods

This paper reviews the prospects for marketing ethnic greens and herbs to Asian and Hispanic consumers. These specific ethnic markets were chosen for their strong recent growth, which is predicted to continue (Humphreys 2018; Specialty Food Association 2019). The top two subgroups within each of these sections were i) Asian sub-groups (Chinese and Asian Indian) and ii) Hispanic sub-groups (Puerto Rican and Mexican). The geographical focus included Washington D.C. and 16 states along the East Coast region of the U.S.

The project team consulted advisory board members and used online focus group bulletin board sessions and telephone surveys to collect data from the target market. Focus group participants were selected at random from a recognized panel of participants managed by Survey Sampling International, LLC (Shelton, CT), a provider of sampling solutions for survey research. To participate in the bulletin board sessions, panelists clicked on a hyperlink at the bottom of the consent statement, directing them to the welcome screen. Each morning the moderator would email panelists reminding them to log in to the system, respond to new questions, and review and respond to other panelists' comments posted on the previous day. In total, of the 44 panelists who accessed the bulletin boards, 38 consumers completed the study: 11 in the "Chinese" ethnicity focus group session, 10 in the "Asian Indian" session, nine in the "Mexican" session, and eight in the "Puerto Rican" session. During the sessions, participants responded to questions about their shopping habits, preferences, perceptions, and demographic characteristics. Bulletin board focus group responses were then used to construct a telephone survey of ethnic consumers.

A preliminary list of ethnic greens and herbs important to the four ethnic groups was compiled based on responses gathered from online focus group bulletin board session participants and informal market research. To determine which of these crops to incorporate in the telephone survey, a panel of marketing, crop specialists, and field/extension faculties reviewed the list of ethnic greens and herbs to eliminate those with existing production barriers that could impede their marketplace success and/or local production (Figure 1, Govindasamy et al. 2007). These data were then used to estimate ethnic consumers' buying behaviors, such as buying frequency, quantities of ethnic greens/herbs bought during each visit, and to estimate the overall market size of the top 10 greens/herbs consumed by respondents who identified with the four ethnicities.

A separate detailed survey for each ethnic group was developed based on input from all specialists and consumer representatives from each of the four groups. The crop list was further refined through a selection method based on expenditures, quantities, and appropriate production considerations for the local market demand and supply factors (Appendix I). A telephone survey of consumers residing in states along the East Coast region (Maryland, Massachusetts, Delaware, Florida, New Hampshire, Georgia, Maine, New Jersey, New York, North Carolina, Pennsylvania, Rhode Island, South Carolina, Vermont, Virginia and Washington, D.C.) of the U.S. was conducted by Perceptive Marketing Research, Inc. (Gainesville, FL), a market research firm. The survey gathered information to assist small and medium farmers with a better understanding of consumer insights and factors that drive ethnic greens and herbs markets, specifically attitudes and behaviors of Asian Indian, Chinese, Mexican, and Puerto Rican consumers. Interviews were conducted using the computer-assisted telephone interviewing system (CATI) with interview times averaging between 20 and 23 minutes.

The survey was pre-tested and then launched from 11 May to 22 Oct. 2010, with a total of 7.678 leads to meet the required samples. Around 195 households refused to answer any questions, and 2.457 of them reported no answer. A total of 3.217 household calls were unsuccessful, 516 respondents were not available during initial and follow-up attempts, and 49 telephone call interviews were interrupted during the survey. A total of 1.244 responses were collected. Of these, 1.117 respondents qualified as they indicated they were responsible for at least half of the food shopping for the household (Chinese-276, Asian Indian-277, Puerto Ricans-284, and Mexicans-280). The remaining 127 respondents did not have a role in purchasing food items for the household, and their responses were removed from the data set (Chinese-21, Asian Indian-45, Puerto Ricans-37, and Mexicans-24). Detailed information, including price and quantity, was obtained to measure retail sales of each produce item based on information provided by ethnic respondents who purchased each particular item (Bernstein 2006; Govindasamy, et al. 2007).

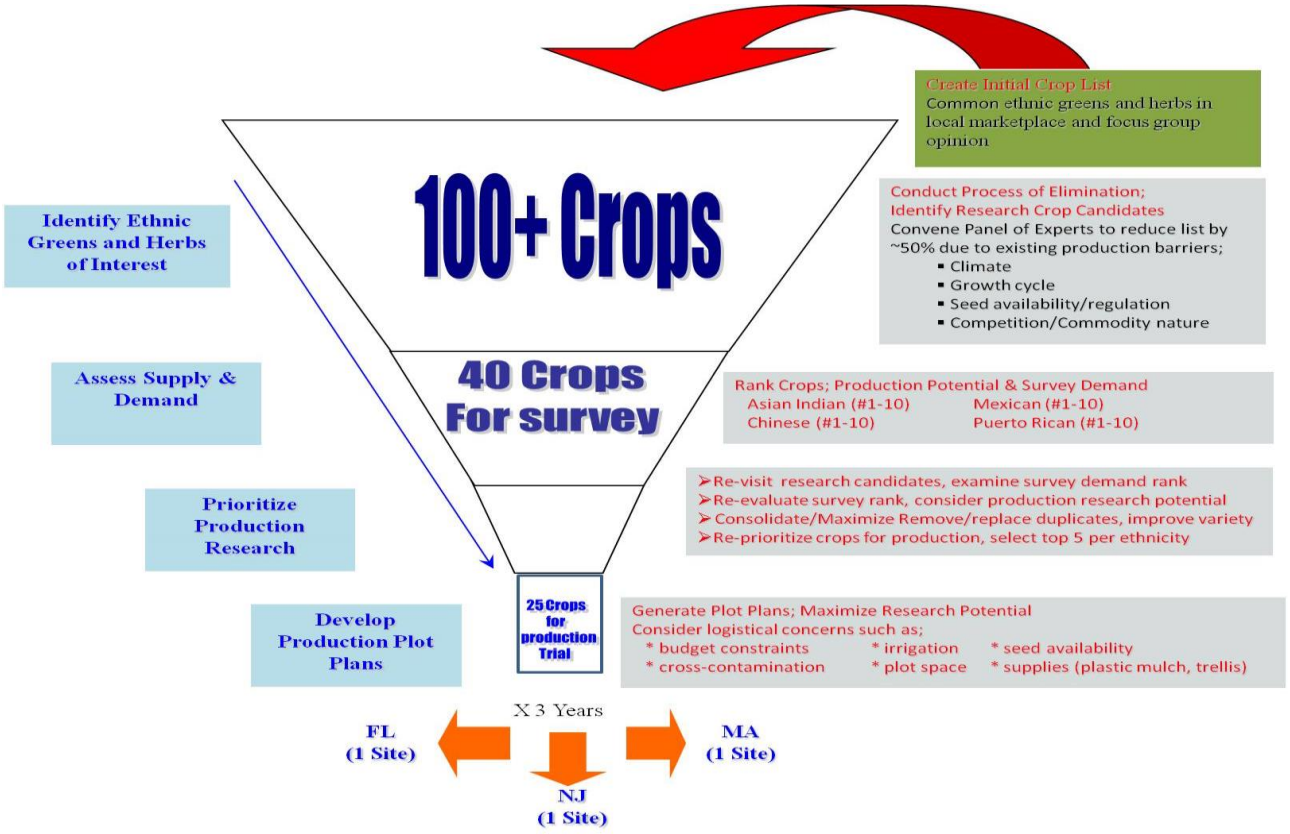

Figure 1. Ethnic Greens and Herbs Selection Process - Govindasamy et al. (2007).

2.1. Two-way contingency and chi-square independence test for ethnic consumer clusters

A cluster analysis including a two-way contingency table and Chi-square independence tests for four ethnic groups were performed. The Chi-square independence test tested whether two variables are associated. In the case of the state variable, our hypotheses were:

H_0 : Ethnic consumer clusters are not associated.

H_1 : Ethnic consumer clusters are associated.

The Chi-square independence test compares the observed frequencies with the expected frequencies Equation (1) is the test statistic used for this comparison. ***E*** represents the expected, whereas ***O*** refers to observed frequencies. Equation (2) was used to estimate ***E***.

$$\chi^2 = \sum (O - E)^2 / E \quad (1)$$

$$E = (Row\ total\ X\ Column\ total) / n \quad (2)$$

The two-way contingency table shows the distribution of the data in each group, which allows us to compare the difference in the levels in the categorical variables in each group. Based on the two-way contingency table, Chi-square tests were derived to test if each of the variables is associated with the response variable [39, 40]

3. Results

Among Asian Indians who responded (Table 1), Radish Greens, Turmeric, and Fenugreek were the top three most popular items purchased, with 74, 73, and 72% of the respondents purchasing these three ethnic crops, respectively. In addition, more than half of the respondents purchased Indian Sorrel Spinach. In contrast, 10% or less of Asian Indians purchased Indian Sorrel and Amaranth (Purple). Regarding purchasing frequency, Asian Indian participants bought most ethnic greens and herbs regularly, one to five times per month routinely with Radish Greens being the most purchased item, 38% of these respondents bought the item regularly and 36% purchased it seasonally.

Of those who purchased Fenugreek, 40% purchased it routinely and 33% purchased it seasonally (Table 2).

Among Chinese respondents (Table 3), Shanghai Bok Choy was the most frequently purchased green/herb of the 10 presented to participants, with 86% of participants indicating that they purchased the item. Both Chinese Broccoli and Spinach were purchased by 72% of these participants.

Among the respondents who purchased Shanghai Bok Choy, 72% bought this crop regularly, and the remaining 14% indicated that they bought it seasonally. Fifty-five percent of Chinese regularly purchased Chinese Broccoli and Spinach (Table 4).

For Mexican respondents (Table 5), ethnic green/herb purchases were widely distributed. Relative to the other items, Roselle was purchased the most, with 51% of Mexicans doing so. Slightly less than half, 48, of Mexicans purchased Purslane, and 44% purchased Epazote.

As Table 6 shows, among the respondents who purchased Roselle (referring to the leaves rather than the more common calyx of hibiscus), 30% of them purchased this item regularly. Of the 48% of Mexicans who purchased Purslane, 23% did so regularly, and 24% did so seasonally. For the 44% who bought Epazote, 25% did so regularly, and 19% on a seasonal basis.

Lettuce was the most popular ethnic green/herb among Puerto Rican participants, purchased by 95% of these participants (Table 7). Slightly fewer, 88%, of Puerto Ricans purchased Culantro, and 72% purchased Garlic Chives.

Among the 95% of respondents who purchased Lettuce, 82% purchased it regularly and 13% of them bought it seasonally (Table 8). The percentage of those who bought Culantro and Garlic chives regularly was lower, 71 and 56%, respectively.

Table 1. Top 10 greens and herbs bought by Asian Indian respondents

Crops	Asian Indian			
	Ethnic Greens and Herbs Purchasing Behavior			
	Yes		No	
	Number	Percent	Number	Percent
Radish Greens (n= 277)	205	74%	72	26%
Turmeric (n= 277)	203	73%	74	27%
Fenugreek (n= 276)	199	72%	77	28%
Indian Sorrel Spinach (n= 275)	163	59%	112	40%
Amaranth (Green) (n= 277)	60	22%	217	78%
Nightshade (n= 277)	50	18%	227	82%
Purslane/Veradolga (n= 277)	35	13%	242	87%
Amaranth (Purple) (n= 277)	29	10%	248	90%
Indian Sorrel (n= 275)	19	7%	256	92%

Note: Percentage calculated based on a total of 277 respondents and the total below 100% indicates non-response.

Table 2. Asian Indians purchasing behavior of ethnic greens and herbs

Crops	Asian Indian					
	Ethnic Greens and Herbs Purchasing Frequency					
	Routinely		Seasonal		Total	
	Number	Percent	Number	Percent	Number	Percent
Radish Greens (n= 277)	106	38%	99	36%	205	74%
Turmeric (n= 277)	133	48%	70	25%	203	73%
Fenugreek (n= 276)	110	40%	90	33%	200	72%
Indian Sorrel Spinach (n= 275)	107	39%	56	20%	163	59%
Amaranth (Green) (n= 277)	28	10%	32	12%	60	22%
Nightshade (n= 277)	15	5%	35	13%	50	18%
Malabar Spinach (n= 277)	15	5%	31	11%	46	16%
Purslane/Veradolga (n= 277)	17	6%	18	7%	35	13%
Amaranth (Purple) (n= 277)	16	6%	13	5%	29	11%
Indian Sorrel (n= 275)	8	3%	11	4%	19	7%

Note: Percentage calculated based on a total of 277 respondents and the total below 100% indicates non-response.

Table 3. Top 10 greens and herbs bought by Chinese respondents

Crops	Chinese			
	Ethnic Greens and Herbs Purchasing Behavior			
	Yes		No	
	Number	Percent	Number	Percent
Shanghai Bok Choy (n= 276)	238	86%	38	14%
Spinach (n= 276)	200	72%	76	28%
Chinese Broccoli (n= 276)	199	72%	77	28%
Sugar Pea tops/bean (n= 276)	114	41%	162	59%
Chives & Flowers (n= 276)	108	39%	168	61%
Garland Chrysanthemum (n= 276)	85	31%	191	69%
Yen Choy (n= 276)	78	28%	198	72%
Malabar Spinach (n= 276)	56	20%	220	80%
Potherb Mustard (n= 276)	47	17%	229	83%
Lycium Leaf (n= 276)	20	7%	256	93%

Note: Percentage calculated based on a total of 276 respondents.

Table 4. Chinese respondents purchasing behavior of ethnic greens and herbs

Crops	Chinese					
	Ethnic Greens and Herbs Purchasing Frequency					
	Routinely		Seasonal		Total	
	Number	Percent	Number	Percent	Number	Percent
Shanghai Bok Choy (n= 276)	198	72%	40	14%	238	86%
Spinach (n= 276)	152	55%	48	17%	200	72%
Chinese Broccoli (n= 276)	151	55%	48	17%	199	72%
Sugar Pea tops/bean (n= 276)	76	28%	38	14%	114	41%
Chives & Flowers (n= 276)	66	24%	42	15%	108	39%
Garland Chrysanthemum (n= 276)	30	11%	55	20%	85	31%
Yen Choy (n= 276)	51	18%	27	10%	78	28%
Malabar Spinach (n= 276)	39	14%	17	6%	56	20%
Potherb Mustard (n= 276)	29	11%	18	7%	47	17%
Lycium Leaf (n= 276)	10	4%	10	4%	20	7%

Note: Percentage calculated based on a total of 276 respondents and the total below 100% indicates non-response.

Table 5. Top 10 greens and herbs bought by Mexican respondents

Crops	Mexican			
	Ethnic Greens and Herbs Purchasing Behavior			
	Yes		No	
	Number	Percent	Number	Percent
Roselle (n= 280)	143	51%	137	49%
Purslane/Verdolaga (n= 280)	133	48%	147	53%
Epazote (n= 280)	123	44%	157	56%
Swiss Chard (n= 280)	104	37%	176	63%
Vine Vegetables (n= 280)	94	34%	186	66%
Lambsquarter (n= 280)	85	30%	195	70%
Lippia (n= 280)	65	23%	215	77%
Papalo (n= 280)	60	21%	220	79%
Amaranth (n= 280)	34	12%	246	88%
Lemon Verbena (n= 280)	21	8%	259	93%

Note: Percentage calculated based on a total of 280 respondents.

Table 6. Mexican respondents purchasing behavior of ethnic greens and herbs

Crops	Mexican					
	Ethnic Greens and Herbs Purchasing Frequency					
	Routinely		Seasonal		Total	
	Number	Percent	Number	Percent	Number	Percent
Roselle (n= 280)	84	30%	59	21%	143	51%
Vine Vegetables (n= 280)	84	30%	10	4%	94	34%
Epazote (n= 280)	71	25%	52	19%	123	44%
Purslane/Verdolaga (n= 280)	65	23%	68	24%	133	48%
Swiss Chard (n= 280)	41	15%	63	23%	104	37%
Lambsquarter (n= 280)	36	13%	49	18%	85	30%
Lippia (n= 280)	36	13%	29	10%	65	23%
Papalo (n= 280)	25	9%	35	13%	60	21%
Amaranth (n= 280)	18	6%	16	6%	34	12%
Lemon Verbena (n= 280)	14	5%	7	3%	21	8%

Note: Percentage calculated based on a total of 280 respondents and the total below 100% indicates non-response.

Table 7. Top 10 greens and herbs bought by Puerto Rican respondents

Crops	Puerto Rican			
	Ethnic Greens and Herbs Purchasing Behavior			
	Yes		No	
	Number	Percent	Number	Percent
Lettuce/Lechuga (n= 284)	271	95%	13	5%
Culantro (n= 284)	251	88%	33	12%
Garlic Chives (n= 284)	204	72%	80	28%
Spanish Oregano (n= 284)	135	48%	149	52%
Wild Garlic (n= 284)	62	22%	222	78%
Lemon Balm (n= 284)	37	13%	247	87%
Lambsquarter (n= 284)	30	11%	254	89%
Purslane (n= 284)	30	11%	254	89%
Dandelion greens (n= 284)	27	10%	257	90%
Tarragon (n= 284)	12	4%	272	96%

Note: Percentage calculated based on a total of 284 respondents.

Table 8. Puerto Rican respondents purchasing behavior of ethnic greens and herbs

Crops	Puerto Rican					
	Ethnic Greens and Herbs Purchasing Frequency					
	Routinely		Seasonal		Total	
	Number	Percent	Number	Percent	Number	Percent
Lettuce/Lechuga (n= 284)	234	82%	37	13%	271	95%
Culantro (n= 284)	203	71%	48	17%	251	88%
Garlic Chives (n= 284)	159	56%	45	16%	204	72%
Spanish Oregano (n= 284)	93	33%	42	15%	135	48%
Wild Garlic (n= 284)	49	17%	13	5%	62	22%
Lambsquarter (n= 284)	21	7%	9	3%	30	11%
Lemon Balm (n= 284)	19	7%	18	6%	37	13%
Purslane (n= 284)	17	6%	13	5%	30	11%
Dandelion greens (n= 284)	17	6%	10	4%	27	10%
Tarragon (n= 284)	9	3%	3	1%	12	4%

Note: Percentage calculated based on a total of 284 respondents and the total below 100% indicates non-response.

The average number of times that participants shopped for ethnic greens and herbs was 4.2 times per month, but this varied by ethnic group (Table 9). Asian Indians shopped 3.7 times per month, while the number of visits was higher for the other three ethnic groups: 4.7 times for Chinese, 4.2 times for Mexicans, and 3.8 times for Puerto Ricans. The expenditure for ethnic greens and herbs were summarized for each ethnic group: $24 expenditures for Asian Indians, $25,70 for Chinese, $23 for Mexicans, and $22,70 for Puerto Ricans. Asian Indians spent over $100 on ethnic greens and herbs monthly. Meanwhile, the other three subgroups spent around $79 to $86,70 on ethnic green and herbs per month. However, for total produce expenditure per month, $142,9 to $210,90 were spent among these four ethnicities. On average, around $42,90 were spent on the 10 crops, which were selected by a systematic process. Respondents lived within approximately 8 miles of ethnic markets.

As can be seen in Table 10, on average, Puerto Ricans lived at the current location for 17.94 years; similarly, the Chinese have lived 13.7 years at the current location. For Asian Indians, the living period was slightly shorter at 11.13 years while 9.71 years for Puerto Ricans. Household sizes were similar among these four ethnic groups. On average, 3.7 members lived in one family among all ethnicities, 3.6 members within Asian Indian families on average, 3.4 for Chinese, 4.9 for Mexicans, and 3 for Puerto Ricans. On average, the number of household members under17 years of age was 1.2 for all four ethnic groups. Mexicans had the highest number of members under 17 years old compared to the other three groups.

3.1. Ethnic greens and herbs consumer clusters

Figure 2 depicts the dendrogram for cluster analysis. From the dendrogram, it is unclear how many clusters are appropriate, as data could be segmented into two, three, or four distinct groups. It can be cut at 2, 3, or 4 clusters. To decide the optimal number of clusters, we created an elbow plot, as shown in Figure 3.

The optimal number of clusters was identified by using Eigenvalues (above 1), as shown in Figure 3 which clearly suggests a four-cluster solution for further analysis, one for each of the four ethnic groups.

Table 9. Average visits, expenditures on greens and herbs, proximity and family size by ethnicity

Household Average Figures	Ethnicity				All Ethnicities
	Asian Indian	Chinese	Mexican	Puerto Rican	
Visits to an Ethnic Market in a Month (Number)	3.71	4.73	4.23	3.81	4.22
Ethnic Greens/Herbs Expenditure per visit	$24,04	$25,70	$23,00	$22,67	$23,88
Expenditures on Ethnic Greens/Herbs per Month	$111,97	$86,72	$84,57	$79,02	$86,85
Total Produce Expenditure per Month	$179,76	$210,90	$142,85	$169,77	$174,55
Total 10 Crops Expenditures per Month	$41,73	$42,54	$44,12	$43,37	$42,93
Proximity to the Nearest Ethnic Grocery Store (Miles)	12.75	11.57	3.39	4.63	8.11
Average Household Size (Number)	3.57	3.41	4.91	3.00	3.73

Table 10. Average household size, years at the residence, and age ranges of residents

Average Figures	Ethnicity				All Ethnicities
	Asian Indian	Chinese	Mexican	Puerto Rican	
Average Number Years Living at Current Location (Number)	11.13	13.69	9.71	17.94	13.13
Average Number of People in a Household Age 17 Years or Younger (Number)	1.01	0.92	2.00	0.89	1.21

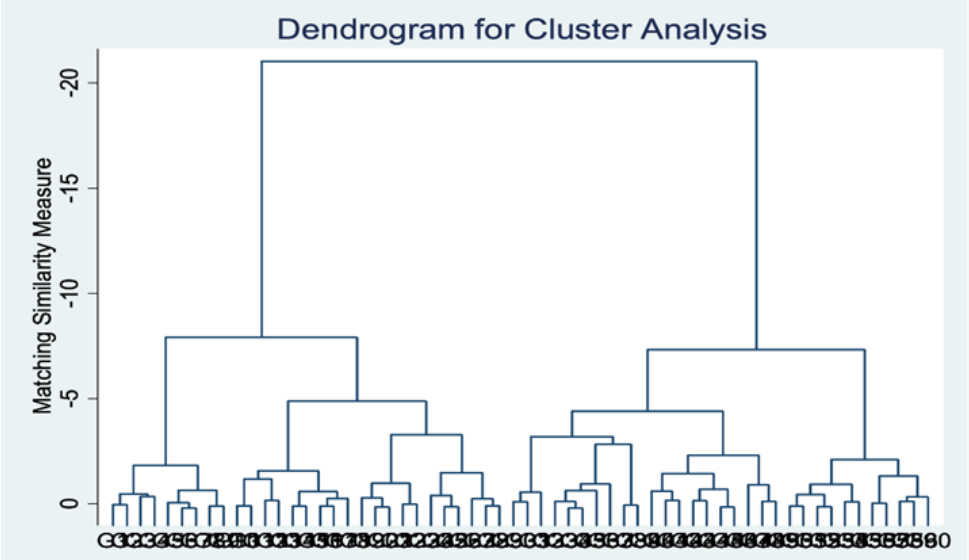

Figure 2. Dendrogram of cluster analysis.

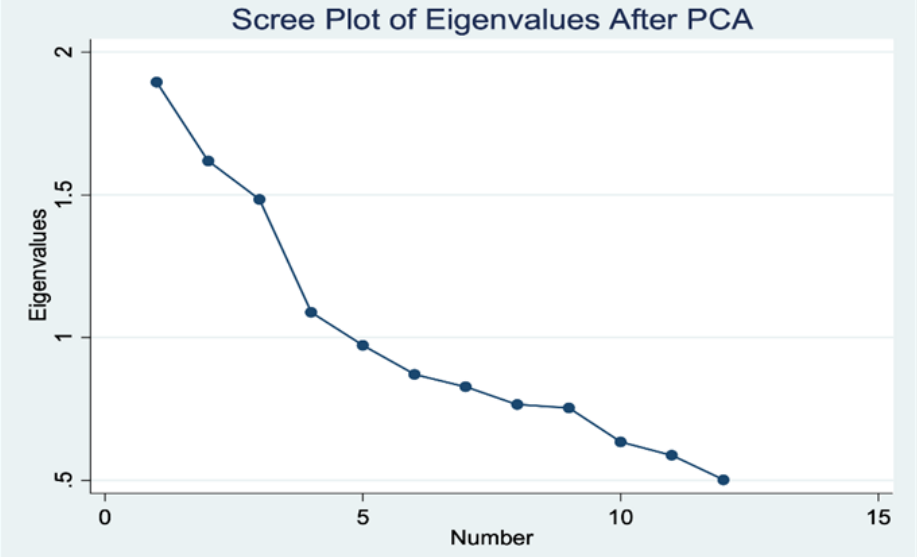

Figure 3. Elbow plot of the optimal number of clusters.

Table 11 contains variables that tested statistically significant. The variables include retail outlet options, green & herbs attributes, consumer preference/support, age, education, family income, gender, and marital status associated with the market segments derived from cluster analysis.

Table 12 shows the summary of consumer profiles, with all four ethnicities, studied showing basic consistencies in terms of greens and herbs purchases. For instance, most Asians frequently purchased greens and herbs from ethnic grocery stores, whereas Hispanics prefer American grocery stores. The freshness and quality are consistently deemed the most important by the sample respondents in each group. Hispanic respondents indicated that price is an important factor in their purchasing decision. Most purchasers in each ethnic group were willing to purchase ethnic greens and herbs, which were locally and organically grown. In addition, the Indian ethnic group reported that they were willing to buy ethnic greens and herbs to support local farmers. The predominant age group that preferred greens and herbs was 36 to 65 years of age for Asian Indians, Chinese, and Puerto Ricans; however, most young Mexicans were willing to buy greens and herbs.

Most of the Mexican and Puerto Rican respondents had up to 2 years of college education, while a majority of Asian Indian and Chinese respondents had a postgraduate degree. More Asian Indian and Chinese respondents had annual household incomes between $60,000-$100,000, while the two Hispanic groups had annual household incomes of less than $59,999. Pertaining to gender and marital status, the target market for all four ethnic groups was more likely to be female and married; however, Mexican and Puerto Rican participants were also likely to be single.

Table 11. Contingency Table and Chi-Square Independence Test of Ethnic Consumer Clusters

	CLUSTER					Chi-Square
Particulars	**Indian (n= 277)**	**Chinese (n= 276)**	**Mexican (n= 280)**	**Puerto Rican (n= 284)**	**Total (n= 1117)**	**P-Value**
A. Retail outlets (Yes / No) #						
American grocery stores	14%	10%	20%	19%	63%	0.000***
Ethnic grocery stores	24%	24%	22%	19%	88%	0.000***
Community farmers' market	12%	5%	10%	9%	36%	0.000***
On-farm markets or roadside stands	4%	2%	7%	6%	19%	0.000***
Pick your own farms	2%	2%	4%	3%	10%	0.009***
B. Green & Herbs Attributes (Yes / No) #						
Availability	15%	14%	17%	16%	62%	0.156 NA
Freshness	24%	24%	22%	23%	93%	0.000***
Quality	24%	24%	22%	24%	93%	0.000***
Price	12%	13%	20%	20%	66%	0.000***
Packaging	7%	4%	14%	12%	38%	0.000***
Information on the package	10%	5%	13%	15%	43%	0.000***
C. Preference /support (Yes / No)#						
Locally grown	23%	22%	20%	23%	88%	0.000***
Organically grown	20%	17%	19%	19%	75%	0.027**
Genetically modified	4%	4%	7%	5%	19%	0.000***
Labeled according to country of origin	16%	16%	17%	16%	65%	0.939NA
New herbs & greens	16%	15%	15%	11%	56%	0.000***
Support local farmer	13%	8%	12%	11%	44%	0.000***
D. Age Range						
Less than 35 years	7%	4%	14%	6%	29%	0.000***
36-65 years	16%	18%	11%	15%	60%	
Over 65 years	2%	2%	0%	5%	10%	
E. Education						
Up to 2 Years of college	5%	8%	25%	23%	60%	0.000***
4 Years college degree	8%	7%	0%	2%	18%	
Postgraduate degree	12%	10%	0%	1%	23%	
F. Annual Household Income						
Less than $59,999	7%	8%	27%	25%	66%	0.000***
$60,000-$100,000	9%	11%	1%	2%	23%	
Over $100,000	7%	4%	0%	0%	11%	
G. Gender						
Female	14%	16%	18%	18%	66%	0.000***
Male	11%	9%	7%	7%	34%	
H. Marital Status						
Married	22%	20%	16%	9%	67%	0.000***
Single	2%	3%	5%	9%	18%	
Divorced	0%	1%	0%	3%	4%	
Widower	0%	1%	0%	2%	4%	
Other	0%	1%	3%	2%	7%	

Note: # denotes multiple responses * Significant at 10%, **significant at 5% and ***significant at 1%.

Table 12. Profile of Ethnic Greens and Herbs Consumer Clusters

Particulars	Asians		Hispanic	
	Indian	**Chinese**	**Mexican**	**Puerto Rican**
Retail outlets	Ethnic grocery stores	Ethnic grocery stores	American grocery stores	American grocery stores Ethnic grocery stores
Green & Herbs Attributes	Freshness and Quality	Freshness and Quality	Freshness, Quality and Price	Freshness, Quality and Price
Preference/support	Locally Grown and Organically Grown and Support Local Farmer	Locally Grown and Organically Grown	Locally Grown and Organically Grown	Locally Grown and Organically Grown
Age Range	Mid-age (36-65 Years old)	Mid-age (36-65 Years old)	Youngest (Less than 35 Years old)	Mid-age (36-65 Years old)
Education	Postgraduate degree	Postgraduate degree	Up to 2 Years College Degree	Up to 2 Years College Degree
Annual Household Income	$60,000-$100,000	$60,000-$100,000	Less than $59,999	Less than $59,999
Gender	Female	Female	Female	Female
Marital Status	Married	Married	Married & Single	Married & Single

5. Discussion

This study investigated preferences for ethnic greens and herbs among Asian Indians, Chinese, Mexicans, and Puerto Ricans who reside along the East Coast region of the U.S., as determined by using online focus group bulletin board sessions and telephone surveys. The geographical focus included Washington D.C. and 16 states from the East Coast region of the US.

Ethnic consumers are often looking for produce with specific attributes and flavors (Bhugra 1999; Arumugam et al. 2016; Lindgren 2018). For instance, Radish Greens, Turmeric, Fenugreek, and Indian Sorrel Spinach were the most popular items purchased by Asian Indians who participated in the study. However, 10% or less of them purchased Indian Sorrel and Amaranth (Purple). These greens and herbs are often used by Asian Indians as food ingredients as well as in medicinal applications, (Hillier et al. 2011; Banerjee et al. 2015). Among Chinese respondents, Shanghai Bok Choy, Chinese Broccoli, and Spinach were the most important green/herbs based on purchase amounts. Again, though they are used as ingredients in meals, these greens and herbs have been used to improve cognitive performance in elderly subjects (Sarkar et al 2015; Monterrosa et al. 2020).

Mexican respondents purchased a wider array of greens and herbs compared to the other three groups. Relative to the other items, Roselle, Purslane, and Epazote were the most purchased greens/herbs. These items have also been used to prevent a wide range of health-related problems (Nurk et al. 2010; Da-Costa-Rocha et al. 2014; Thomsen et al. 2016; Dhakal and Khadka 2021). Lettuce, Culantro, and Garlic Chives were the foremost popular ethnic greens/herbs among Puerto Rican participants. Consumption of these greens and herbs has continued to grow due to the consumers' interest in the role of food in keeping and improving human well-being (Valerino-Perea et al. 2019; Vadiveloo et al. 2020).

There appear to be differences between ethnic groups regarding where they purchase food for their household. One study reported that Hispanic households purchased most of their food products from grocery stores, while Asian households chose to shop for food at wholesale markets/stores (Da-Costa-Rocha et al. 2014). Wholesale stores and supermarkets offer a wider variety of grocery items including cereals, pulses and fruits, vegetables, and bulk produce.

A family's socioeconomic and demographic characteristics are known to influence food purchasing behaviors, nutritional quality, and health outcomes (Valerino-Perea et al. 2019). In our study, respondents traveled about 8 miles to reach grocery stores, however, few studies show that using the nearby grocery store in terms of distance to shopping for healthier options is flawed (Ragaert et al. 2004 and Drewnowski et al. 2012) as households usually do not shop at the grocery store that is closest to them (Ledoux and Vojnovic 2013 and Sohi et al. 2014). However, this situation persists based on actual food budget expenditure data. Our results revealed that the average ethnic group expenditure per visit was $24 for Asian Indians, $25,70 for Chinese, $23 for Mexicans, and $22,70 for Puerto Ricans. The total produce expenditure per month, $142,90 to $210,90 was spent among these four ethnicities. On average, around $42,90 were spent on the 10 crops which were selected by a systematic process. The expenditure at stores also depends on the presence of a child in the family, ownership of a personal vehicle, education, employment, and marital status (Da-Costa-Rocha et al. 2014). In rural areas, food expenditure was highest at convenience stores, while families with access to personal vehicles were more likely to purchase foods at wholesale locations (Da-Costa-Rocha et al. 2014). Food movements are built on multiple values that address how to grow, transport, source or buy and cook foods. Some values encompass ethical and moral reasons, which create a strong emotional connection with consumers (Da-Costa-Rocha et al. 2014).

6. Conclusions

This study revealed that the preferences for ethnic produce are different among ethnic groups. Consumers make food decisions based on cultural background and lifestyle. Specifically, food safety, wider variety, affordable price, freshness, and quality of the ethnic greens and herbs are important factors that impact purchasing (Lang 2010). This study revealed that participants purchased a wide variety of ethnic greens and herbs and that growing and providing more species/varieties could entice consumers to visit markets to buy these items, which could benefit retailers as well as growers (Lang 2010; Simon et al. 2012; Lee et al.2014).

The ethnic grocery store/market distance and choices of fresh ethnic produce may facilitate consumers to purchase ethnic items. Since distance is one of the important factors, the grocery store providing ethnic greens and herbs should be located near

the neighborhood, which provides each ethnic group a greater opportunity to buy ethnic produces. Furthermore, developing market intelligence can assist growers in tailoring their products and promotional activities to better meet the needs of the ethnic greens and herbs purchaser, allowing these consumers to be able to purchase authentic ethnic produce from local farms, which will enable them to satisfy their social as well as community needs. The ethnic consumer profile cluster for all four ethnicities showed basic consistencies in terms of purchasing greens and herbs. The overall study results will help stakeholders discover potential changes in ethnic markets that could be beneficial to increasing the farm operational profit of small and medium-sized growers in the region. Further, the researchers need to explore the field-level production trials of more ethnic greens and herbs to introduce new produce in specific market segments.

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Appendix 1: Common and scientific names of ethnic vegetables and herbs listed in this study

Common Name	**Scientific Name**	**Common Name**	**Scientific name**
Amaranth (Purple)	*Amaranthus tricolor*	Lycium leaf	*Lycium chinense*
Amaranth (Green)/Yen Choy	*Amaranthus* spp.	Malabar spinach	*Basella alba "Rubra"*
Swiss Chard	*Beta vulgaris cicla*	Nightshade	*Solanum nigrum*
Chinese broccoli	*Brassica oleracea var. alboglabra*	Papalo	*Porophyllum ruderale*
Chives & flowers	*Allium schoenoprasum*	Potherb mustard/Mizuna	*Brassica rapa var. lacinifolia*
Culantro	*Eryngium foetidum*	Purslane/Verdolaga	*Portulaca oleracea*
Dandelion greens	*Taraxacum officinalle*	Radish greens	*Raphanus sativas*
Epazote	*Chenopodium ambrosioides*	Roselle/Indian sorrel	*Hibiscus sabdariffa*
Fenugreek	*Trigonella foenum-graecum*	Shanghai bok hoy	*Brassica rapa var. chinensis*
Garland chrysanthemum	*Chrysanthemum coronarium*	Spanish oregano	*Plectranthus amboinicus*
Garlic chives	*Allium tuberosum*	Spinach	*Spinacea oleracea*
Indian sorrel spinach	*Rumex vesicarius* spp.	Sugar Pea tops/bean	*Pisum sativum*
Lambsquarter	*Chenopodium album*	Tarragon	*Artemisia dracunculus*
Lemon balm	*Melissa officinalis*	Turmeric	*Curcuma longa*
Lemon verbena	*Aloysia triphylla*	Vine vegetables	*Cucurbita* spp.
Lettuce/Lechuga	*Lactuca sativa*	Wild garlic	*Allium vineale*
Lippia	*Lippia graveolens*		

Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 167-173
DOI: 10.29136/mediterranean.1095291

Assessment of water stress effects on red beet under the Mediterranean conditions

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ARTICLE INFO

Received: March 29, 2022
Received in revised form: July 18, 2022
Accepted: September 28, 2022

***Keywords*:**

Red beet
Water stress
Water use efficiency
Yield
Yield response factor

ABSTRACT

Although there are numerous scientific data on the response of various plants to water stress, there are few studies on red beet in the literature, and non-specifically under the Mediterranean conditions. This study aimed to investigate the effects of water stress (WS) levels (control-WS_0, low-WS_{20}, medium-WS_{40}, high-WS_{60}, and extreme-WS_{80}) on water use, growth, yield parameters, and yield response factor of red beet (*Beta vulgaris*) in Mediterranean conditions. During the growing season, the highest daily evapotranspiration values were 3.7, 2.8, 2.1, 1.4, and 0.7 mm for the control treatment, low, medium, high, and extreme water stresses, respectively. Soil salinity, plant height, fresh leaf yield, and storage-root yield values were decreased as water stress increased. However, there were no significant differences in soil pH, taproot length, and plant water use efficiency between treatments. Significantly important strong- or moderate-positive linear correlations were observed between soil salinity, evapotranspiration, plant height, fresh leaf yield, and storage-root yield values. The yield response factors for red beet storage-root and fresh leaf yields were found to be 0.88 and 0.98, respectively. The results revealed that red beet is slightly tolerant to water stress, with comparatively lower storage-root and fresh leaf yield reductions under the reduced evapotranspiration caused by water stress.

1. Introduction

Abiotic stresses, such as high levels of light, radiation, temperature, water (drought, flooding, and submergence), chemical factors, salinity, essential nutrients, gaseous pollutants, and mechanical factors (Pereira 2016), are the leading causes of crop failure worldwide, limiting average yields by more than 50% (Wang et al. 2003) or as much as 70% (Boyer 1982). Many food crop yields will continue to decline in the future, due to the reduced water supplies and increased global warming trends and climate change in many locations (Lobell et al. 2011). According to Hasanuzzaman et al. (2013), worldwide crop production is expected to decrease around 30% by 2025 compared to current productivity.

Due to their great magnitude of impact and global spread, drought and salinity are two of the most important abiotic stresses (Bartels and Sunkar 2005; Shrivastava and Kumar 2015). More than a quarter of the world's land area is believed to be dry, with roughly a third of the world's cultivable land suffering from water scarcity (Kirigwi et al. 2004). Water extraction by root and water transport within the plant are reduced when soil moisture is consistently low, and a drought-like state prevails. Plants respond to drought stress by improving water extraction efficiency and root water usage efficiency while simultaneously lowering transpiration rates. These two abiotic stresses, in general, cause dehydration or osmotic stress by reducing the availability of water for important cellular activities and turgor pressure maintenance.

Irrigation is critical for food security, employment, and economic development in semi-arid regions of the world, particularly in the Mediterranean region. In such places, it is necessary to irrigate more areas using limited irrigation practices. Because plants' ability to endure water stress varies between species and among populations of the same species, understanding the water-yield function, which is the crop yield response to water stress, is critical in determining and evaluating reduced irrigation management applications (Doorenbos and Kassam 1986; Allen et al. 1998).

Despite extensive research on the response of practically all cultivated plants to water stress, there is little information in the literature about red beet as a vegetable. Therefore, the growth and yield parameters, evapotranspiration, water use efficiency, and water-yield response function of red beet were investigated in this study under various water regimes.

2. Materials and methods

2.1. Experimental site

The experiment was carried out under a polyethylene-covered rain-out shelter with uncovered sides at the Agricultural Research and Implementation Area of Akdeniz University, in Antalya, Turkey. The experimental area with an average altitude of 54 meters is located at 36° 53' 15" north latitude and

30° 38' 53" east longitude. The Mediterranean climate prevails in the area, with hot, dry summers and mild, wet winters. The long-term annual average temperature is 18.8°C, with the lowest average temperature of 10.0°C and a temperature difference (T_{max} - T_{min}) of 8.9°C in January and the highest average temperature of 28.4°C with a temperature difference of 11.4°C in July. The total annual precipitation is 1059 millimeters, 538 millimeters falling between January and April, 61 millimeters between May and September, and 460 millimeters between October and December (Anonymous 2021).

2.2. Plant material

The cultivar *Beta vulgaris* var. *Conditiva* Alef. was used as a red beet plant material in the experiment. The plants' taproot can grow to a depth of 30-40 cm. It grows best in well-drained loam, sandy, or clayey loam soils at 15-18°C as a cool climate vegetable. This plant consumed the most water during the period that storage-root began to develop. Fresh leaves have higher levels of K, Mg, Na, P, and vitamin A and C than storage roots. Although fresh beet leaves are used as a filling ingredient of pastry, the storage roots are the most commonly consumed part of the plant, whether as a canned or pickled (Şalk et al. 2008).

2.3. Experimental design and treatments

The experimental design was a randomized complete block with four replications per treatment. There were four water stress (WS) levels including WS_{20} (low stress) WS_{40} (medium stress), WS_{60} (high stress), and WS_{80} (extreme stress), in addition to the control treatment (WS_0). To remove large particles, the soil used in the experiment was sieved with a 4 mm screen. A 33 kg air-dried soil was placed in lysimeter pots with a capacity of 36 dm^3. Table 1 shows the properties of the experimental soil used in the experiment.

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

Physical Properties			
Particle size distribution		Soil water contents (dry weight basis)	
Sand (%)	58.7	Saturation (%)	31.9
Silt (%)	20.7	Field capacity (%)	16.0
Clay (%)	20.6	Wilting point (%)	9.0
Bulk density (g cm^{-3})	1.4		
Chemical Properties			
Electrical cond. (paste) (dS m^{-1})	0.4		
pHe (paste)	7.7		

Before the experiment began, the soil in each lysimeter was saturated with tap water and then covered to prevent evaporation. After the lysimeters' drainage stopped, the weights were assumed to be field capacity. Similarly, weight of the lysimeters at wilting point were calculated by using the wilting point of the experimental soil given in Table 1. Throughout the experiment, all treatments were irrigated when 45 to 55% of available water in the control treatment was utilized. To keep track of the soil water status, replications of the control treatment were weighed every other day. The amount of applied irrigation water (AIW) was calculated by using the equation (1) (Duzdemir et al. 2009a, 2009b; Cemek et al. 2011; Kurunc et al. 2011; Ünlükara et al. 2015; Hancioglu et al. 2020):

$$AIW = \frac{W_{fc} - W_a}{\rho_w} xP_I \quad (1)$$

where: W_{fc} and W_a are the weights of lysimeter at field capacity and right before irrigation (kg), ρ_w is bulk density of water (1 kg L^{-1}) and P_I is the water application rate, which is 1.0, 0.8, 0.6, 04, and 0.2 for WS_0, WS_{20}, WS_{40}, WS_{60}, and WS_{80} treatments, respectively. To capture possible drainage water, a drainage container was placed under each lysimeter pot. After each irrigation practice, the amount of drainage water volume, (if any) was measured as leachate and considered in the calculation of crop evapotranspiration.

At the end of October, three red beet seeds were sown directly into each lysimeter pot and 1.5 L of water was applied. One month after sowing, two seedlings were removed and only one seedling remained in each pot, then the experiment was initiated by saturating all treatments. After the experiment was initiated, 5 irrigation practices were realized during the experimental period. Irrigation practices were performed in intervals between 11- to 21-days. To meet the plant nutrition needs, 3.45 g of potassium nitrate and 2.9 g of MKP (mono-potassium phosphate) were applied to each lysimeter at the beginning of the experiment and 0.7 g of ammonium nitrate 1.5 months later (Şalk et al. 2008).

2.4. Analyses and measurements

The amount of crop evapotranspiration (ET_v) between two-sequenced irrigation applications was calculated by using the following water balance equation (2):

$$ET_v = \frac{(W_n - W_{n+1})}{\rho_w} + (AIW - DW) \quad (2)$$

where: W_n and W_{n+1}, are the weights of lysimeter before n^{th} and $n+1^{th}$ irrigation application (kg), ρ_w is bulk density of water (1 kg L^{-1}) and AIW and DW are amounts of applied and if any drainage water (L). The daily ET_d (mm day^{-1}) was calculated by ET_v divided to the surface area of soil in the lysimeter and the number of days between the two-sequenced irrigation applications.

Plant heights were measured on a weekly basis. At the end of February, the harvested plants were cleaned, leaves and storage roots were weighed and the taproot lengths were measured in the laboratory. Soil samples were taken from the lysimeters immediately after the harvest. These samples were air-dried and sieved. Saturation extracts were obtained from saturated soil pastes, then electrical conductivities of the extracts (EC_e) and pH values (pH_e) were measured by using an EC and pH meter (Richards 1954; Carter et al. 2007).

2.5. Water use efficiency and yield response factor

The water use efficiency, or the amount of consumed water to produce one-unit storage-root yield, was calculated by using the equation (3):

$$WUE = \frac{Y}{ET_s} \quad (3)$$

where: Y is the fresh leaf yield or storage-root yield (g) and ET_s is seasonal evapotranspiration (mm $season^{-1}$).

The response of yield to the water supply was quantified through the yield response factor (k_y) by using the following water production function equation (4) (Stewart and Hagan 1973);

$$k_y = \left(1 - \frac{Y_a}{Y_m}\right) \Big/ \left(1 - \frac{ET_a}{ET_m}\right) \quad (4)$$

where: Y_m and Y_a are the maximum and actual storage-root yields (g), ET_m and ET_a are the maximum and actual seasonal evapotranspiration (mm season^{-1}) from the control (non-stress) and water stress treatments, respectively (Doorenbos and Kassam 1986).

2.6. Statistical analysis

SPSS statistical analysis software (IBM SPSS Inc. 2012) was used to analyze the obtained data at $P<0.01$ significance level. Where appropriate, mean separations of the data were realized by the Duncan test at a $P<0.05$ level of significance. Considering correlation coefficient (r) values, the strengths of the linear relationships between investigated parameters were evaluated as strong ($r \geq 0.8$), moderate ($0.5 < r < 0.8$), and weak ($r \leq 0.5$) (Peck and Devore 2012).

3. Results

Table 2 shows the statistical analysis results for the studied parameters including evapotranspiration; electrical conductivity and pH of saturated paste extract plant height; taproot length, fresh leaf weight, storage-root yield, and irrigation water use efficiency. In general, soil pH_e, taproot length, and water use efficiency values were not affected by the water stress treatments. However, soil EC_e ($P<0.05$) and evapotranspiration, plant height, fresh leaf yield, and storage-root yield values ($P<0.01$) showed significant differences between treatments.

3.1. Soil salinity and pH

The highest EC_e value was determined for the control (0.63 dS m^{-1}), low stress (0.61 dS m^{-1}), and medium stress (0.54 dS m^{-1}) treatments, whereas the lowest value was observed for extreme stress treatment (0.47 dS m^{-1}), which did not differ statistically from the high and medium stress treatments (Table 2). Compared to the extreme water stress treatments, the increases in EC_e values for low stress and control treatment were calculated to be 30 and 34%, respectively. Despite the fact that pH_e levels ranged from 8.00 to 8.13, there was no significant difference observed between treatments (Table 2).

3.2. Crop evapotranspiration

Throughout the experiment, changes in daily ET values (mm day^{-1}) of the treatments were calculated and are presented in Figure 1. In general, daily ET values for all treatments showed increased in the middle of the growing season around the third irrigation application and then decreased. As expected, the daily water consumption values for the control treatment were the highest during the whole growing season, but the lowest for the extreme water stress treatment. The highest daily ET values obtained in the middle of the growing season were 3.7, 2.8, 2.1, 1.4, and 0.7 mm for the control treatment, low, moderate, and extreme water stresses, respectively. The greatest variation in daily plant water consumption was again observed for the control while the smallest change occurred under extreme water stress (Figure 1).

According to the statistical analysis results, the seasonal ET values of red beet were strongly affected by water stress levels at the 0.01 probability level. Seasonal ET values were calculated as 241 (WS_0), 187 (WS_{20}), 140 (WS_{40}), 93 (WS_{60}), and 47 (WS_{80}) mm and they were significantly different from each other (Table 2). Compared to the control treatment, seasonal ET values for the low, medium, high, and extreme water stress treatments were reduced by 22, 42, 61, and 81%, respectively.

3.3. Growth and yield parameters

Throughout the growing season, changes in red beet plant heights under varied water stress levels were presented in Figure 2. During the first two weeks of the experiment, there was no significant difference in plant heights between treatments. Following this, differences in plant heights began, particularly for the high and extreme water stress treatments and the plants in these treatments remained stunted. At the end of the experiment, the control treatment had the highest average plant height (41.3 cm), but it was not substantially different from the low (38.0 cm) and medium stress (36.8 cm) treatments. On the other hand, it can be said that high and extreme water shortages in irrigation levels caused significant decreases in plant height (Table 2 and Figure 2). Compared to the control treatment, decreases in plant heights for WS_{60} and WS_{80} treatments were calculated as 35% and 45%, respectively.

Table 2. Effect of water stress on water use, growth, yield parameters of red beet

Analysis	Water stress levels					P>F
	WS_0	WS_{20}	WS_{40}	WS_{60}	WS_{80}	
Sat. paste extract EC_e (dS m^{-1})	0.63$^{\#}$ a$^{£}$	0.61 a	0.54 ab	0.50 b	0.47 b	*
Sat. paste extract pH_e	8.02	8.10	8.13	8.02	8.00	ns
ET (mm season^{-1})	241 a	187 b	140 c	93 d	47 e	**
Plant height (cm)	41.3 a	38.0 a	36.8 a	27.0 b	22.8 b	**
Tap root length (cm)	16.0	15.3	15.0	13.0	11.3	ns
Fresh leaf yield (g plant^{-1})	134.7 a	116.5 a	79.3 b	49.0 bc	29.0 c	**
Storage-root yield (g plant^{-1})	169.0 a	157.0 ab	116.8 bc	82.5 c	34.8 d	**
Water use efficiency (g mm^{-1})	0.70	0.84	0.83	0.89	0.74	ns

#: each value is the mean of four replications; £: within rows, means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.05 significance level; **: significant at the 0.01 probability level; *: significant at the 0.05 probability level; ns: non-significant.

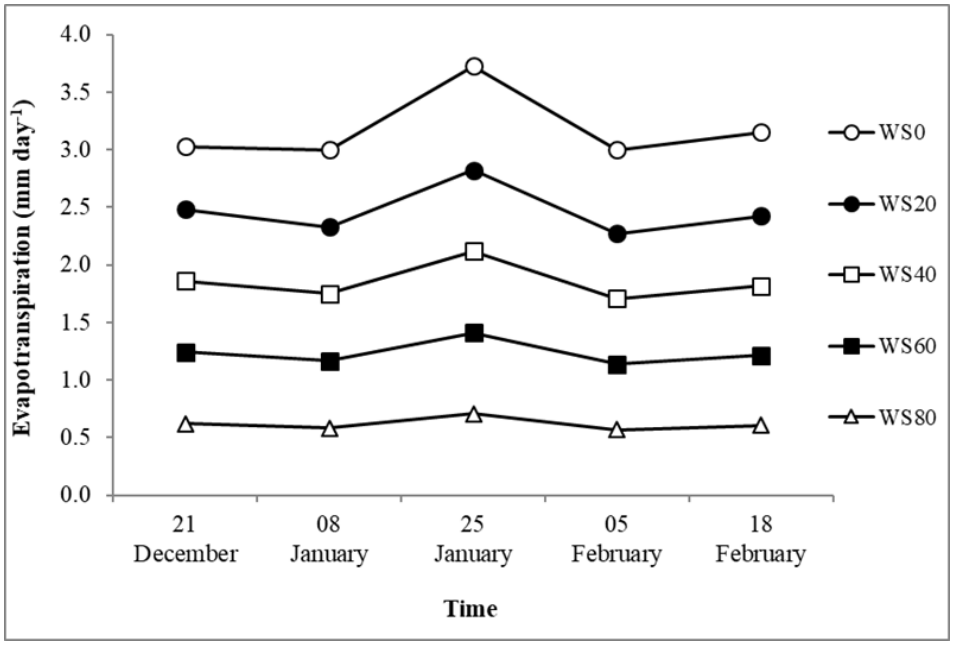

Figure 1. Changes on daily ET of red beet throughout the growing season.

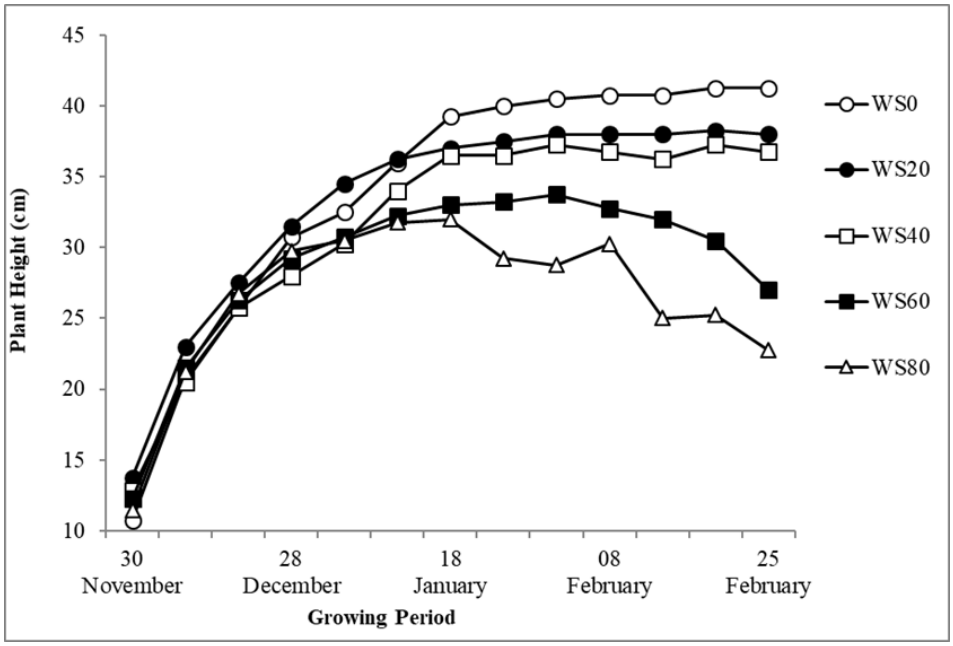

Figure 2. Changes on red beet plant heights throughout the growing season.

The statistical analyses revealed that increasing or decreasing water stresses did not cause a significant difference in red beet taproot lengths. On the other hand, fresh leaf and storage-root yields were significantly influenced by water stress levels (P<0.01). In general, increased water stresses resulted in considerable reductions in fresh leaf and storage-root yields. For these two yield parameters, the highest value was obtained from the control (134.7 and 169.0 g plant^{-1}, respectively) and low stress (116.5 and 157.0 g plant^{-1}, respectively) whereas the lowest value was observed from the extreme stress (29.0 and 34.8 g plant^{-1}, respectively) treatment, but the fresh leaf yield value was not significantly different from that of the high water stress treatment (49.0 g plant^{-1}) (Table 2). Considering the control treatments, calculated decreases were 41, 64, and 88% in fresh leaf yields and 31, 51, and 79% in storage-root yields for the medium, high, and extreme water stresses, respectively.

3.4. Plant water use efficiency and yield response factor

The statistical analysis showed that water stress levels had no effect on red beet WUE, despite the fact that they ranged from 0.70 to 0.89 g mm^{-1}. The relative evapotranspiration deficit (1–ETa/ETm) corresponding to the relative yield decrease (1–Ya/Ym) for each replication of the experiment was plotted to determine k_y values for fresh leaf and storage-root yield parameters. As shown in Figure 3, there are strong correlations between relative evapotranspiration deficit versus relative fresh leaf yield (R^2= 0.98) and storage-root yield (R^2= 0.94). The

calculated k_y coefficients were 0.98 and 0.88 for fresh leaf and storage-root yields, respectively.

3.5. Relationship between parameters

Table 3 shows the statistical evaluation (*r* and *P* values) of the linear relationships between parameters. There were significantly important ($P<0.01$) strong-positive linear correlations between ET values versus plant height, fresh leaf yield, and storage-root yield values; plant height values versus fresh leaf and storage-root yield values; fresh leaf yield values versus storage-root yield values. Similarly, there were moderate-positive linear correlations between soil EC_e values versus ET, plant height, fresh leaf yield ($P<0.05$) and storage-root yield values; ET values versus taproot length values ($P<0.05$); taproot length values versus fresh leaf yield values ($P<0.05$) were observed. However, neither the soil pH_e nor the WUE values showed a strong or moderate linear relationship with any of the other parameters (Table 3).

4. Discussion

In this study, the effects of water stress on growth (plant height and taproot length), yield parameters (fresh leaf and storage-root yields), water consumption, and water use efficiency, of red beet were investigated. Although the salinity of the irrigation water used in all treatments was the same, the soil salinity values showed statistically significant differences between them. This is because the salinity in the crop root zone may have increased due to an evapo-concentration process driven by ET under non-leaching conditions in the soil because pure water is evaporated from the wet soil surfaces and is transpired from crop leaves and the amount of salt taken up by the plants is negligible in comparison to the amount of salt in the soil and that added by irrigation water (Hanson et al. 2006). Duzdemir et al. (2009a, 2009b), Kurunc et al. (2011) and Ünlükara et al. (2015) claimed that if salts are not leached out of the crop root zone, the amount of salt delivered to the soil increases as the amount of applied water increases depending on the salt concentration of irrigation water. They also reported that EC_e values were higher in control treatments with more water was delivered to the soil than in limited water treatments, as expected.

Initial, crop development, mid-season, and late-season are the four key stages of a typical K_c curve. The K_c coefficient increases with increasing plant growth during the crop development period then becomes stationary in the mid-period and subsequently drops till the harvest (Allen et al. 1998). However, in this experiment, the K_c curve did not have a stable pattern in the mid-season, because the number of irrigation

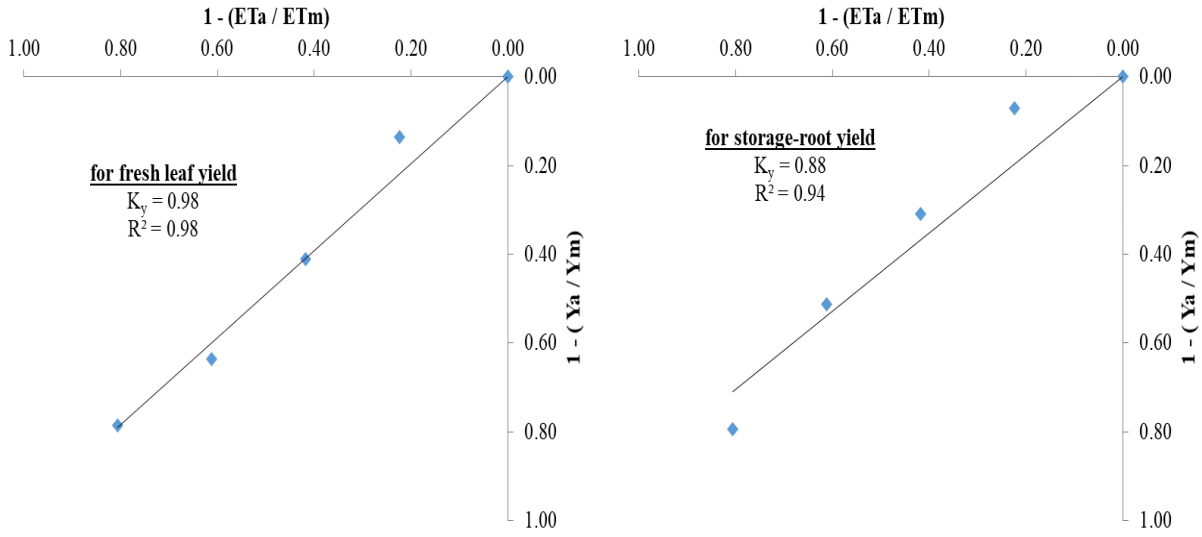

Figure 3. Yield response factors for storage-root and fresh leaf yields of red beet.

Table 3. Relationship between investigated parameters

	EC_e	PH_e	ET	PH	TRL	FLY	SRY
pH_e	0.29^{ns}						
ET	0.67**	0.19^{ns}					
PH	0.61**	0.36^{ns}	0.88**				
TRL	0.34^{ns}	-0.01^{ns}	0.54*	0.34^{ns}			
FLY	0.55*	0.16^{ns}	0.94**	0.88**	0.53*		
SRY	0.64**	0.26^{ns}	0.90**	0.81**	0.44^{ns}	0.81**	
WUE	-0.13^{ns}	0.07^{ns}	-0.12^{ns}	-0.09^{ns}	-0.23^{ns}	-0.22^{ns}	0.28^{ns}

EC_e: Electrical cond. of soil saturated paste extract; pH_e: pH of soil saturated paste extract; ET: evapotranspiration; PH: plant height; TRL: tap root length; FLY: fresh leaf yield; SRY: storage-root yield; WUE: water use efficiency; **: significant at the 0.01 probability level; *: significant at the 0.05 probability level; ns: non-significant.

practices throughout the season was limited to 5. The changes in daily evapotranspiration and K_c curves of the water regime treatments can be seen in Figure 1. As a result, it can be claimed that red beet, as a late autumn plant, exhibits a partially conventional K_c use curve.

In a study, investigating the effects of three different water application levels (100%, 50%, and 30%) on red beet, Stagnari et al. (2014) found that storage-root and dry leaf yields decreased with increasing water stress. They calculated that, compared to the control, the reductions in dry storage-root yield were 62 and 75%, whereas in leaf dry yields 45 and 69% for 50 and 30% stress treatments respectively. Our findings (respectively 31, 51, and 79% decreases in storage-root and similarly 41, 64, and 88% reductions in fresh leaf yields for 60, 40, and 20% water applications) are consistent with Stagnari et al. (2014). These findings reveal that a significant reduction in irrigation water has a negative impact on the red beet plant's growth and yield parameters. It was shown that increased water stress adversely affected the growth and yield parameters of different plants such as cowpea (Duzdemir et al. 2009b), bell pepper (Kurunc et al. 2011), and long pepper (Ünlükara et al. 2015). The decrease in growth and yield parameters could be attributed to biomass production primarily taking place in the roots under water stress conditions (Albouchi et al. 2003) or a decrease in chlorophyll content and hence photosynthetic activity (Viera et al. 1989).

The yield response factor is used to assess a plant's water stress tolerance (Doorenbos and Kassam 1986). If $k_y \leq 1$, the plant is tolerant to water stress; otherwise it is sensitive. In this study, the yield response factors for storage-root and fresh leaf yields were determined to be 0.88 and 0.98, respectively. It may be inferred that red beet is slightly tolerant to water stress, with comparatively lower yield reductions when water consumption is reduced due to stress. Stagnari et al. (2014) stated that red beet plants can show high adaptation to water stress based on changes in growth and physiological characteristics that modify the yield and quality.

5. Conclusions

In this study, the effects of irrigation water regime on growth (plant height and taproot length), yield parameters (fresh leaf and storage-root yields), and irrigation water use efficiency of red beet plant were investigated. In general, increasing water stress significantly decreased soil salinity, plant height, fresh leaf yield, and storage-root yield, but had no influence on soil pH, taproot length, and plant water use efficiency. Under high water stress, the smallest variation in daily plant water consumption was recorded, whereas the largest change occurred under the control treatment. Significantly important strong- or moderate-positive linear correlations were found between soil salinity, evapotranspiration, plant height, fresh leaf yield, and storage-root yield values; however, soil pH_e and WUE values showed no strong or moderate linear relationship with any of the other investigated parameters. The yield response factors for fresh leaf and storage-root yields were found to be 0.98 and 0.88, respectively, indicating that the red beet plant is slightly tolerant to water stress.

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Research Article

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

MEDITERRANEAN AGRICULTURAL SCIENCES
(2022) 35(3): 175-181
DOI: 10.29136/mediterranean.1067571

Morphological, physiological, cytological characteristics and agricultural potential of colchicine induced autotetraploid plants in safflower

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ARTICLE INFO

Received: February 3, 2022
Received in revised form: July 22, 2022
Accepted: July 23, 2022

***Keywords*:**

Carthamus tinctorius
Autotetraploidy
Colchicine induction
Flow cytometer
Trait evolution

ABSTRACT

Safflower (*Carthamus tinctorius* L.) is one of the important oilseed and bio-energy crops. All of the safflower cultivars in the world have diploid genomes (2n= 2x= 24). In this research, autotetraploidy induction in safflower was performed by colchicine treatments to the emerging shoot tips at the cotyledonary stage. As a result of flow cytometric analyses performed in the C_2 progenies, autotetraploids (4x= 48) had DNA content of 4.88 pg $2C^{-1}$, while diploids (2x= 24) had 2.29 pg $2C^{-1}$. The autotetraploids in C2 generation exhibited bigger stomata size (33.40 µm to 46.90 µm in length) and a higher chloroplast number (9.5 to 17.2 in the guard cells), but less stomatal density (17.98% to 16.67% in index) compared to their diploid counterparts. However, autotetraploidy reduced the pollen viability from 80.24% to 16.20%, and seed set rate from 35.06% to 7.01% per capitula. As a result, autotetraploid plants were able to produce very few seeds despite the high unit seed size and weight in their heads. While oil content of the large-seeded autotetraploids was significantly lower, by two-fold, (26.37% to 13.23% in the whole seeds) than the small-seeded diploids, fatty acid composition was not significantly influenced by autopolyploidization.

1. Introduction

Safflower is one of the oldest cultivated annual crops in semi-arid and arid regions of the world for its seeds rich in omega fatty acids and flowers with high dyeing power. *Carthamus tinctorius* L., the only cultured species of safflower, carries the diploid genomes of its putative wild diploid progenitors, *C. oxyacanthus* Bieb (Knowles 1969) or *C. palestinus* Eig (Chapman and Burke 2007) without polyploidy. Hence all safflower varieties cultivated today have a diploid chromosome number (2n= 2x= 24) (Estilai 1971; Sheidai et al. 2009; Uysal et al. 2018). Although an *in vitro* study was carried out by Moghbel et al. (2015) to obtain artificial polyploid safflower, the results obtained from this research remained only at the experimental level.

Polyploidy refers to the presence of more than two complete sets of chromosomes per cell nucleus (Simmonds 1980). Polyploid plants carry multiple copies of alleles which can help increase allelic diversity and confer various evolutionary and adaptive advantages in comparison with diploid plants (Parisod et al. 2010). Based on numerous studies on artificially induced polyploidy by a potent mitotic inhibitor colchicine after the first treatment by Blakeslee and Avery (1937), it is most likely expected to be an enlargement or increment in both vegetative and generative cells, tissues and organs of the putative polyploids with a phenomenon described as gigas effect (Sattler et al. 2016).

However autoploidy induction can lead to high rates of multivalent pairing during meiosis and an associated reduction in fertility due to the production of aneuploid gametes (Acquaah 2012). Therefore autopolyploidy breeding is usually applied to the crops cultivated for their vegetative organs and those with vegetative propagation, due to the low rates of viable pollen and seed production (Levin 1983; Paterson 2005; Wang et al. 2016). The reduction in fertility is a hindrance to the use of induced autopolyploids, especially when the organs of interest are reproductive such as fruits or seeds (Dewey 1980). For example, incomplete or weak seed set remains the main constraint to the utilization of autotetraploid barley (Evans and Rahman 1990) and rye (Pfahler et al. 1987).

Carthamus tinctorius L. has less chromosome number (2n= 2x= 24) and haploid genome size (1.07 Gb) than many other plant species (Wu et al. 2021). The aim of the study was to establish an efficient system for generation of autotetraploid *Carthamus tinctorius* plants through colchicine induction and to identify their phenological, morphological, physiological, cytological and agricultural characteristics compared with its diploid parent. The findings from our study presented in this paper are also expected to be a fruitful resource for extensive investigation on evolutionary plant breeding.

2. Materials and Methods

2.1. Plant material

The seeds of a safflower (*Carthamus tinctorius* L.) cultivar "Olein" registered by the first author's breeding team in 2019 were used as genetic material. This diploid cultivar (2n= 2x= 24) was characterized with spiny capitulum, red flower color and oleic acid type.

2.2. Colchicine treatments and induced autopolyploidy

Safflower seeds were immersed in 70% ethanol for 1 min, followed by 10 mins of 10% NaOCl and then rinsed three times with sterile distilled water. The sterilized seeds were sown carefully in plastic seedling trays filled with sterile peat and grown in the growth chamber at 25±1°C until the emergence of cotyledeonal leaves. Small cotton swabs soaked in aquaepus colchicine solutions at the concentrations of 0% (used only pure water), 0.25%, and 0.5% (w/v) were placed on the emerging apical tip between two cotyledonary leaves, and the solution added in drops at regular intervals for 3 days, with 6 hours duration each day according to the cotton swab method explained in detail by Kushwah et al. (2018).

Colchicine treated and untreated (control) seedlings were transplanted in plastic pots with a 3:1 mixture of peat and perlite and cultured in a greenhouse under relatively consistent environmental conditions. From each colchicine treatments, 80 healty seedlings, at 4-6 true leaf stage, were selected and then transplanted to the experimental field on 22 March 2020. A total of 22 colchicine treated plants survived at only 0.25% concentration in C_1 generation, these were self-pollinated during the flowering period and then harvested at maturity stage for C_2 generation. The seeds of the individual C_1 plants were separately sown in 22 rows with 4 m length at 50x25 cm spacing on 29th April 2021. Thus diploid and putative autotetraploid plants were grown in the rows side by side based on the practices recommended for safflower cultivation. Each row was represented by 10 plants for identification and confirmation of autopolyploidy in C_2 generation.

2.3. Identification of autotetraploids by flow cytometric analysis

The ploidy levels and nuclear DNA contents of C_2 plants and their diploid parent "Olein" were detected by using a flow cytometry (CyFlow® Ploidy Analyser, Partec, Germany) equipped with an HBO lamp for UV. The fresh leaf tissues (0.5 cm^2) were evaluated for the flow cytometric analysis by using a commercial kit, the 'CyStain PI absolute P' nuclei extraction and staining kit (Partec GmbH, Munster) according to the manufacturer's instructions. The absolute core DNA content (pg) of the samples was calculated using the fluorescent intensities of the G1 peaks of the sample and the internal standard of common vetch (*Vicia sativa*). An ANOVA was performed on nuclear DNA content data by using the SPSS Ver. 15.0 software for Microsoft Windows (Nizam et al. 2020).

2.4. Confirmation of tetraploids by chromosome counting

Freshly grown root tips from the germinated seeds of diploid and autotetraploid plants, confirmed by flow cytometric analysis, were pretreated with 2 mmol L^{-1} of 8-hydroxyquinoline solution at 20°C for 2 h, and fixed in ethanol:acetic acid (3:1, v/v) at 4°C for 24 h. The fixed tips were then washed thoroughly in distilled water to remove the fixative solution, and hydrolyzed in 1 N HCl at 60°C for 5 min. After hydrolysis, root tips were stained with 2% aqueous aceto orcein (Sheidai et al. 2009; Uysal et al. 2018). The chromosomes at the metaphase plate were observed under an optical microscope (Zeiss Axiostar, Jena, Germany) with a 100x oil immersion objective.

2.5. Cytological observation of stomata, chloroplasts and pollens

The abaxial epidermis layers of diploid and autotetraploid leaves were observed for stomata measurements such as density, length and width (µm) through the light microscope (Nikon SE, Tokyo, Japan) at 400× magnification. The number of epidermis and stomata cells within a visual field of 0.196 mm^2 (objective 40×) were counted at the randomly selected 10 unit areas per leaf sample (Boso et al. 2016). Stoma index (SI) as a percent was calculated by the following formula: SI= [(Number of stomata per unit area) / (Number of stomata per unit area) + (Number of epidermal cells per unit area)] × 100 (Meidner and Mainsfield 1969). After the abaxial epidermal layers were placed on a microscope slide into a drop of 1% $AgNO_3$ with a cover glass, chloroplasts were counted in 10 pairs of stomatal guard cells at 400× magnification (Monakhos et al. 2014).

The length and width (µm) of pollen grains from the diploid and autotetraploid flowers were recorded on a microscope slide with the help of an ocular micrometer at 400x magnification (Pei et al. 2019). The Hemocytometric Thoma slide was used to estimate the pollen number per anther by way of a method explained in detail by Eti (1990). Pollen viability was carried out using 1% 2,3,5-triphenyltetrazolium chloride (TTC) stain. Among the pollen grains that were kept in a few drops of TTC on a slide for 3-4 hours, the reddish stained ones were recorded as viabile (Norton 1966).

2.6. Analysis of physiological, morphological and agricultural characteristics

In order to determine the leaf chlorophyll density at the rosette and anthesis stages, SPAD measurements with Minolta SPAD-502 (Chlorophyll Meter, Minolta Co. Ltd., Japan) were made on 10 randomly selected leaves from diploid and autotetraploid plants (Jiang et al. 2017). On the basis of changes in morphological and agricultural characteristics including plant height (cm) stem thickness (mm), leaf length and width (cm), corolla tube length (mm), flower weight per capitula (g), caputula number per plant, capitula size (cm), seed size (mm), seed weight per plant (g),1000 seed weight (g), seed hull thickness (mm) and seed hull content (%) were determined in both diploid and autotetraploid plants.

2.7. Determination of seed oil content and fatty acid composition

The seed oil content and fatty acid composition of diploid and autotetraploid plants were determined by Nuclear Magnetic Resonance (NMR, Bruker: mq one Total Fat Analyzer) and Gas Chromatography (GC-FID, Shimadzu GC-2025 with Teknokroma TR-CN100 capillary column) according to the methods described in detail by Erbas et al. (2016).

2.8. Statistical analysis

An analysis of Student's t-test (two sample t-test) was performed at the 5% significance ($P \leq 0.05$) level to compare diploid and autotetraploid plants (SAS 1998). Standard deviations (SD) of the means were calculated using Microsoft Excel 2010.

3. Results

As illustrated in Figure 1, two fold fluorescence intensity appeared in autotetraploid plants compared to that of G_1 nuclei of diploid plants. While diploid displayed a peak of DNA content at 518.0, the major peak representing the DNA content in autotetraploid was around 714.3. 2C nuclear DNA contents 2x and 4x plants were 2.29 and 4.89 pg $2C^{-1}$, respectively, suggesting that the induction of autotetraploidy was successful in *Carthamus tinctorius*. As a result of flow cytometric analyses performed in C_2 progenies of 22 putative C_1 plants, only one autotetraploid group, which was represented by 10 progenies, was detected in C_2 generation. So autopolyploidization efficiency in C_2 generation was 4.5% (1 of 22 C_1 plants from only 0.25% colchicine treatment).

Mitotic analysis confirmed that chromosome numbers in the somatic cells of diploids and autotetraploids were 2n= 2x= 24 and 2n= 4x= 48, respectively. However, counting 48 chromosomes in autotetraploids was very tedious due to the overlapping of chromosomes (Figure 1). Stomata, chloroplast and SPAD measurements were also performed for identification and confirmation of autopolyploidy as presented in Table 1. The autotetraploids in C_2 generation exhibited bigger stomata size (33.40 µm to 46.90 µm in length) and a higher chloroplast number (9.5 to 17.2 in the guard cells), but less stomatal density (17.98% to 16.67% in index) compared to their diploid counterparts ($P \leq 0.05$). Although autotetraploid plants had significantly higher leaf SPAD values at rosette leaf stage, the values at anthesis stage were not significantly different between both ploidy levels (Table 1).

When diploid and autotetraploid plants in C_2 generation were grown in the rows side by side in the same environmental conditions, autopolyploids exhibited thicker stems and leaves, longer and wider leaves, but produced a lower number of leaf per plant and the same height of the main stem compared to diploids (Table 2). Chromosome doubling caused 1.6 and 1.1 fold increase in leaf width and length, respectively as illustrated in Figure 2-C.

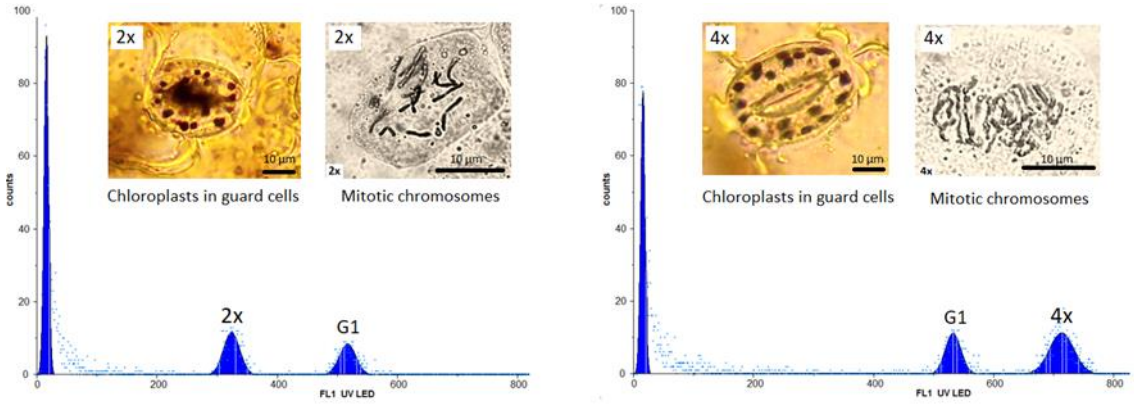

Figure 1. Flow cytometric histograms of the nuclear DNA contents, mitotic chromosomes in somatic cell and chloroplasts in guard cells in diploid (2n= 2x= 24) and autotetraploid (2n= 4x= 48) plants of *Carthamus tinctorius* L.

Table 1. Stoma and chloroplast characteristics, leaf SPAD values in diploid and autotetraploid plants

Characteristics	Ploidy levels		t- value
	Diploid (2x= 24)	Tetraploid (4x= 48)	
Stoma number in an unit area	23.30 ± 1.89§	10.20 ± 1.32	25.58*
Stoma index (%)	17.98 ± 0.89	16.67 ± 0.74	-4.73*
Stoma length (400x) (µm)	33.40 ± 2.91	46.90 ± 2.56	9.64*
Stoma width (400x) (µm)	24.80 ± 3.55	34.50 ± 2.07	10.69*
Chloroplast number in guard cells	9.50 ± 1.41	17.20 ± 1.40	13.13*
Leaf SPAD value at rosette stage	61.64 ± 4.54	83.51 ± 3.08	13.18*
Leaf SPAD value at anthesis stage	80.39 ± 4.63	87.34 ± 12.53	1.83

§ All values are means ± standard deviation (n= 10). * $P \leq 0.05$ according to Student's t-test.

Table 2. Certain plant and leaf characteristics of diploid and autotetraploid plants

Characteristics	Ploidy levels		t-value
	Diploid (2x= 24)	Tetraploid (4x= 48)	
Plant height (cm)	60.50 ± 3.02§	60.75 ± 6.41	0.09
Main stem thickness (mm)	7.66 ± 0.78	9.25 ± 1.04	2.91*
Leaf number per plant	24.63 ± 0.92	23.13 ±1.46	-5.85*
Leaf length (cm)	14.13 ± 1.43	15.58 ± 2.56	4.38*
Leaf width (cm)	4.51 ± 0.56	7.08 ± 0.38	10.12*
Leaf thickness (mm)	0.25 ± 0.06	0.35 ± 0.05	3.79*

§ All values are means ± standard deviation (n= 10). * $P \leq 0.05$ according to Student's t-test.

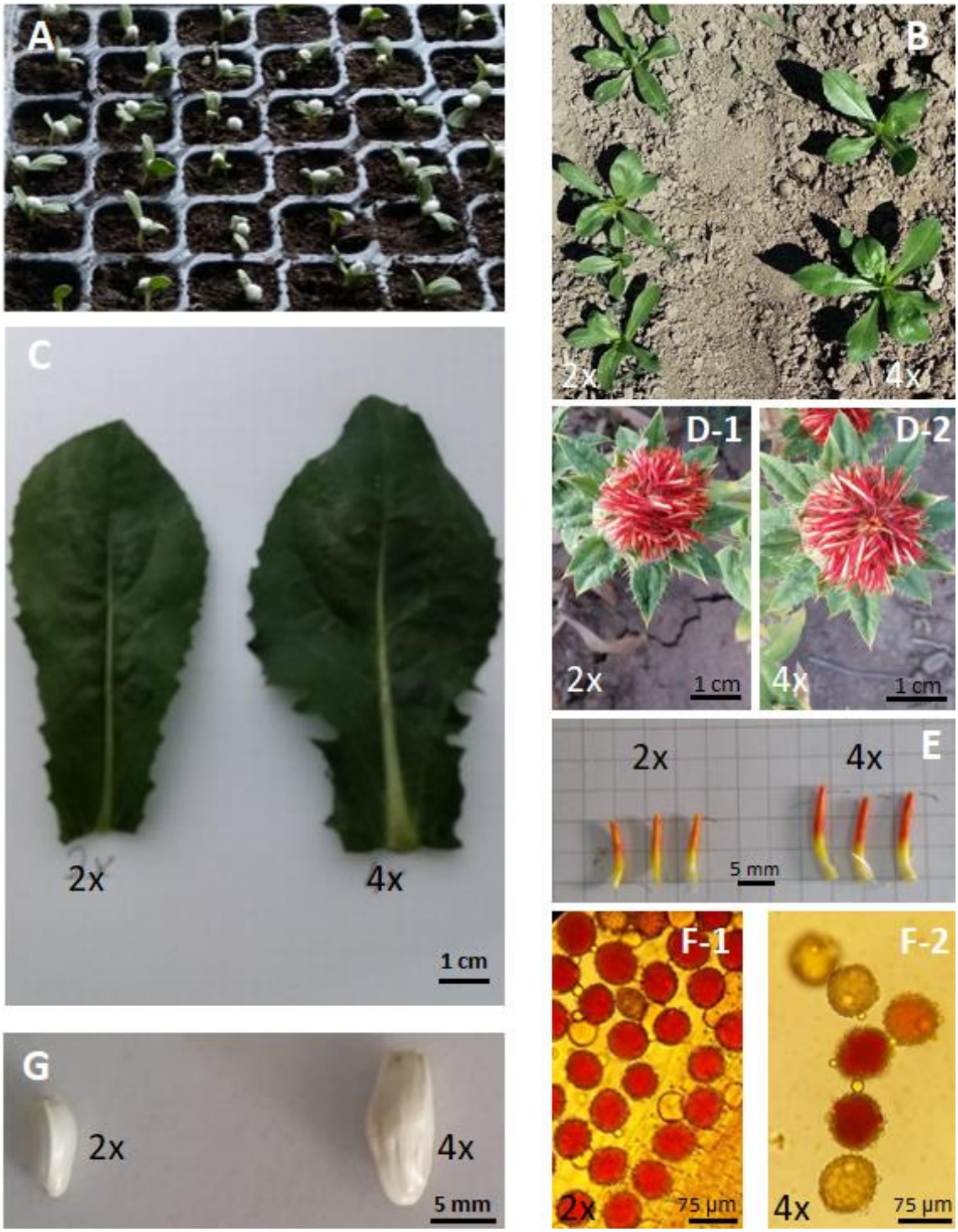

Figure 2. Cotton swap method (A), rosette growth stage (B), leaf size (C), capitulum size (D-1, D-2), corollo lenght (E), pollen size and viability in TTC test (F-1, F-2), and seed size (G) in diploid (2x) and tetraploid (4x) plants, respectively.

The autotetraploid plants remained longer in the rosette stage and started to bloom 2-3 days later than the diploids (Table 3). While there were not significant differences in capitulum number per plant (with an average of 5-6 heads) and flower number per capitulum (with an average of 100 flowers), the autotetraploid plants exhibited wider and longer flower parts. As a result, autotetraploid flowers had higher dry weight of flowers compared to diploids. For example, the length of corolla tube in the diploid and autotetraploid flowers was 12.43 mm and 16.80 mm, respectively (Table 3; Figure 2- E).

The pollen viability and productivity showed significant ($P \leq 0.05$) decreases with chromosome doubling in safflower (Table 3; F-1 and F-2 in Figure 2). The pollen viability as tested by 1% TTC was calculated as 80.24% for diploid and 16.20% for autotetraploid plants. In addition, the colchicine-induced autotetraploid plants showed significantly lower pollen grains per

anther than their diploids counterparts ($P \leq 0.05$). Another finding was that autoteraploid plants produced larger pollen than diploid plants (75.7 µm and 69.63 µm, respectively) (Table 3; F-1 and F-2 in Figure 2).

Although autotetraploid plants produced almost the same number but larger capitulum (D-1 and D-2 in Figure 2), diploid plants produced 79.04% more seeds and 93.15% more seed yield than autotetraploids (Table 4). Autotetraploid seeds were 2.7 times heavier, 1.5 times longer and 1.4 times wider than diploid seeds (Figure 2-G). In addition, higher hull (shell) thickness and lower seed germination rate of the autotetraploid safflower seeds were detected (Table 4).

The seed oil content of autotetraploid plants was significantly lower (26.37 to 13.23% in the whole seeds and 54.33 to 45.20% in the kernels) than the diploid ones ($P \leq 0.05$) (Table 5). The dramatic decrease in the oil content of large-seeded autotetraploids compared to small-seeded diploids was mainly due the the significant increase in the seed hull (achene shell) content (50.30 to 71.60%) (Table 4). However, there were not any significant differences among the fatty acid compositions of diploid and autotetraploid plants. The safflower oil contained two main unsaturated fatty acids, oleic and linoleic, which represent circa 90% of the total fatty acid content, while the remaining circa 10% corresponds to saturated fatty acids, palmitic and stearic. Oleic acid was the most abundant fatty acid which comprised 79.37% in the seed oil of diploid plants and 78.11% in the seed oil of autotetraploid plants. On the other hand, linoleic acid was found at the rates of 10.97% and 9.29% in the seed oils of diploid and autotetraploid plants, respectively (Table 5).

4. Discussion

Autopliploidization was successfully performed with colchicine treatment at 0.25% to the apical shoot tips between two cotyledonary leaves of the safflower seedlings. After confirmation of putative autotetraploids in C_2 generation, by way of flow ctytometric analysis and mitotic chromosome counting, polyploidization efficiency was only 4.5%. Autotetraploid genome (4x= 48) had about two fold higher DNA content than that of diploid genome (2x= 24). Autotetraploid safflower plants

Table 3. Certain floral characteristics of diploid and autotetraploid plants

Characteristics	Ploidy levels		t-value
	Diploid (2x= 24)	Tetraploid (4x= 48)	
First flowering days after sowing	59.6 ± 1.26[§]	62.8 ± 1.03	5.08*
Flower number per capitulum	97.01 ± 6.95	101.67 ± 7.64	0.64
Flower weight per capitula (g)	0.12 ± 0.02	0.18 ± 0.04	3.77*
Corolla tube length (mm)	12.43 ± 0.55	16.80 ± 0.10	7.74*
Pollen diameter (400x) (µm)	69.63 ± 3.93	75.7 ± 5.08	3.82*
Pollen viability (%)	80.24 ± 7.00	16.20 ± 6.30	-25.87*
Pollen number per anther	22000 ± 3771	9200 ± 2700	-7.56*

[§] All values are means ± standard deviation (n= 10). *$P \leq 0.05$ according to Student's t-test.

Table 4. Certain capitulum and seed characteristics of diploid and autotetraploid plants

Characteristics	Ploidy levels		t- value
	Diploid (2x= 24)	Tetraploid (4x= 48)	
Capitulum number per plant	5.13 ± 1.13[§]	5.63 ± 1.30	1.00
Capitulum diameter (cm)	2.53 ± 0.21	2.83 ± 0.10	3.38*
Seed number per capitula	34.01 ± 5.51	7.13 ± 4.02	-11.72*
Seed set rate (%)	35.06 ± 6.23	7.01 ± 5.49	-10.32*
Seed germination rate (%)	96.00 ± 3.27	66.67 ± 9.43	-3.19*
Seed yield per plant (g)	7.73 ± 2,63	0.53 ± 0.29	-7.10*
1000 seed weight (g)	27.75 ± 4.13	73.94 ± 24.64	5.19*
Seed length (mm)	7.10 ± 0.13	10.61 ± 0.50	20.51*
Seed width (mm)	3.94 ± 0.19	5.39 ± 0.48	7.89*
Seed hull content (%)	50.30 ± 4.04	71.60 ± 2.81	7.85*
Seed hull thickness (mm)	0.38 ± 0.02	0.75 ± 0.06	15.23*

[§] All values are means ± standard deviation (n= 10). *$P \leq 0.05$ according to Student's t-test.

Table 5. Seed oil and fatty acids content in diploid and autotetraploid plants

Characteristics	Ploidy levels		t- value
	Diploid (2x= 24)	Tetraploid (4x= 48)	
Oil content (%) in whole seed	26.37 ± 3.01[§]	13.23 ± 1.00	-13.03*
Oil content (%) in kernel	54.30 ± 2.56	45.20 ± 1.13	-7.81*
Palmitic acid (%)	7.27 ± 1.14	8.29 ± 0.37	1.37
Stearic acid (%)	1.56 ± 0.16	1.68 ± 0.35	0.49
Oleic acid (%)	79.37 ±3.10	78.11 ± 2.79	0.48
Linoleic acid (%)	10.97 ± 1.91	9.29 ± 1.59	-1.33
Linolenic acid (%)	0.81 ± 0.32	2.61 ± 1.42	2.25

[§] All values are means ± standard deviation (n= 10). *$P \leq 0.05$ according to Student's t-test.

had a smaller number of stomata per unit epidermal area and a greater number of chloroplasts in larger stomatal guard cells. These findings revealed that stoma size, stomata density and chloroplast number seem to be important morphological markers in order to distinguish ploidy levels. However, these morphological measurements may not always give accurate results to screen out pure tetraploids because of the chimeras (Jaskani et al. 2004). The SPAD value was not a reliable criterion to estimate the ploidy level in *Carthamus tinctorius*. To confirm this assumption, no significant differences were detected in the chlorophyll contents and net photosynthetic rates of diploid, autotetraploid and octoploid *Jatropha jurcas* plants (Niu et al. 2016).

Since autotetraploids produced similar main stem height and leaf number per plant, they exhibited more leaves, thicker stems and leaves plus longer and wider leaves. This result indicates that autopolyploidy promotes cell expansion rather than cell elongation in the leaves just as in the plant stem. The primary cell wall is largely composed of polysaccharides, such as cellulose, hemicellulose, lignin and pectin. A comparison of euploid series (2x, 4x, 6x, and 8x) in *Arabidopsis thaliana* showed that induced polyploidy had slower growth, enlarged cell size, and lower lignin and cellulose but higher pectin and hemicellulose in the stem (Corneillie et al. 2019). Autotetraploid safflower plants remained longer in the rosette stage, and started to bloom 2-3 days later than their diploid counterparts. Similar findings were obtained in the studies on colchicine-induced autotetraploid sunflower and barley (Evans and Rahman 1990, Srivastava and Srivastava 2002). It was expressed by Pei et al. (2019) that differences in endogenous phytohormone levels and flowering genes expression gave rise to delay flowering and bolting in polysomic tetraploids of radish (*Raphunus sativus*).

While there were not significant differences in capitulum number per plant and flower number per capitulum, autotetraploid flowers having larger and longer stamens and pistils had higher dry weight of flowers compared to diploids. Similarly, polyploid plants in *Arabidopsis thaliana* increased petal and sepal sizes in their flowers compared to diploids (Corneillie et al. 2019). Since safflower is a crop that is cultivated, not only for its seeds, but also for its flowers, autoteraploid varieties may be more advantageous than diploids in the safflower cultivation for only flower production.

Although colchicine-induced autotetraploid plants produced larger anthers and pollen grains, pollen numbers per anther were very low compared to diploids. On the other hand, the viability rates of the few pollens produced by tetraploids were also very low. Similarly, tetraploid plants of *Raphanus sativus* and *Helianthus annuus* had larger flowers and pollen grains, but lower pollen viability and germination rate than diploid plants (Limera et al. 2016; Srivastava and Srivastava 2002). The reduction in pollen fertility is a common consequence of autopolyploidy and may result from issues concerning the multivalent formation and meiotic irregularities (Acquaah 2012). Low pollen productivity and consequently low seed formation of autopolyploids can be eliminated by self-pollination and selection methods in advanced C generations (Baghyalakshmi et al. 2020; Singh 1992; Srivastava and Srivastava 2002).

As a result of the decrease in pollen number and viability by the autopolyploidy, it resulted in 80% lower seed set rate or seed forming efficiency. Similarly, the seed setting rate was reduced from 86% to 29% in sunflower autotetraploids due to the irregular pairings such univalents and multivalent of four homolog chromosomes during meiosis (Srivastava and Srivastava 2002). However, the seeds of autotetraploid safflower plants were heavier, longer and wider than the seeds of diploids. The seed weight of autotetraploids in *Ricunus communis* was also higher than their diploid counterparts (Baghyalakshmi et al. 2020).

In addition, the hull (shell) content and germination rate of achenes decreased with the chromosomal doubling in safflower. Perhaps the polysaccharides, such as cellulose, hemicellulose, lignin and pectin, that cause stem and leaf thickening caused similar thickening in the seed hull. Due to the high hull thickness and low seed germination rate of the large-seeded tetraploid plants, their seeds germinated on an average of 3 days later than the small-seeded diploids under the same field conditions. In fact, one reason for late flowering in the autotetraploid plants may be related to the thick-hulled large seeds which need more water absorption than the thin-hulled small seeds of diploid plants.

Safflower is primariliy a cultivated crop because of the high quality of edible oil in its seeds. Another area of interest of this research was to observe how the oil content and fatty acid composition would be in the seeds of the autotetraploids as compared to the diploid ones. Although autotetraploid plants produced a larger leaf area, higher chloroplast number and higher SPAD values, it was an unexpected result that their seed oil content was significantly lower by two fold in the whole seeds and 1.2 fold in the kernels than the diploid plants. This result is a considerable disadvantage for the industrial value of autotetraploid safflower. However, the fatty acid composition was not significantly affected by the autopolyploidization. Similarly, the autotetraploid plants of *Jatropha curcas* contained less oil in their seeds than diploids, mainly due to energy deficiency or unbalanced distribution (Niu et al. 2016). Since seed oil content has significant negative correlations with seed hull percentage (Rao et al. 1977) and seed size (Claassen et al. 1950), autotetraploid safflower seeds with higher hull contents had less crude oil than the diploid seeds.

5. Conclusion

After the identification and confirmation of autopolyploidy in C_2 generation by flow cytometric and cytological analysis, various phenological, morphological, physiological and agricultural characteristics of diploid and autotetraploid plants were comparatively evaluated during the growth and development stages. In brief, the results obtained from this research showed that the low seed yield and oil content of autotetraploids reduced their agricultural and industrial potential. These negative features can be eliminated by self-pollination and effective selection methods in advanced C generations. Also, further genetic studies, such as interspecific gene transfer and ploidy manipulation through genetic engineering, marker-assisted selection (MAS), and CRISPR/Cas9 genome editing, are needed to improve their agricultural prospects. Consequently, our research provides important findings in terms of revealing possible problems that may be encountered in the process of safflower breeding with chromosome doubling.

Acknowledgments

We would like to thank Prof. Dr. Metin Tuna from Tekirdağ Namık Kemal University for flow cytometry analysis.

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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 35.

Alagoz, Zeki
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Karagüzel O (2003) Farklı tuz kaynak ve konsantrasyonlarının Güney Anadolu doğal *Lupinusvarius*'larının çimlenme özelliklerine etkisi. Akdeniz Üniversitesi Ziraat Fakültesi Dergisi 16: 211-220.

Keeve R, Loupser HL, Kruger GHJ (2000) Effect of temperature and photoperiod on days to flowering, yield and yield components of *Lupinusalbus* (L.) under field conditions. Journal of Agronomy and Crop Science 184: 187-196.

Book:

Taiz L, Zeiger E (2002) Plant Physiology. 3rd Edition, Sinauer Associates, Massachusetts.

Book chapter:

Van HartenAM (2002) Mutation breeding of vegetatively propagated ornamentals. In: Vainstein A (Ed), Breeding for ornamentals: Classical and Molecular Approaches. Kluwer Academic Publishers, Dordrecht, pp. 105-127.

Institution publications with unknown author name(s):

TSI (2005) Agricultural Structure.T.C. Prime Ministry State Institute of Statistics, Publication No. 1579, Ankara.

DOI and received information from the internet:

Gulsen O, Kaymak S, Ozongun S, Uzun A (2010) Genetic analysis of Turkish apple germplasm using peroxidase gene-based markers. doi:10.1016/j.scienta.2010.04.023.

FAO (2010) Statistical database.http://faostat.fao.org/site/339/default.aspx. Accessed 27 July, 2010.

Theses:

Sever Mutlu S (2009) Warm-season turfgrass species: Adaptation, drought resistance and response to trinexapac-ethyl application. PhD Thesis, The University of Nebraska, Nebraska.

Girmen B (2004) Gazipaşa yöresinde doğal yayılış gösteren hayıtların (*Vitexagnus-castus* L.) seleksiyonu ve çoğaltılabilme olanakları. Yüksek Lisans Tezi, Akdeniz Üniversitesi Fen Bilimleri Enstitüsü, Antalya.

Full-text congress/symposium book:

Hawkes JG (1998) Current status of genetic diversity in the world. In: Zencirci N, Kaya Z, Anikster Y, Adams WT (Eds), The Proceedings of International Symposium on *In Stu* Conservation of Plant Genetic Diversity. CRIFC, Ankara, Turkey, pp. 1-4.

Kesik T (2000) Weed infestation and yield of onion and carrot under no-tillage cultivation using four crops. In: 11th International Conference on Weed Biology. Dijon, France, pp. 437–444.

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

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