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Phone: +90 242 310 2412
Fax: +90 242 310 2479
E-Mail: masjournal@akdeniz.edu.tr
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Determination of endogenous zeatin/IAA levels in selected fortune mandarin mutants against *Alternaria alternata* pv. citri

Ertugrul TURGUTOGLU[1], Ibrahim BAKTIR[2]

[1]Bati Akdeniz Agricultural Research Institute, Antalya, Türkiye
[2]Faculty of Agricultural Sciences and Technologies, Cyprus International University, Nicosia, Cyprus

Corresponding author: E. Turgutoğlu, e-mail: ertugrulturgutoglu@gmail.com
Author(s) e-mail: ibrahim.baktir@gmail.com

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ABSTRACT

Alternaria brown spot is a serious disease in mandarins and mandarin hybrids. In this particular research, 9 Alterneria tolerant and 2 susceptible mutant individuals obtained from a series of *in vitro* and *in vivo* studies were used. Endogenous indole acetic acid (IAA) and zeatin hormone levels of the individuals were noted before and after the Alternaria disease inoculations in order to determine the hormonal variations during the research. It was determined that endogenous zeatin levels decreased after the inoculation compared to its initial levels. The indole acetic acid levels of the individuals increased after inoculation except for some individuals.

1. Introduction

Alternaria brown spot disease is caused by a necrotypic fungus *Alternaria alternata pv.* citri. Alternaria brown spot was found for the first time in Australia on the 'Emperor' tangerines cultivar in 1903 (Pegg 1966). Since then the disease has been found in many countries in different continents including South Africa (Schutte et al. 1992), Israel (Solel 1991), Cuba (Herrera 1992), Colombia (Castro Caicedo et al. 1994), Turkey (Canıhoş et al. 1997), Argentina (Peres et al. 2003) and Peru (Marin et al. 2006). Alternaria brown spot is an important disease because of its effects on leaves, branches and unripe fruits of the tangerine and its hybrids (Pegg 1996; Canıhoş et al. 1999). Among tangerine cultivars and their hybrids, particularly 'Dancy' and, to a lesser extent, 'Fortune' are the most susceptible to the disease (Nemsa et al. 2012). Similarly, Peever et al. (2000) reported that 'Minneola', 'Orlando', 'Sunburst' and 'Nova' hybrids were also very sensitive to this pathogen. In the meantime, the toxic substance secreted by the same pathogen was found to be effective on mandarin 'Dancy' cultivar and its hybrids as well as mandarin x grapefruit hybrids and mandarin x orange hybrids (Vicent et al. 2007).

Nowadays, Alternaria brown spot disease is considered to be the most detrimental fungal disease on tangerine and its hybrids. This particular disease causes serious problems especially for the late season mandarin cultivars such as Minneola tangelo and Fortune in Turkey.

A number of interior and environmental factors, which work together in complex synergisms and antagonisms, regulates resistance responses of plants to the disease. The plant growth substances have vital importance among these factors (Pieterse et al. 2009; Santner et al. 2009; Jaillais and Chory 2010). The interactions between salicylic acid and jasmonic acid/ethylene (SA-JA/ET) are accepted as the backbone of immunity in plants (Pieterse et al. 2012). These hormones are considered to be stress hormones (Baktır 2015). However, traditional plant growth regulators such as auxins, gibberellic acids, cytokines and abscisic acid protect the plants against invasive hazardous pathogens or increase the immunity systems of the plants (Pieterse et al. 2012; Naseem et al. 2012).

Skoog and Miller (1957) described opposite behaviors of auxins and cytokinins in root and shoot developments of these plants, respectively. Consequently, auxins are accepted as rooting and cytokinins as shooting hormones (Baktır 2015). Auxins suppress the response of salicylic acid on plant immunity systems and this situation partially strengthens the role of jasmonic acid (Robert-Seilaniantz et al. 2011; Naseem and Dandekar 2012). Naseem et al. (2012) determined that external cytokinin applications prevented the development of the pathogens from the research they conducted on the interaction between cytokinins and salicyclic acid related to SA-biosynthesis in the mutants (sid2). This finding showed that cytokinin signals increase the resistance or immunity of the plants in comparison with salicyclic acid inductions in hormone/disease networks.

Cytokinins come into interaction with salicyclic acid sensitivity factor TGA3 to activate the transcriptional regulator ARR2 (*Arabidopsis* response regulator) which promotes salicyclic acid stimulation (Choi et al. 2011). Therefore, cytokinins can act synergistically on the salicyclic acid excitation pathway (Galis et al. 2004).

In this research, the variations in levels of auxin and zeatin in 9 Alternaria resistant (M_1V_3) and 2 Alternaria sensitive (M_1V_3) Fortune mandarin mutants were determined both before and at

the end of the research (Table 1). The mutants used in this research were obtained through the artificial irradiation method.

Table 1. The sensitivity of Fortune mandarin mutants to the Alternaria brown spot disease

Genotype	Tolerance
7-4-1	Tolerant
1A	Tolerant
2A	Tolerant
1-4-1	Tolerant
2B	Tolerant
6B	Tolerant
4-3-6	Tolerant
5-3-2	Tolerant
6D	Tolerant
3A	Susceptible
5-3-5	Susceptible

2. Materials and Method

After the application of acute gamma rays of 50 and 60 gray doses into "Fortune" budwoods, the radiated budwoods were grafted onto common sour oranges (M_1V_1). Following the grafting, the plants were vegetatively grown and brought to M_1V_2 and M_1V_3 stages. In this particular stage, 9 tolerant mutant individuals (M_1V_3) and 2 sensitive mutant individuals (M_1V_3) were used (Turgutoğlu and Baktır 2019).

The genotypes were carefully pruned in order to encourage the growth of new shoots and leaves. The fungus culture were conducted as described by Diaz et al. (2018). Following the pruning, when new leaves reached to lengths of 1 to 3 cm, each leaf was inoculated with an Alternaria spore suspension containing $5x10^5$ spores per ml (Azevedo et al. 2010). Perez-Jimenez and Perez-Tornero (2021) reported that the application of the toxin to the excised and wounded leaves seemed to be the most reliable method among the test methods to analyze sensitivity to Alternaria of 'Fortune' explants cultured *in vitro*. Following the inoculation, the plants were transferred into polyethylene bags in order to preserve the humidity and prevent the leaves drying out. According to Dalkilic et al. (2005) symptoms of the disease usually appear 24 hours after the inoculation. For this reason, symptoms of the disease were observed and examined at the indicated time on the inoculated plants. Physical conditions for the plants were adjusted to 26±2°C and 80-85% humidity rate in the growing rooms during the research.

Extraction and chromatographic analyses were done in the leaves before both the inoculation and 24 hours after the inoculations when the signs of disease appeared on the leaves in order to determine the variations of endogenous auxin and cytokinin levels in the plants. The extraction and purification processes were made in accordance with Kuraishi et. al. (1991), Battal and Tileklioğlu (2001), Erez (2009) and Atmaca (2015).

Extractions of the sampled leaves were chopped for 10 minute in a homogenizer. The leaf samples were treated with 80% methyl alcohol at 4°C before the homogenization. The homogenized material was kept for 24 hours at 4°C in the dark.

The residue remaining on the filter was discarded while the aqueous portion was removed after the samples were filtered through Whatman No: 1 filter paper. The methyl alcohol remaining in aqueous portion was evaporated at 45°C through the evaporator. The extract freed from methyl alcohol and dissolved with 0.1 M KH_2PO_4 (pH 8.0) was taken from the round bottom flask and centrifuged at 6000 rpm for an hour at 4°C.

The samples were placed into a beaker by discarding the sediment part in the tubes and were shaken for 1-2 minutes in order to separate phenolic and colored compounds after adding 1 gr PVPP (polyvinyl poly pyrrolidone, Sigma) (Erez 2009). It was then filtered through Whatman No: 1 filter paper. The cartridge was conditioned by passing 2.5 ml of methanol (80%) and 2.5 ml of distilled water through the filtrate cartridge before applying the filtrate to the Sep-Pak C 18 Cartridge. After this application, the filtrate was passed through the cartridge to keep the hormones in the cartridge. The derived hormones adsorbed in the cartridge were taken into vials using 5 ml of methanol (80%), and then injected into High Performance Liquid Chromatography (HPLC, LC 20 AT model). IAA and Zeatin analysis were done in HPLC according to Morris et al. (1990).

The following analysis systems were applied during the hormone analysis in HPLC:

Detector-DAD (Diode Array Detector, SPD–M20A)

Column-Inerstil C_{18} (5 µm, 250 x 4.6, GL Science, Tokyo, Japan)

Floe rate: 1 ml min^{-1}

Mobile phase: Methanol and 0.1 M acetic acid (55/45 v/v) (Ülger et al. 1999)

Wavelengths: 276 nm for IAA and 272 nm for Zeatin

The experimental data were statistically analyzed with the general linear model (GLM). Means were compared using LSD's Multiple Range Test.

3. Results and Discussion

In the study, 9 tolerant and 2 susceptible individuals from Fortune mandarin mutants were used for both *in vivo* and *in vitro* evaluations (Table 1).

It was determined that the zeatin level decreased in disease inoculated types except 3A genotype after the inoculation compared to pre-inoculated ones. The highest zeatin level was determined in 6D genotype with 3.48 ppm before the inoculation. The level of zeatin decreased to 2.71-ppm level in the same genotype after the inoculation. The lowest zeatin level with 0.58 ppm was found in 4-3-6 tolerant genotype after the disease inoculation. In general, zeatin levels were found to have decreased after the disease inoculations compared to pre-disease inoculations (Table 2).

IAA levels decreased in five of the genotypes (7-4-1, 2A, 1-4-1, 2B and 6B) obtained through mutations compared to pre-inoculated ones. On the other hand, IAA levels increased in genotypes 1A, 4-3-6, 5-3-2, 6D and 5-3-5 after the inoculations. The highest level of IAA was detected in genotype 5-3-2 with 2.12 ppm after the inoculation while the lowest IAA level was detected in genotype 7-4-1 with 0.04 ppm after the inoculation (Table 3).

The individual roles of auxin and cytokinin differ according to the plant and pathogen systems (Navarro et al. 2006; Wang et al. 2007; Choi et al. 2010; Choi et al. 2011). Kazan and Manners (2009) conducted research on *Arabidopsis* by inoculating *Pseudomonas syringae pv. tomato* DC3000 (Pto) in order to find out possible effects of auxins in *Arabidopsis*. They reported that auxins in general increased plant sensitivity and suppressed PR1 genes, related to increasing auxin levels. Despite this report,

Table 2. Changes in endogenous zeatin (Z) levels in Fortune mandarin mutants before and after the inoculations with *Alternaria alternata pv. citri*.

Genotypes	Zeatin (Z) levels (ppm)		Average Z value of the genotypes
	Before inoculation	After inoculation	
7-4-1	1.36ı*	0.60pq	0.98 ± 0.144h
1A	1.36ı	0.42r	0.89 ± 0.178ı
2A	1.92f	0.64p	1.28 ± 0.242f
1-4-1	1.10l	0.22s	0.66 ± 0.167j
2B	1.99e	1.50h	1.75 ± 0.093d
6B	1.85g	0.99n	1.42 ± 0.163e
4-3-6	3.06b	0.58q	1.82 ± 0.469c
5-3-2	1.06lm	0.88o	0.97 ± 0.036h
6D	3.48a	2.71c	3.10 ± 0.146a
3A	1.17k	2.36d	1.77 ± 0.225d
5-3-5	2.71c	1.33ı	2.02 ± 0.261b
Fortune	1.24j	1.03mn	1.14 ± 0.036g
Average value of the applications	1.86 ± 0.121A	1.11 ± 0.117B	

*The differences are statistically important between different letters (P<0.05). LSD (0.05), Genotype: 0.0355, Application: 0.0145, Genotype x Application: 0.0502.

Table 3. Changes in endogenous IAA levels in Fortune mandarin mutants before and after the inoculations with *Alternaria alternata pv. citri*.

Genotype	IAA levels (ppm)		Genotype average
	Before inoculation	After inoculation	
7-4-1	0.63l*	0.04r	0.34 ± 0.112j
1A	0.25p	0.54m	0.40 ± 0.056ı
2A	2.05b	1.50f	1.78 ± 0.105a
1-4-1	0.38o	0.05r	0.22 ± 0.063k
2B	1.04h	0.89ı	0.97 ± 0.031f
6B	1.99c	0.21p	1.10 ± 0.337e
4-3-6	0.48n	0.72k	0.60 ± 0.047h
5-3-2	1.23g	2.12a	1.68 ± 0.169b
6D	0.14q	1.48f	0.81 ± 0.254g
3A	0.84ıj	0.80j	0.82 ± 0.014g
5-3-5	0.56m	1.85d	1.21 ± 0.244d
Fortune	0.83j	1.68e	1.26 ± 0.036c
Application averages	0.87 ± 0.095B	0.99 ± 0.105A	

*The differences are statistically important between different letters (P<0.05). LSD (0.05), Genotype: 0.0355, Application: 0.0145, Genotype x Application: 0.0502.

a number of researchers indicated that high levels of cytokinin activated PR1 genes and induced increments of gene resistance (Naseem et al. 2012; Choi et al. 2010; Choi et al. 2011). It has been known that higher zeatin levels increase resistance of plants against some viral diseases and harmful insects (Ballare 2011).

Auxin was analyzed and tested by Kazan and Manners (2009) to identify its effect on endurance dynamic interactions of plant pathogens. It was proven that *Pseudomonas syringae pv. tomato* DC3000 (Pto) increased auxin biosynthesis during its infection period in tested plants (Chen et al. 2007). Meantime, it was reported that the roles of phytohormones auxin and cytokinin were independent in plant immunity (Robert-Seilaniantz et al. 2011). In another study, the same researchers show that Pto increased auxin accumulation and decreased cytokinin levels relative to baseline levels (Robert-Seilaniantz et al. 2011).

Considering the results obtained in this study, significant differences were detected in both zeatin and auxin levels between genotypes before and after disease inoculation. The IAA levels increased in most of genotypes after disease infections while zeatin levels decreased. There seems to be many important dynamics in the growth and development of plants related to interactions between auxin and cytokinin. Nevertheless, there is a combination of different hormonal networks as well as auxin and cytokinin concerning plant resistance to the disease infections in Fortune mandarin.

4. Conclusion

In this particular research, 9 *Alterneria alternata pv. citri* tolerant and 2 susceptible mutant individuals obtained from a serious of *in vitro* and *in vivo* studies were inoculated with the disease. The IAA levels increased in most of the genotypes after disease infections, while/whereas zeatin levels decreased. The differences in hormone levels likely occurred in mutant individuals due to some possible changes in their genetic structures.

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Phenotypic and genotypic characterisation of pepper genotypes for *Tomato Spotted Wilt Orthotospovirus* reaction and resistance

Omer Faruk OZDEMIR, Eminur ELCI

Niğde Ömer Halisdemir University, Agricultural Sciences and Technologies Faculty, Plant Production and Technologies Department, 51240, Niğde, Türkiye

Corresponding author: E. Elci, e-mail: eminur@gmail.com
Author(s) e-mail: omerfaruk3307@gmail.com

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ABSTRACT

In order to determine the effects of *Tomato spotted wilt orthotospovirus* (TSWV) on the yield and fruit quality parameters of some common local and commercial pepper (*Capsicum annuum* L.) genotypes under greenhouse conditions, mechanical inoculations were performed, and virus infections were tested by DAS-ELISA and RT-PCR after the inoculations. Based on the DAS-ELISA results, 95% of the inoculated plants were found to be positive for TSWV, in accordance with the expected amplicon size (276 bp) obtained by RT-PCR results. Infection of TSWV caused significant ($p \leq 0.05$) reduction in fruit number (45.97-100%), fruit weight (66.38-98.77%), fruit width (63.77-96.52%), fruit length (31.97-93.514%), flesh thickness (28.64-82.41%), fruit firmness (5.82-94.43%), fruit colour (1.62-7.79%), and total yield (68.62-100%) in infected plants. The best performance against TSWV was observed on cv. Yalova Çarliston 341, while the lowest was observed on cv. Bora 77 among the tested pepper cultivars Yalova Çarliston 341, Yalova Tatlı Kıl, Yalova Yağlık 28, Mazamort, Sera Demre 8, Üçburun, Geyikboynuzu, Bor Biberi, Bora 77 and 153-227. Moreover, the incidence of *Tsw* resistance gene was investigated by molecular analysis using CAPS marker $SCAC_{568}$ with *Taq*I restriction enzyme digestion; however, *Tsw* gene could not be detected in any of the tested cultivars except wild type *C. chinense* and resistant genotype cv.153-227. This study reveals the effects of TSWV in common pepper genotypes and will be important for virus resistant breeding studies.

1. Introduction

Vegetables play an important role as a source of vitamins and minerals in the nutrition of people. Due to the fact that the genetic centre of many important vegetable crops can be found in Turkey, vegetable production is an important sub-sector of farming in Turkey (Turhan and Korkmaz 2006; Çelik et al. 2018). Pepper is an important crop both for human nutrition and commercially, with its rich nutrient content, wide usage areas and large production volume. Pepper is produced in many regions of the world and additionally pepper production is carried out in Turkey with high production volumes over large areas of production. Turkey is one of the most important pepper suppliers as its production is around 2600000 tons annually and, ranked 3rd in the pepper production after China and Mexico (TÜİK 2020). There are many disease factors that negatively affect pepper production. It is known that viruses cause diseases in pepper as well as many plants and restrict pepper production (Çelik et al. 2010). It is reported that among these viral diseases, *Tomato spotted wilt orthotospovirus* (TSWV) is one of the most important 10 viruses that cause a high loss of yield (Scholthof et al. 2011).

TSWV firstly appeared on tomato plants in Australia Brittlebank (1919). It spread rapidly from Australia and was found in many countries in America, then in Europe, Asia, and Africa (Adkins 2000). In Turkey, TSWV was initially reported in lettuce plants in Mersin (Tekinel et al. 1969), after that, it was detected in Çanakkale, Balıkesir, Manisa, Uşak, Şanlıurfa, Samsun and the Mediterranean Region (Azeri 1981). Worldwide, there are more than 1000 plant species that are the host plants of TSWV, causing the virus to spread rapidly (Margaria et al. 2015). Another factor that causes the spread of TSWV is thrips, the vector of this virus. While TSWV is transmitted both circulatively and propagatively by 9 thrips species of 3 genus (Thrips, Frankliniella, Scirtothrips) belonging to the Thysanoptera order and Thripidae family, *Frankliniella occidentalis* has been reported as the most important vector of TSWV (Ullman et al. 1992; Şevik 2008). While cultural and chemical management are not effective in controlling TSWV due to the biological structures of viruses, the wide host plants range of TSWV and its transportation via thrips, utilising resistant varieties is the most effective method of controlling its spread. Both classical and molecular breeding methods are used in the mes development of resistant varieties. However, the marker assisted selection (MAS) method, which is one of the molecular breeding methods, is more reliable, faster and is the most widely used alternative and auxiliary method in recent years (Şimşek et al. 2015). *Tsw* gene provides resistance to viral disease caused by TSWV in pepper plants. Lines developed from *Capsicum chinense* Jacq. show hypersensitivity resistance against TSWV in pepper and are used as a source of resistance in breeding programmes. Pepper varieties which have the *Tsw* gene do not

show symptoms after mechanical inoculation, they show hypersensitivity resistance by shedding their leaves, after forming local lesions (Boiteux 1995). Studies on the *Tsw* gene have shown that this gene is located on the same loci (chromosome 10) of some *Capsicum chinense* Jacq. lines (Black et al. 1991; Boiteux 1995; Moury et al. 1997).

RAPD, SCAR, and CAPS markers are used for the detection of *Tsw* gene (Welsh and McClelland 1990; Williams et al. 1990, Lefebvre et al. 1997). In addition, CAPS markers are the most commonly used markers for detecting *Tsw*. Marker assisted selection is not always possible with RAPDs, because RAPDs designed for one population are not always polymorphic or not reliable for other populations (Paran and Michelmore 1993). It is possible to covert a RAPD piece into a SCAR marker to overcome these problems. SCAR markers are based on the sequencing of RAPD fragments and higher identification of more specific primers. But these identified primers often lead to monomorphic amplifications and loss of polymorphism. To achieve this polymorphism CAPS markers can be obtained by enzymatic restriction of SCAR (Konieczny and Ausubel 1993, Moury et al. 2000).

Various studies have been carried out in Turkey aiming to develop resistant pepper lines to TSWV. Çelik et al. (2018) aimed to breed lines that can be used as parents in order to develop new pepper varieties resistant to TSWV. In the study, they used a variety sensitive to TSWV, three genotypes resistant to TSWV, and reported that they obtained 10 lines resistant to TSWV with features that can be used in pepper breeding studies. Şimşek et al. (2015) used 12 TSWV resistant pepper genotypes, 6 TMV and PMMoV resistant pepper genotypes, and one superior pepper genotype in terms of quality characteristics. As a result of the study, it was reported that 3 genotypes had the desired resistances, and were determined as candidate varieties.

The aim of this current study, was to determine the effects of tomato spotted wilt orthotospovirus on the yield and fruit quality parameters of some local and commercial pepper genotypes that are widely used in the Central Anatolian Region, and screening for *Tsw* resistance gene incidence.

2. Materials and Methods

2.1. Materials

In this study, the most common local cultivars were selected based on their known superior fruit characteristics and yield in the Central Anatolian and Southern Mediterranean regions. In total ten different pepper genotypes (*Capsicum annuum L.*) and one wild type of pepper (*Capsicum chinense*) were used. The cultivars Üç Burun, Yalova Yağlık 28, Bora 77, Mazamort, Sera Demre 8, Yalova Çarliston 341, Yalova Kıl Tatlı are commercially used in the Central Anatolian region, and two of them are local genotypes called Bor pepper and Geyikboynuzu (Samandağ- Southern Mediterranean region). The resistant varieties used in this study were *Capsicum chinense L.* which is a wild type of pepper genotype and 153-227 which was produced by the company Yüksel® Seeds. In addition, TSWV infected pepper plants were supplied by the T.R. Ministry of Agriculture and Forestry, Ankara Directorate of Agricultural Quarantine and were used as inoculum sources of the virus.

2.2. Methods

2.2.1. Experimental design

This study was conducted under greenhouse conditions during the 2020 summer period in the Niğde province. Seeds were germinated in seed trays and seedlings transplanted to plastic pots after germination. While the seed trays were filled with a mixture of peat and perlite (3:1), a mixture of soil and peat-perlite (3:1) was used in the plastic pots. No fertiliser was applied to the plants during the daily irrigation. An equal number of plants were inoculated with TSWV, and mock-inoculated plants were used for each genotype as a control. Each genotype contained a total of 50 plants, including 25 virus inoculated plants and 25 mock-inoculated (buffer inoculated) plants.

2.2.2. Mechanical inoculations of pepper seedlings

Mechanical inoculation of TSWV was performed to the pepper plants twice at 15-day intervals using inoculation buffer (pH: 7.4) including 0.199 g l KH_2PO_4, 1.14 g l Na_2HPO_4, 0.1% Na_2SO_3 and 1% PVP-40 (1:10 sample dilution). The first inoculation was carried out 1 week after transplanting, when the plants were at the 5-6 leaf stage.

2.2.3. Confirmation of TSWV infections

The DAS–ELISA method was performed 30 days after inoculations to check TSWV infections according to Clark and Adams (1977) and instructions of the antisera's manufacturer for the monoclonal antisera of TSWV. After the ELISA test, the plate was read by the ELISA reader, and numerical results of the ELISA test were obtained. In addition to the serological detection, molecular tests were also performed to confirm the presence of TSWV in the tested pepper plants. A Plant/Fungi RNA Isolation Kit was used for isolation and purification of total RNA, according to the manufacturer's instructions (Norgen Biotek Corp., Canada). cDNA was synthesized by reverse transcription of obtained total RNAs using random hexamer primers and the cDNA synthesis Kit instructions (OneScript plus cDNA synthesis Kit, abm good, Canada). RT-PCR was performed using the obtained cDNA samples and PCR was carried out with 2 µl of cDNA, 0.5 µl of 10 mM dNTP, 1 µl of 25 mM $MgCl_2$, 2.5 µl of 5x PCR buffer and 0.5 µl of 10 µM of each virus specific primers (TSWV RdRp, F: 5′-ATCAGTCGAAATGGTCGGCA-3′, R: 5′-AATTGCCTTGCA ACCAATTC-3′, amplicon size: 276 bp, Perez et al. (2014), with 0.25 µl of 5 units µl Taq DNA polymerase. PCR was performed under the following conditions: denaturation 94°C 5 min, 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and a final extension for 10 min at 72°C. The PCR products were visualised under UV light after electrophoresis on 1.5% agarose gel and stained with ethidium bromide under a UV-transilluminator.

2.2.4. Molecular screening of TSWV resistance in pepper varieties

The cetyl trimethylammonium bromide (CTAB) extraction method (Doyle and Doyle 1987) was used to extract DNA from pepper leaves to check the incidence of resistance genes. The *Tsw* resistance gene CAPS markers (SCAC568: F: GTGCCAGAGGAGGATTTAT, R: GCGAGGTGGACACTG ATACT were used for PCR analysis (Moury et al. 2000). The PCR was carried out with 2 µl of diluted DNA, 1 µl of 10 µM dNTP mix, 2 µl of 25 mM $MgCl_2$, 2.5 µl of 10X PCR buffer, and 0.5 µl of 10 µM of each primer with 0.2 µl of 5 U µl Taq DNA polymerase. Reactions were incubated at 94°C for 5 min and following 40 amplification cycles (30 s at 94°C, 45 s at 50°C, and 1 min at 72°C) was performed and a final extension for 10 min at

72°C. The final PCR products were visualised under UV light after electrophoresis on ethidium bromide-stained 3% agarose gels. The obtained PCR amplicons were used for digestion. The mixture prepared by using 1 µl *Taq*I enzyme, 1 µl of 10x Cutting Buffer, 2 µl of nuclease-free sterile water and 7 µl of PCR product was incubated for 2 hours at 65°C (Moury et al. 2000). The final products were visualised under UV light after electrophoresis on ethidium bromide-stained 3% agarose gels.

2.2.5. Fruit quality and yield analysis

Fruits of both the infected and uninfected plants were harvested after maturation. All fruit quality traits were evaluated for ripen fruits per plant. Fruit number (FN) of each plant was recorded. Fresh weight (FW) (g) of each fruit was measured by a precise scale. Fruit width (FWth) (mm) and flesh thickness of fruits (FT) were determined by caliper. Fruit length (FL) (mm) of each fruit was measured by ruler. Also, fruit colours (FC) were determined, and different measurements were taken from two different parts of each fruit by the colorimeter during this process. Firmness of fruits (FF) was measured with a penetrometer by taking two different measurements from two different faces of each fruit.

2.2.6. Statistical analysis

Analysis of variance (ANOVA) statistical tests were performed using the statistical package JMP 16 (SAS, USA). Duncan multiple comparison test is used to compare the differences between the averages which are statistically significant according to the variance analysis results.

3. Results and Discussion

3.1. Virus symptoms

Plants were inoculated twice with an interval of 15 days using TSVW isolates. Although symptoms were observed as a result of the first inoculation in some plants, they were obtained after the second inoculation in most of the plants. TSWV symptoms varied according to genotypes, and they were mostly observed on leaves. Symptoms such as necrotic ringspot, concentric ringspot, chlorotic ringspot, yellowish or brownish ringspots with bronzing, mosaic, mottle, leaf curving and deformity were observed on the leaves of the pepper plants. No symptoms were observed on the fruits, but some symptoms were visible on the stems. Common symptoms of TSWV such as stunting, wilting, and die back at the tips of shoots were observed on the pepper plants.

While symptoms such as necrotic, chlorotic, and concentric ringspots, wilting, stunting and die back were observed in all genotypes, in addition to these symptoms, bronzing in Geyikboynuzu and Yalova Çarliston 341, and leaf deformity in Üçburun were also observed (Figure 1).

Roggero et al. (2002) reported that TSWV causes chlorotic and necrotic ringspots, wilting of the shoots and deformity, while Ferrand et al. (2019) reported that TSWV causes concentric and chlorotic ringspots, mosaic, mottling and deformity. These symptoms reported as a result of different studies are similar to the symptoms obtained in our study.

To show the effect of TSWV on pepper plants, a scale was created by ranking the plants from mild to severe (1 to 5). While creating the disease scale, the least affected plant was numbered as 2, while the most affected plant was numbered as 5. Plant number 1 was chosen from mock-inoculated (healthy) plants that did not show stunting, had green leaves, and produced fruit. Plant number 2 was chosen from infected plants that did not show stunting, yellowed leaves, and produced less or no fruit. Plant number 3 was selected from infected plants with stunting, hardly any TSWV symptoms on leaves, and no fruit. Plant number 4 was selected from infected plants with stunting, TSWV symptoms frequently on leaves, and no fruit. Plant number 5 was selected from infected plants with stunting, complete deformation of leaves and die back on shoot tips (Figure 2).

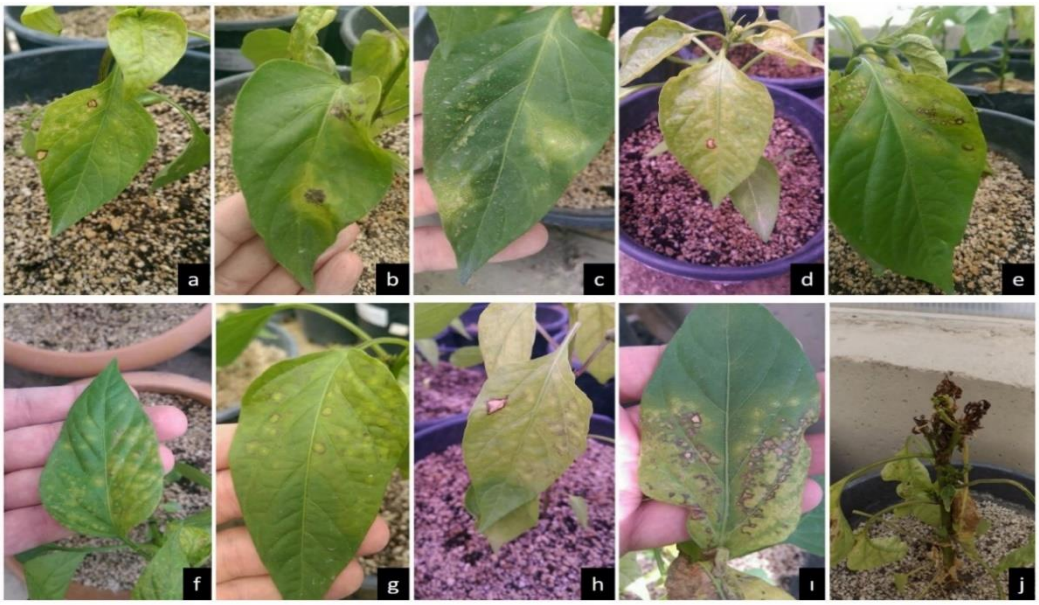

Figure 1. Symptoms are observed as necrotic and concentric ringspots on cv. 153-227 (a), necrotic ringspots on Sera Demre 8 (b), concentric ringspots on Bora 77 (c), bronzing and necrotic ringspots on Geyikboynuzu (d), concentric ringspots on Yalova Yağlık 28 (e), concentric and chlorotic ringspots on Bor (f), concentric and chlorotic ringspots on Mazamort (g), bronzing, concentric and necrotic ringspots on Yalova Çarliston 341 (h), concentric and necrotic ringspots with deformity of leaves on Üçburun (I), wilting, stunting and die back at the tips of shoots on Yalova Kıl Tatlı (j).

3.2. TSWV infections

All samples were tested by the DAS-ELISA method to detect the presence of TSWV. Test results were evaluated both visually and numerically. The evaluation was made according to the value of the negative control. While values greater than twice the value of the negative control were considered positive. As a result of the DAS-ELISA test, 95% of the inoculated plants were determined as TSVW positive. Also, TSWV infections were molecularly detected by the RT-PCR analysis. The test was performed with two samples from each pepper genotype and a TSWV-specific primer, which amplify a region of 276 bp in size. As a result, amplicons with the expected size were obtained from the samples tested by the primer pairs used (Figure 3). The presence of TSWV in mechanically inoculated pepper plants was confirmed via RT-PCR. These results, obtained from the RT-PCR process, agree with studies of Bozdoğan and Kamberoğlu (2015) and Keleş Öztürk and Baloğlu (2019) and show that symptoms detected on inoculated plants were caused by TSWV infection.

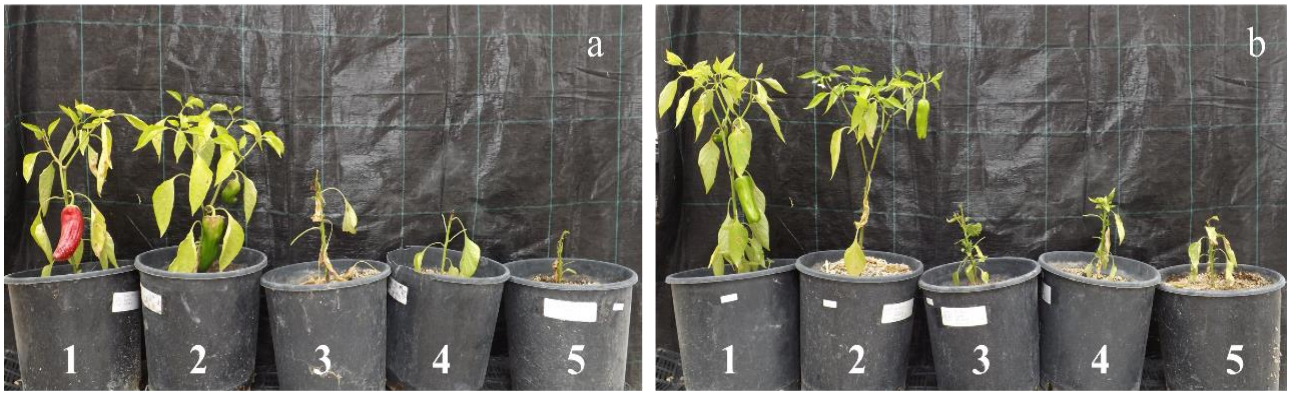

Figure 2. The effects of TSWV infections on pepper plants. The scale created by ranking the plants from healthy and mild to severe (left to right); Yalova Yağlık 28 (a) and Mazamort (b).

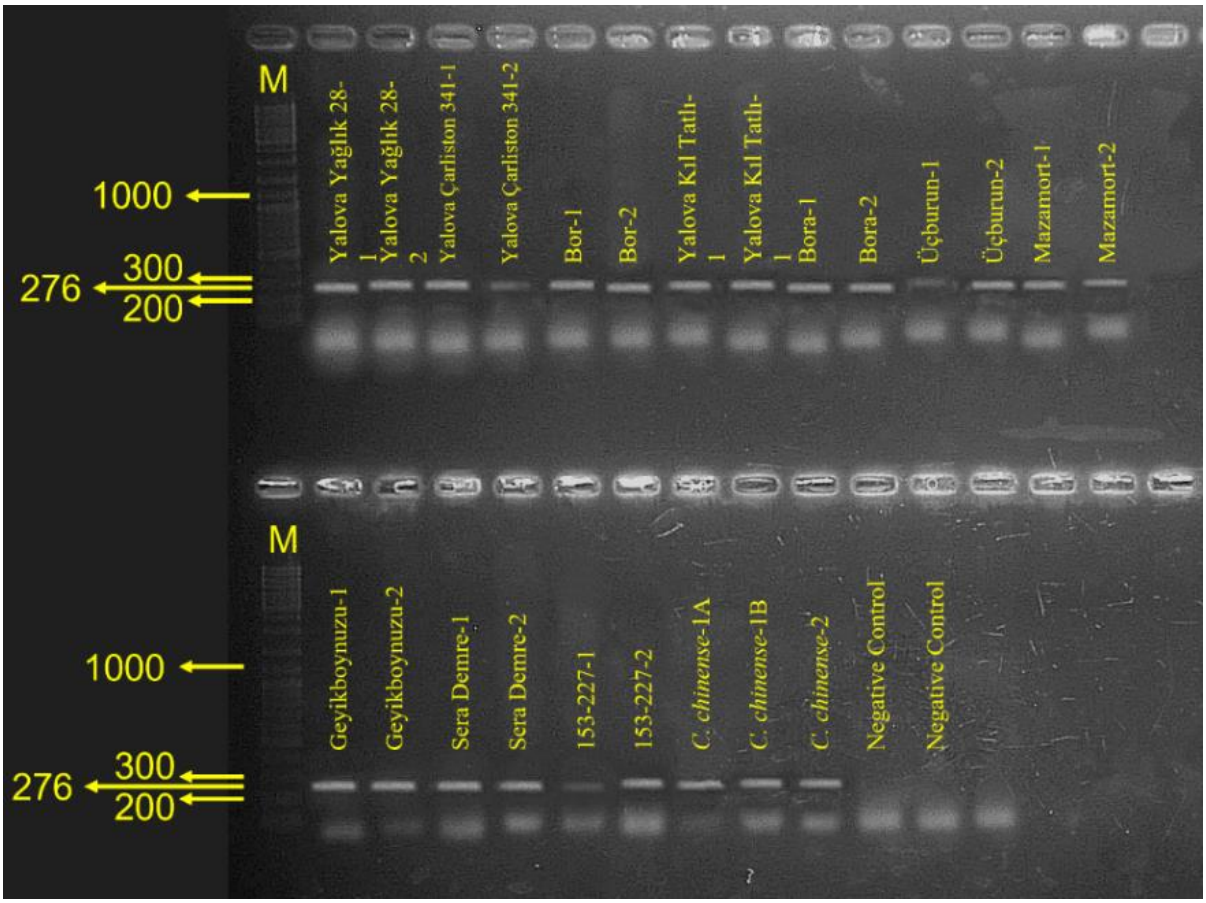

Figure 3. Results of PCR test using TSWV-specific primers (TSWV RdRp, 276 bp). As a result, bands with the expected size (276 bp) were obtained from the samples tested by the primer pairs used. The presence of TSWV in mechanically inoculated pepper plants was molecularly confirmed with these results. M: DNA Ladder.

3.3. Genotypic Characterisation

In order to determine the resistance level of the genotypes against TSWV, *Tsw* gene was investigated in these genotypes. PCR test was performed using DNAs isolated from these genotypes and using a CAPS marker ($SCAC_{568}$). PCR products were digested by using *Taq*I restriction enzyme.

As a result of PCR, amplicons approximately 568 bp in size were obtained and these samples were digested by the *Taq*I enzyme. Two amplicons were obtained approximately 200 and 300 bp in size in cvs. Yalova Yağlık 28, Yalova Çarliston 341, Yalova Kıl Tatlı, Bora 77, Bor, Üçburun, Mazamort, Geyikboynuzu and Sera Demre, while only a single amplicon 568 bp in size was obtained as expected for the resistant variety 153-227 and wild type *C. chinense* (Figure 4). The results were in accordance with the previous results in which the $SCAC_{568}$ marker was developed (Moury et al. 2000). In addition, completely similar results were obtained with the results of the studies conducted by Polat et al. (2012), Silvar and García-González (2017), Çelik et al. (2018) and İkten (2019).

Based on these results, *Tsw* gene was detected in cv. 153-277 pepper genotypes which is known to be a resistant variety. Although 153-227 carries the resistance gene, it was found to be infected by TSWV and showed a weak reaction according to our phenotypic analysis. The reason for these contradictory results obtained from molecular and phenotypic analysis could be due to the mechanical inoculations with a high concentration of the virus. Also, it is known that single resistance genes have a mostly temperature-dependent manner. The resistance gene *Tsw* does not provide resistance at high temperature conditions (Moury et al. 1997); Roggero et al. 1996, 2002).

3.4. Phenotypic Characterisation

According to the results of variance analysis for yield and quality parameters of pepper genotypes, it was determined that there are significant differences ($P \leq 0.05$) between all mock-inoculated and infected plants. Infected plants of Yalova Yağlık 28, Mazamort, Geyikboynuzu and Bor genotypes did not produce fruit and data could not be obtained from these groups. These groups were not included in the evaluations except for total yield and fruit number.

No fruit was obtained in the genotypes of Yalova Yağık 28, Mazamort, Geyikboynuzu and Bor infected with the TSWV. The highest fruit number decrease was obtained in these genotypes with 100%. Bora 77 (96.37%) was the group with the highest decrease after the groups that did not produce fruit. The least decrease was observed in Yalova Çarliston 341 with 45.97% (Table 1). The highest total yield loss was obtained from Yalova Yağlık 28, Mazamort, Geyikboynuzu and Bor with 100%. After the groups that did not produce fruit, the highest percent reduction was calculated for Bora 77 (98.85%), while the lowest percent reduction was calculated for Yalova Çarliston 341 (68.62%) (Figure 5 and Table 1). The highest fruit weight reduction was obtained in genotype Bora 77 with 98.77%, and the lowest reduction was obtained in genotype Yalova Çarliston 341 with 66.38% (Table 1). The genotype with the highest fruit width decrease was Bora 77 (96.52%) and the genotype with the lowest decrease was Yalova Çarliston 341 with 63.77% (Table 1). The highest percent fruit length reduction was calculated for Bora 77 (93.51%), while the lowest percent reduction was calculated for Yalova Çarliston 341 (31.97%) (Table 2). 153-227 (82.41%) was the group with the highest flesh thickness decrease, and the least decrease was seen in Sera Demre 8 with 28.64% (Table 2). The genotype with the highest fruit firmness decrease was Bora 77 (94.43%), while the genotype with the lowest decrease was Yalova Çarliston 341 with 5.82% (Table 2). While the highest fruit colour (L value) decrease was obtained in Bora 77 (7.79%), the lowest decrease was obtained in Yalova Çarliston 341 with 1.62% (Table 2). In addition, according to the results obtained from the Yalova Çarliston 341 in fruit firmness and fruit colour, no significant difference was found between the infected and mock-inoculated groups.

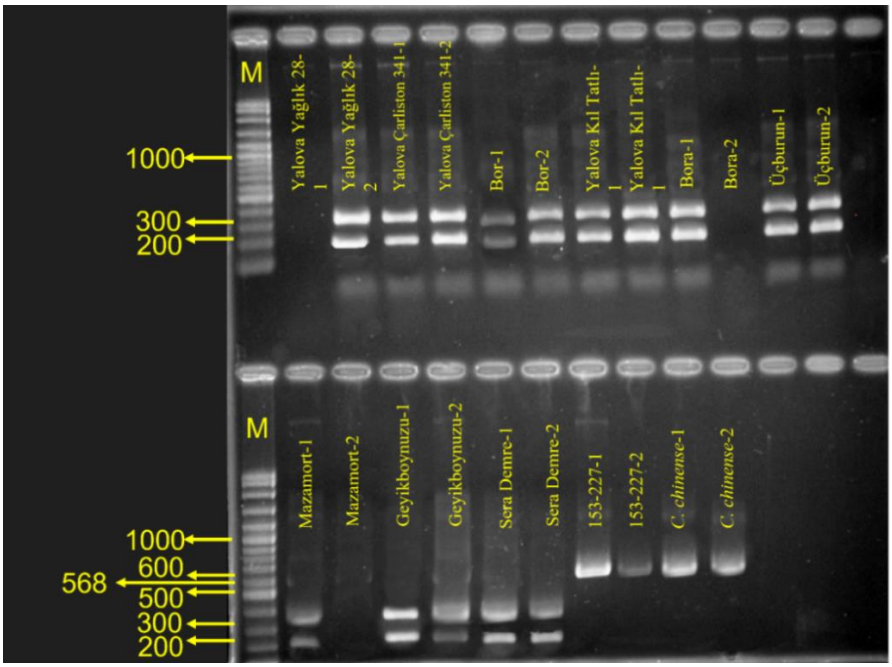

Figure 4. *Tsw* resistance gene screening results. Agarose gels indicate two bands each approximately 200 and 300 bp in cvs. Yalova Yağlık 28, Yalova Çarliston 341, Yalova Kıl Tatlı, Bora 77, Bor, Üçburun, Mazamort, Geyikboynuzu and Sera Demre, while a single band was obtained at 568 bp as expected for the resistant variety 153-227 and wild type *C. chinense*. M: DNA Ladder.

Table 1. Effects of TSWV infections on total yield, fruit number, fruit weight and fruit width of pepper genotypes

Genotypes	Total yield			Fruit Number			Fruit Weight (g)			Fruit Width (mm)		
	TSWV Infected	Mock-Inoculated	Effect of TSWV (%)	TSWV Infected	Mock-inoculated	Effect of TSWV (%)	TSWV Infected	Mock-inoculated	Effect of TSWV (%)	TSWV Infected	Mock-inoculated	Effect of TSWV (%)
Yalova Çarliston 341	9.58d	30.53c	-68.62	0.47def	0.87bcd	-45.97	3.19cde	9.49bc	-66.38	5.13fg	14.16de	-63.77
Yalova Tatlı Kıl	2.89d	31.42c	-90.80	0.40def	1.93a	-79.27	0.98de	9.39bc	-89.56	1.87g	9.66ef	-80.64
Yalova Yağlık 28	0d	93.50a	-100	0f	0.93bcd	-100	0e	27.02a	-100	0g	29.724a	-100
Mazamort	0d	26.26c	-100	0f	0.73cdef	-100	0e	8.75bcd	-100	0g	13.81de	-100
Sera Demre 8	3.97d	31.53c	-87.40	0.67cdef	1.40abc	-52.14	0.97de	8.51bcd	-88.60	4.18fg	13.27de	-68.50
Üç Burun	3.67d	50.89b	-92.78	0.27def	1.53ab	-82.35	1.22de	13.39b	-90.86	3.22g	25.15ab	-87.19
Geyikboynuzu	0d	24.86c	-100	0f	1.27abc	-100	0e	8.29bcd	-100	0g	14.08de	-100
Bor Biberi	0d	29.98c	-100	0f	1bcd	-100	0e	11.66b	-100	0g	18.95cd	-100
Bora 77	0.32d	27.84c	-98.85	0.07ef	1.93a	-96.37	0.10e	8.19bcd	-98.77	0.70g	20.15bc	-96.52
153-227	5.7d	43.75b	-86.97	0.07ef	0.80bcde	-91.25	1.92cde	14.58b	-86.83	1.56g	14.47cde	-89.21
Std. Deviation		**23.707**			**1.083**			**11.362**			**11.552**	

Table 2. Effects of TSWV infections on fruit length, fruit thickness, fruit firmness and fruit colour of pepper genotypes

Genotypes	Fruit Length (cm)			Flesh Thickness (mm)			Fruit Firmness (kg)			Fruit Colour (L value)		
	TSWV Infected	Mock-Inoculated	Effect of TSVW (%)	TSWV Infected	Mock-inoculated	Effect of TSVW (%)	TSWV Infected	Mock-inoculated	Effect of TSVW (%)	TSWV Infected	Mock-inoculated	Effect of TSVW (%)
Yalova Çarliston 341	10cd	14.70ab	-31.97	1.22fg	2.57ab	-52.52	5.66bc	6.01bc	-5.82	71.04a	72.21a	-1.62
Yalova Tatlı Kıl	6.70ef	14.64ab	-54.23	0.80gh	1.66def	-51.80	3.13de	6.59bc	-52.50	61.07e	65.77bc	-7.14
Yalova Yağlık 28	0g	13.5abcb	-100	0i	3.12a	-100	0f	6.54bc	-100	0f	62.56d	-100
Mazamort	0g	8.16de	-100	0i	1.91cde	-100	0f	6.70bc	-100	0f	64.61c	-100
Sera Demre 8	8.04de	16.02a	-49.81	1.32efg	1.85def	-28.64	5.39bcd	6.82bc	-20.96	61.83de	63.36cd	-2.41
Üç Burun	3.12fg	9.10de	-65.71	0.81gh	2.51abc	-67.72	2ef	6.29bc	-68.20	67.85b	64.05cd	+5.60
Geyikboynuzu	0g	11.32bcd	-100	0i	1.64def	-100	0f	4.80cd	-100	0f	60.90e	-100
Bor Biberi	0g	8.52de	-100	0i	2bcd	-100	0f	7.62ab	-100	0f	68.20b	-100
Bora 77	1.03g	6.78ef	-93.51	0.41hi	2.25bcd	-81.77	0.51f	9.16a	-94.43	58.81ef	63.78cd	-7.79
153-227	3.09fg	14.12abc	-78.11	0.54hi	3.07a	-82.41	0.91ef	7abc	-87	64.91c	67.85b	-4.33
Std. Deviation		**5.247**			**1.108**			**3.400**			**27.268**	

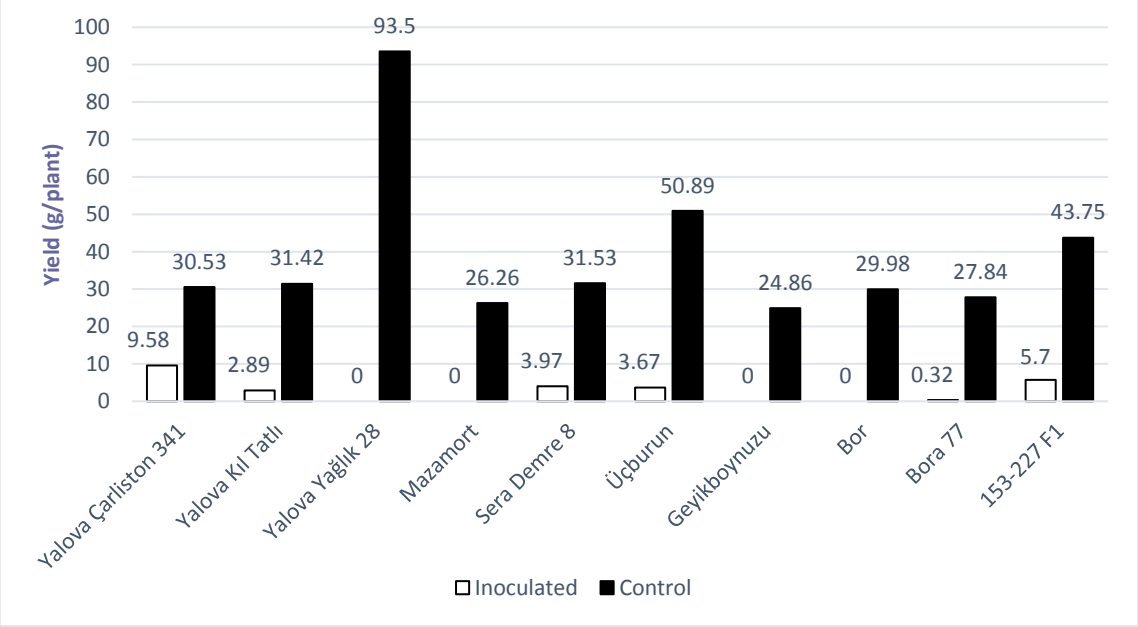

Figure 5. Total yield of TSWV infected and mock-inoculated pepper plants. The bars show the mean values. The difference between inoculated and mock-inoculated groups of each variety is shown.

4. Conclusions

According to the results of this study, it was determined that there were significant decreases in total yield and fruit quality parameters in plants infected with TSWV compared to uninfected controls. No fruit was obtained from the infected groups of Yalova Yağlık 28, Mazamort, Geyikboynuzu and Bor genotypes, and these genotypes were determined as the genotypes most sensitive to TSWV infection. It was determined that Bora 77 was the genotype with the highest decrease in yield, fruit number, fruit weight, fruit width, fruit length and fruit firmness, after the genotypes without fruit, and the genotype that was most affected by TSWV infection. Yalova Çarliston 341 was the genotype with the least decrease in yield, fruit number, fruit weight, fruit width, fruit length and fruit firmness, and it was determined as the least affected genotype among the genotypes. In conclusion, the results have shown that the performance of Bora 77 was poor among the genotypes and that this genotype was highly susceptible to TSWV. The best performing Yalova Yağlık 28 suggests that it could be tolerant to TSWV. In addition, *Tsw* gene that provides resistance to TSWV in pepper was not detected in common genotypes tested in this study.

Although the selected genotypes used in this study have superior fruit characteristics and high yields, they were found to be sensitive to TSWV. TSWV is the most destructive viral disease in pepper plants. According to these results, in order to use the superior characteristics of these varieties and to obtain products with the desired characteristics, it is recommended to apply the virus control methods completely in order to prevent the transmission or spread of virus infection, or to transfer the resistance gene to these varieties with the breeding programmes, which are the most effective methods against viruses. The results of this study will also assist in the development of sustainable virus resistant varieties through screening/selection of tolerant/resistant varieties for the management of TSWV.

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Disease complex of *Rhizoctonia solani* and *Meloidogyne hapla* Chitwood, 1949 (Nemata: Meloidogynidae) on tomato

Fatma Gul GOZE OZDEMIR[1], Serife Evrim ARICI[1], Ibrahim Halil ELEKCIOGLU[2]

[1]Isparta University of Applied Sciences, Faculty of Agriculture, Department of Plant Protection, Isparta, Türkiye
[2]Çukurova University, Faculty of Agriculture, Department of Plant Protection, Adana, Türkiye

Corresponding author: F. G. Goze Ozdemir, e-mail: fatmagoze@isparta.edu.tr
Author(s) e-mail: evrimarici@isparta.edu.tr, halile@cu.edu.tr

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ABSTRACT

The root rot disease complex of *Meloidogyne hapla* Chitwood, 1949 (Nemata: Meloidogynidae) and *Rhizoctonia solani* Kühn was investigated in 2021 under controlled conditions with different applications on tomato. Three week-old seedlings (cv. Alberty F1) were inoculated with *M. hapla* and/or *R. solani* according to priority of the applications. After sixty days, the parameters of plant and root height, plant and root wet weight, numbers of gall and egg masses, disease severity, *M. hapla* second stage juvenile density and *R. solani* density in the soil were recorded. The plant growth parameters were more adversely affected in plants inoculated with *R. solani* 2 weeks after the *M. hapla* application, whereas the number of galls and egg masses in the roots were negatively affected in only *M. hapla* inoculation 2 weeks after the inoculation of *R. solani* application. The disease severity of *R. solani* inoculation 2 weeks after *M. hapla* application (44.7%) and simultaneous *M. hapla* and *R. solani* application (33.6%) were found to be higher than only *R. solani* application (21.6%) and *M. hapla* inoculation 2 weeks after *R. solani* application (22.9%). In this study, it was found that *M. hapla* infestation of tomato increased root rot disease caused by *R. solani*.

1. Introduction

Tomato is one of the most commonly produced, consumed and traded agricultural products in the world. Turkey is third in the world in tomato production with 12.8 million tons (Anonymous 2021). Plant parasitic nematodes are one of the main pests that cause the damage losses in tomato production and quality. This damage caused by plant parasitic nematodes is estimated to be US$173 billion per year (Elling et al. 2013). Karajeh et al. (2008) stated that, about 5% of the world crop production is destroyed annually by *Meloidogyne* species. Similar research indicated that *Meloidogyne* species cause yield losses of up to 80% in tomato growing areas in the Western Anatolia region of Turkey (Kaşkavalcı 2007). *Meloidogyne hapla* Chitwood, 1949 (Nemata: Meloidogynidae) called "Northern Root-Knot Nematode", is one of the four most common root-knot nematode species worldwide and causes significant economic losses in various vegetables, fruit trees and pasture crops, including tomatoes (Moens et al. 2009). *Meloidogyne hapla* is seen more widely in temperate climates. Although it grows north of 39°N, it can be encountered in high altitudes of tropical regions as well (Whitehead 1969; Taylor and Buhrer 1958; Moens et al. 2009). It is possible to find it in northern Europe, northern Asia, southern Canada, and North America (Al Abadiyah Ralmi et al. 2016), and is detected on pepino, kiwi, tomato, pepper, and eggplant in Turkey (Özarslandan et al. 2005; Akyazı et al. 2012; 2017; Kepenekçi et al. 2014; Uysal et al. 2017). *Meloidogyne hapla* infection initially causes the development of small galls in the secondary roots (Gugino et al. 2006), and is more widespread in cool and temperate regions than tropical (Seid et al. 2015). In a study conducted in the vegetable producing areas of Isparta and Burdur Provinces in Turkey, 83 (51.8%) of 160 samples were found to be infected with root-knot nematodes, whereas 68 samples of root-knot nematodes were identified, and 22 of them were *M. hapla* (Uysal et al. 2017).

There are many soil-borne plant pathogens that affect the yield and quality of tomatoes (Bruehl 1987). *Rhizoctonia solani* Kühn. (Teleomorph: *Thanatephorus cucumeris*), one of these disease factors, is an important fungal disease that causes tomato wilt and root rot. *R. solani*, which is very difficult to control and causes serious damage both on seedlings and in the later stages of the plant (Solanki et al. 2011). The pathogen has infected at least 200 plant species with a wide host range (Lehtonen et al. 2008). This pathogen causes post emergence damping-off of seedlings and a weakening of seeds or seedlings before or after they germinate. Infected plants have cankers with red-brown spots on the stems and roots (Parmeter 1970). The fungus can survive for a long time in plant material or in soil as sclerotia (Anderson 1982). In many studies conducted throughout the world, including Turkey, it has been reported that *R. solani* is intensely isolated from tomato plants. These isolates belong to many anastomosis groups, but AG-4 isolate is encountered more frequently (Demirci and Döken 1995; Yıldız and Döken 2002; Kuramae et al. 2003; Taheri and Tarighi 2010; Bayar 2018; Demirer Durak and Ok 2019).

Recently, nematode-fungi interactions and their damage to many economically important crops have attracted the attention of scientists (Back et al. 2002). The interaction between root-knot nematodes and the root rot caused by *R. solani* has been studied on different hosts, and most of these studies have reported a synergistic interaction between these two important pathogens (Powell 1971; Mai and Abawi 1987; Shahzad and Ghaffar 1992; Evans and Haydock 1993; Bhagawati et al. 2007; Mokbel et al. 2007; Al Hazmi and Al Nadary 2015). *Meloidogyne incognita* (Kofoid and White 1919) Chitwood, 1949 (Nemata: Meloidogynidae) and *Rhizoctonia solani* interaction in tomato was reported in previous studies (Golden and Van Gundy 1974; Goswami et al. 1975; Mehta et al. 1995; Kumar and Haseeb 2009; Sagar et al. 2012). However, there are not many studies on the interaction of *M. hapla* with *R. solani* although it is the most widespread root knot nematode species, especially in temperate regions. Only Irvine (1964) reported that the highest death rates in alfalfa plants were in the treatment of *M. hapla* and *R. solani* together, followed by *M. hapla* treatment alone.

The root knot nematodes and *R. solani* interaction can cause serious damage to tomatoes, especially in greenhouse conditions. Developing a successful strategy to manage this nematode / fungus disease complex primarily depends on identifying the interaction between these two pathogens. The aim of this study was to investigate the interaction of local *M. hapla* and *R. solani* isolates in tomato root rot disease complex under controlled conditions.

2. Materials and Methods

2.1. Material

This study was carried out on the Alberty F1 tomato variety, which is known to be susceptible to both pathogens. *Rhizoctonia solani* isolate was obtained from tomato roots collected in the Deregümü region of Isparta province and was identified according to Barnett and Hunter (1998). Root knot nematode, DR15 isolate, was previously taken from a tomato greenhouse in the Deregümü region of Isparta province, identified morphologically and molecularly (Uysal et al. 2017) and mass production continued under controlled conditions (24±1°C, 60±5% humidity).

2.2. Nematode inoculum

In this study, an inoculum density of 1200 eggs per seedling was used. Eggs were obtained by soaking approximately 1 cm of diced tomato roots in 1% sodium hypochlorite for 5 minutes (Coolen and D'Herde 1972). First of all, the eggs were poured on a 75 µm sieve, then collected on a 5 µm sieve and washed with tap water to remove sodium hypochlorite (Nico et al. 2004; Liu et al. 2008). Finally, they were washed with sterilised distilled water and adjusted to a suspension of 1200 eggs in tubes containing 10 ml of distilled water (Al Hazmi and Al Nadary 2015).

2.3. Preparation of Inoculum of Rhizoctonia solani

Approximately 10 g of barley seeds were placed in 250 ml flasks and autoclaved with sterilised water for 30 minutes for two consecutive days. After the barley flasks had cooled, they were inoculated with one disc (5 mm in diameter) of 7 day old cultures of *R. solani* on Potato Dextrose Agar (PDA). The flasks were then incubated at 27±20°C for two weeks. During incubation, the flasks were shaken twice a day to ensure proper growth of the fungal mycelium on the barley seeds. Two weeks later, the fungi-colonised barley seeds were mixed in a bowl to ensure homogeneity and the inoculum amount was used at 15 g seedling (Al Hazmi and Al Nadary 2015).

2.4. Determination of the interaction of M. hapla and R. solani on tomato

In the present study, the experiments consisted of 5 different applications involving individual, simultaneous and sequential inoculations of *M. hapla* and *R. solani* on tomato. As a control, plants without nematode and fungi were used. Applications; 1) *M. hapla* only (N); 2) *R. solani* only (F); 3) Simultaneous inoculation of *M. hapla* and *R. solani* (N + F); 4) Fungus inoculation 2 weeks after nematode application (N+2F); 5) Nematode inoculation 2 weeks after fungus application (F + 2N). This study was carried out under controlled conditions (24±1°C, 60±5% humidity) and was designed in a randomised plot design with 10 replications. Three-week-old tomato seedlings were transplanted into 14 cm plastic pots containing approximately 1500 g of soil (68% sand, 21% silt and 11% clay). Inoculations were made 3 days after the seedling transplantation. One thousand two hundred *M. hapla* eggs 10 ml^{-1} and 15 g *R. solani* inoculum per seedling were used as the initial inoculum density, and inoculations were carried out according to the application priority. The nematode inoculum was equally distributed through three small holes made in the soil around the seedling stem and deep enough to contact the roots. Fungus inoculation was made by dispersing the seeds on the soil surface and mixing them well. Then, a small amount of soil was added and irrigated (Al Hazmi and Al Nadary 2015).

The study was completed 60 days after inoculation. After determining the plant height and fresh weight, the plants were uprooted and carefully washed thoroughly with tap water. Root lengths and root fresh weights were measured. Roots were exposed to 0.25% trypan blue for 3 minutes, then the gall and egg masses were counted under a stereomicroscope (Sharma and Ashokkumar 1991). The Baermann funnel technique was used to obtain the second stage juvenile density in the soil. The diseased plant rate at the end of the experiment (number of plants with root rot/number of healthy plants x 100) was calculated and then the *R. solani* density in the soil (cfu) was determined (Sagar et al. 2012).

2.5. Statistical analysis

Statistical analysis of the findings was calculated by using the SPSS (version 20.0) programme and analysis of variance (ANOVA) was used to test the differences between the means. In order to compare the means of different groups, "Tukey" was used when the variances were homogeneous ($P \leq 0.05$).

3. Results and Discussion

In the present study, the interaction effects of *R. solani* and *M. hapla* on plant growth and nematode and fungus density were investigated on the Alberty F1 tomato variety, by seperate and combined applications. Plant and root length and fresh weight values of all applications were found to be lower than the control application. The highest plant height values were found in seperate *M. hapla* (37.6 cm) and *R. solani* applications (39.8 cm) while the lowest values were in simultaneous *M. hapla* and *R. solani* applications (23.9 cm). There was no statistical difference between plant heights of simultaneous inoculations (30.1 cm) and *M. hapla* inoculation (31.0 cm) found 2 weeks after *R. solani*

inoculation (*P≤0.05)*. While plant fresh weight values were found to be close to each other in N+F (simultaneous *M. hapla* and *R. solani* applications), F+2N (*M. hapla* inoculation 2 weeks after *R. solani* application) and N+2F (*R. solani* inoculation 2 weeks after *M. hapla* application) applications, it was observed that it was lower than seperate nematode and fungus applications. While there was a statistical difference between N (*M. hapla* only) and F (*R. solani* only) applications in root length, no statistical difference was found between plant height, plant and root fresh weight values. It was determined that the plant growth parameters in simultaneous nematode and fungus applications were lower than seperate *M. hapla* and *R. solani* applications ($P \leq 0.05$). However, the lowest root fresh weight value was in N+2F (2.2 g) application and there was a statistical difference between N+F (4.0 g) and F+2N (3.3 g) applications ($P \leq 0.05$). It was determined that plant growth was more adversely affected when *R. solani* was inoculated 2 weeks after *M. hapla* inoculation (Table 1).

The lowest gall (83.3/root) and number of egg masses (105.0/root) in roots were determined in F+2N application. There was no statistical difference between the number of gall and egg masses of N, N+F and N+2F applications ($P \leq 0.05$). The second stage juvenile density of N (2949.8/250 cc soil) and N+F (2853.6/250 cc soil) applications in the soil was found to be higher than F+2N and N+2F applications. It was observed that the density of the second stage juvenile in the soil was adversely affected by inoculation with *M. hapla* 2 weeks after *R. solani* inoculation and *R. solani* application 2 weeks after inoculation with *M. hapla*. However, when the number of galls in the roots and the number of egg masses were analysed, the application in which nematode density was most negatively affected was F+2N application (Table 2).

The diseased plant rate was highest in N+2F (44.7%) application, followed by N+F (33.6%). The lowest disease rate was determined in F (21.6%) and F+2N (22.9%) treatments, and no statistical difference was found between them ($P \leq 0.05$). The disease rate in simultaneous nematode and fungus inoculations was found to be higher than the application of *R. solani* only. The highest concentration of *R. solani* in the soil was found in N+2F (2111.4 cfu g^{-1} soil) application, while the lowest was determined in F (1109.4 cfu g^{-1} soil) application. No statistical difference was found between the *R. solani* concentrations in the soil of N+F and F+2N applications ($P \leq 0.05$). It was determined that *R. solani* was more intense on plants inoculated with *R. solani* 2 weeks after *M. hapla* inoculation. It was found that the infection of roots with *M. hapla* contributed to the increase of the disease (Table 3).

Table 1. Effect of the interaction of *Meloidogyne hapla* and *Rhizoctonia solani* on plant growth parameters of Alberty F1 tomato variety

Applications*	Plant length (cm)	Plant wet weight (g)	Root length (cm)	Root wet weight (g)
	Average ± Standard Error			
C	47.5 ± 1.1a**	8.7 ± 0.4a	28.6 ± 1.2a	6.1 ± 0.1a
N	37.6 ± 1.5b	5.4 ± 0.3b	17.0 ± 0.6c	4.3 ± 0.1b
F	39.8 ± 1.5b	5.6 ± 0.3b	24.3 ± 0.7b	4.4 ± 0.2b
N+F	30.1 ± 1.1c	3.8 ± 0.1c	16.3 ± 0.5cd	4.0 ± 0.1bc
F+2N	31.0 ± 0.6c	3.3 ± 0.1c	14.7 ± 0.6cd	3.3 ± 0.2c
N+2F	23.9 ± 0.7d	2.7 ± 0.1c	13.4 ± 1.6d	2.2 ± 0.1d

*N: Nematode inoculation, F: Fungus inoculation, N+F: Simultaneous nematode and fungus inoculation, N+2F: Fungus inoculation 2 weeks after nematode application, F+2N: Nematode inoculation 2 weeks after fungus application, C: Control. ** Lowercase letters indicate statistical differences between applications in the same column (*P≤0.05*).

Table 2. Effect of *Meloidogyne hapla* and *Rhizoctonia solani* interaction on nematode density in Alberty F1 tomato variety

Applications*	Number of galls / root	Number of egg masses / root	2nd stage juvenile density in 100 g soil
	Average ± Standard Error		
C	-	-	-
N	168.6 ± 5.0a**	190.9 ± 3.8a	2949.8 ± 96.3a
F	-	-	-
N+F	176.4 ± 6.0a	196.8 ± 7.3a	2853.6 ± 99.0a
F+2N	83.3 ± 4.3b	105.0 ± 5.0b	2242.0 ± 204.5b
N+2F	160.3 ± 5.5a	178.8 ± 7.4a	1848.4 ± 49.0b

*N: Nematode inoculation, F: Fungus inoculation, N+F: Simultaneous nematode and fungus inoculation, N+2F: Fungus inoculation 2 weeks after nematode application, F+2N: Nematode inoculation 2 weeks after fungus application, C: Control. ** Lowercase letters indicate statistical differences between applications in the same column (*P≤0.05*).

Table 3. Effect of *Meloidogyne hapla* and *Rhizoctonia solani* interaction on disease rate on Alberty F1 tomato variety

Applications*	Disease rate (%)	*Rhizoctonia solani* concentration (cfu g^{-1} soil)
	Average ± Standard Error	
C	-	-
N	-	-
F	21.6 ± 1.7c**	1109.4 ± 34.7c
N+F	33.6 ± 1.8b	1731.6 ± 35.2b
F+2N	22.9 ± 1.5c	1535.9 ± 82.4b
N+2F	44.7 ± 1.7a	2111.4 ± 93.0a

*N: Nematode inoculation, F: Fungus inoculation, N+F: Simultaneous nematode and fungus inoculation, N+2F: Fungus inoculation 2 weeks after nematode application, F+2N: Nematode inoculation 2 weeks after fungus application, C: Control. ** Lowercase letters indicate statistical differences between applications in the same column (*P≤0.05*).

In this study, it was found that the disease rate and soil density of *R. solani* increased in the presence of *M. hapla* on tomato. However, it was determined that the highest increase in disease rate was determined when *R. solani* was inoculated 2 weeks after *M. hapla* inoculation and was followed by simultaneous inoculation. This increase in root rot indicates a synergistic interaction between the two pathogens and greater damage to the plant. When the plant growth parameters were examined, it was determined that the most damage occurred in *R. solani* inoculated 2 weeks after *M. hapla* inoculation. Due to *M. hapla* being a sedentary endoparasite and the physiological and anatomical changes it causes in the root tissues in giant cell formation may be a reason for this damage. The feeding cells of fixed endoparasite nematodes, "syncytia" or "giant cell", contain many golgi apparatus, mitochondria, a dense cytoplasm, and many ribosomes, and have high metabolic activity (Melendez and Powell 1970; McLean and Lawrence 1993; Abdel-Momen and Starr 1998). These nutrient-rich cells are appropriate substrates for fungal colonisation (Porter and Powell 1967; Powell 1968; Batten and Powell 1971; Carter 1981). Many other researchers recorded a synergistic interaction between root knot nematodes and *R. solani* on different hosts (Powell 1971; Mai and Abawi 1987; Evans and Haydock 1993; Bhagawati et al. 2007; Mokbel et al. 2007; Al Hazmi and Al Nadary 2015). Bhattarai et al. (2009) found that *R. solani* damage increased in the combination of *Globodera pallida* with *R. solani* or *G. rostochiensis* with *R. solani* and stem canker index increased significantly in co-inoculation with *G. pallida* and *R. solani* compared with *R. solani* only.

It was determined that *R. solani* inoculations did not increase the number of gall and egg masses in the roots 2 weeks after simultaneous inoculation and *M. hapla* inoculation, and it was in the same statistical group with *M. hapla* application only. However, it was determined that the number of gall and egg masses considerably decreased in the application of *M. hapla* inoculation 2 weeks after *R. solani* inoculation. Root rot caused by *R. solani* may have been affected by the nematode feeding process in root tissues and subsequently negatively affected nematode growth. The existence of a fungal mass that prevents nematode penetration or invading the places that the nematode chooses to feed may cause a decrease in nematode density (Triantaphyllou 1960; Nord-Meyer and Sikora 1983; Mokbel et al. 2007). The decrease in dry and wet weight of the plant as a result of the increase in fungal pathogenicity can reduce the nematode population (Mauza and Webster 1992). In most studies, it has been reported that root gall nematode-induced galling and nematode population decrease in the presence of *R. solani* (Choo et al. 1990; Mehta et al. 1995; Roy and Mukhopadhyay 2004; Kumar and Haseeb 2009; Sagar et al. 2012). Irwine (1964) reported that the highest death in alfalfa plant was in the simultaneous *M. hapla* and *R. solani* applications, followed by *M. hapla* application only. In a study of Göze Özdemir and Arıcı (2021) *in vitro* conditions, they determined that *R. solani* culture filtrates showed toxic effects on *M. hapla* eggs and juveniles which the 2nd stage juvenile death by *M. hapla* in the pure culture filtrate concentration of *R. solani*, live egg and hatching from the egg masses percentages, 83.2%, 76.7% and 54.2%, respectively.

4. Conclusion

In the present study, the results have shown that *M. hapla* infestation of tomato increased root rot disease caused by *R. solani*. With the increase in the disease rate, it was observed that plant growth was negatively affected in simultaneous nematodes and fungi applications. The lowest plant growth and the highest disease severity were determined in the inoculation of *R. solani* 2 weeks after the *M. hapla* inoculation. It was also observed that the density of *R. solani* in the soil was higher in simultaneous nematode and fungus applications than fungus inoculation only. These results indicate that co-infection of *M. hapla* and *R. solani* caused significant losses in yield. To manage this disease complex, the development of a successful strategy must depend on integrated disease management that includes appropriate methods to suppress the populations of both pathogens.

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Molecular characterisation of Alfalfa mosaic virus isolates in potato from the Tokat province, Türkiye

Serife TOPKAYA

Department of Plant Protection, Faculty of Agriculture, Tokat Gaziosmanpasa University, Tokat, Türkiye

Corresponding author: S. Topkaya, e-mail: serife.topkaya@gop.edu.tr

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ABSTRACT

The *Alfalfa mosaic virus* (AMV) was detected in potato fields in the Tokat province. The coat protein (CP) sequences of AMV isolates from the Tokat province were determined and compared with sequences of reference AMV isolates from GenBank. Total nucleic acid (TNA) was extracted from plants with positive results according to serological test results. Then, reverse-transcription polymerase chain reaction (RT-PCR) was performed using primer pair specific to partial the coat protein region, and positive PCR products were sent for sequence analysis in both directions. Two Turkish AMV isolates (AMV-PN3-5 and AMV-PN3-6) had a 96-99% nt homology amongst themselves, according to nucleotides (nt) sequence analysis. Based on the phylogenetic tree obtained from 24 AMV isolates from GenBank for both sequences, the two Turkish AMV isolates were clustered in subgroup I containing Iranian, Canadian, Turkish, Korean, and Serbian isolates, at the nucleotide level. Sequence comparison showed that these two isolates of AMV shared 96% to 99.7% sequence similarity with the twenty-six reported isolates of AMV obtained from GenBank. This is the first report on the genetic variability of AMV isolates infecting potato crops in the Tokat province.

1. Introduction

Potato (*Solanum tuberosum* L.), is one of the most important horticulture crops in the world. Turkey is one of the important potato producers in the Mediterranean region and potatoes can be grown almost anywhere in the country (Yardımcı et al. 2015). Turkey produced 5100674 tons of potatoes in 1389415 decares (da) in 2021 (TUIK 2020). The Tokat province produced 50514 tons of potatoes in a cultivation area of 20291 da.

Multiple virus infections lead to a decrease in yield and tuber quality in potato plants (Kolychikhina et al 2021). Potato is infected by more than 40 viruses such as *Potato virus Y* (Potyvirus, PVY), *Potato virus X* (Potexvirus, PVX), *Potato virus S* (Carlavirus, PVS), *Potato virus A* (Potyvirus, PVA), *Potato virus M* (Carlavirus, PVM), AMV (Alfamovirus), and *Potato leaf roll virus* (Polerovirus, PLRV) and 2 viroids such as *Potato spindle tuber viroid* (PSTVd). These viruses cause significant yield losses in potato crops (Hameed et al 2014, Kolychikhina et al 2021).

The *Alfalfa mosaic virus* (AMV) is a type species of the *Alfamovirus* genus in the *Bromoviridae* family within plant viruses. It has a worldwide distribution and infects more than 600 plant species belonging to the *Solanaceae*, *Fabaceae*, *Umbelliferae*, and *Compositae* families and several vegetables such as potato, tomato, alfalfa, pepper, eggplant, tobacco, clover, legumes, and woody crops (Brunt et al. 1990; Bol 2008). Potato plants infected with AMV show symptoms in the form of bright yellow areas on the leaves called calico and tuber necrosis in tubers (Nie et al. 2015). It has been reported that AMV causes tuber necrosis in potato tubers in Canada (Nie et al. 2015). In recent years, studies conducted by Nie et al (2020) have also shown that the necrosis seen in AMV-infected tubers is dependent on the potato variety rather than the AMV strain/haplotype. The virus is easily transmitted by a minimum of 14 aphid species non-persistently, primarily *Myzus persicae* (Ragsdale et al. 2001). AMV is also transmitted by seeds, pollen, and *Cuscuta* spp. (Bailiss and Offei 1990, Hemmati and McLean 1977).

The genome of AMV is composed of three single strands of positive polarity (+ssRNA) particles. RNA1 (P1 protein) and RNA2 (P2 protein) segments are responsible for viral replicase proteins. RNA 3 encodes the coat protein (CP) gene and the viral movement protein (MP), both of which are required for infection (Tenllado and Bol 2000; Bol 2003, 2008). Furthermore, the P1 and P2 sequences encoded by the AMV genome have low genetic diversity, whereas the CP and MP gene regions have high genetic diversity (Bergua et al. 2014).

In Turkey, AMV infection has been reported in different crops using different detection approaches including mechanical inoculation (biological indexing) and Double Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA), and RT-PCR methods (Sertkaya et al. (2017), Özdemir et al. 2011). Viral pathogen were recorded in eggplant in the Manisa province (Özdemir et al. 2011), in bean in the Burdur province, West Mediterrenean (Çulal Kılıç and Yardımcı 2015), in pepper in Tokat (unpublished), in alfalfa in the Van and Bingöl provinces (Usta and Güller 2020, Güller et al. 2022), and in other provinces (Arlı-Sökmen et al. 2005; Demir 2005; Buzkan et al. 2006; Özdemir and Erilmez 2007; Çetinkıran and Baloğlu 2011).

PVY, AMV, PVS, and PLRV viral agents that cause infections in potato plants have been reported from the Tokat province (Topkaya 2020). Symptoms that may be caused by the AMV agent have been observed in potato plants during field surveys conducted in potato-growing areas. The main aim of this study was to molecularly determine the AMV isolates in potato plants from the Tokat province and compare sequence identities between the Turkish AMV potato isolates and reference AMV isolates reported in GenBank.

2. Material and Method

2.1. Virus source

In 2019, AMV was determined serologically and molecularly in potato growing areas in Tokat by Topkaya (2020). Two samples were chosen for further molecular characterisation.

2.2. Total Nucleic acid (TNA) extraction and complementary DNA (cDNA) synthesis

The total nucleic acid (TNA) extraction of isolates was done using leaves of potato plants according to Astruc et al. (1996) with minor modifications. The isolated TNAs were stored at -20°C until the cDNA synthesis. Total RNAs were used as a template for RT-PCR by using the random hexamer primer (5'-NNNNNN-3'). cDNAs were synthesized in a total reaction volume of 10 µl to be used as a template in amplification studies. For cDNA synthesis, the 2.5 µl of extracted TNA was used as a template and added to a PCR tube, and incubated for 5 min at 65ºC. Then the reaction mix containing 1 µl 10X reaction buffer (WizScript™, Republic of Korea), 0.5 µl 20X dNTP (2.5 mM), 1 µl random hexamer primer, 0.5 µl reverse transcriptase enzyme ((WizScript™, Republic of Korea), 0.25 µl RNAse inhibitor, 1.75 of distilled water added to the tube. RT incubation was performed with min incubation at 25ºC, followed by at 37ºC for two hours and 85ºC for 5 min.

2.3. Polymerase Chain Reactions (PCR)

RT-PCR was performed using the specific primers AMV-CP F (5'- GTGGTGGGAAAGCTGGTAAA-3') and AMV-CP R (5'-CACCCAGTGGAGGTCAGCATT3') (Martínez-Priego et al. 2004) for partial coat protein gene sequence of AMV. Amplification was performed in a final volume of 25 µl containing 2.5 µl of 10× Taq Buffer, 2 µl of $MgCl_2$, 2 µl of cDNA, 0.5 µl of each dNTP (10 mM) mix and, 10 pmol forward and reverse primers with 0.25 µl of Taq DNA polymerase (Fermentas, USA), 18.25 µl of distilled water. After then, PCR products (about 700bp) were electrophoresed in 1.5% agarose gel including ethidium bromide.

2.4. Phylogenetic analysis

The sequence data of the CP gene was subjected to a Blast Nucleotide search (Basic Local Alignment Search Tool) for comparison with references AMV isolates from GenBank. The phylogenetic tree was generated with two Tokat AMV isolates and references AMV isolates derived from the GenBank. The evolutionary relationship was calculated with the maximum likelihood (ML) method of the MEGA7 (Kumar et al. 2016) and Sequence Demarcation Tool Version 1.2 (SDTv1.2) (Muhire et al. 2014) software. The bootstrap values were performed with 1000 replications.

3. Results and Discussion

AMV infects more than 600 plant species worldwide and is transmitted mechanically, by seed/weed seeds, and by aphids in a non-persistent manner (Bol 2003). AMV infection on potato plants has been reported in different countries such as Egypt, Canada (Xu and Nie 2006), Korea (Jung et al. 2000), Iran, and Turkey (Çarpar and Sertkaya 2016, Topkaya 2020) Saudi Arabia (Al-Saleh et al. 2014). In previous studies in Turkey, AMV was reported on various hosts and at different infection rates. AMV was detected in the Hatay province by Sertkaya et al. (2017) at 5.4% and 4.6% rates during the potato production in 2014 and 2015 years, respectively, and 15.3% rates of AMV in *Physalis angulata*. AMV was observed in potato plants in the Tokat province. In a previous study conducted by Topkaya (2020), it was detected at 1.38% rate in the tested samples. Although this infection rate seemed to be lower, it has the possibility to increase because it easily spreads such as by non-persistent transmission with aphids and mechanically. The variation in the rate of AMV from year to year has also been reported among different authors (Wang et al. 2012; Milošević 2013; Rusevski et al. 2011, 2013; Stanković et al. 2014).

As a result of RT-PCR tests with AMV-specific primers, expected bands of around 700 bp were obtained from two samples. PCR products were sequenced by the Sanger method (Atlas Biotechnology- Ankara). Obtained sequence data were analysed with MEGAX software and compared with reference isolates (Table 1) and the obtained phylogenetic tree. Based on the phylogenetic analysis, all AMV strains were clustered in two main groups (Subgroup I and Subgroup II). Subgroup I contained Iranian, Canadian, Turkish, Korean, and Serbian isolates, whereas subgroup II contained strains from France and England. In the phylogenetic tree, two Turkish AMV isolates (PN 3-5 and PN 3-6) were clustered with subgroup I including Chinese, Serbian AMV isolate, and two Turkish Iğdır isolates (Figure 1). In previous studies, AMV isolates were separated into two groups of I and II by Parrella et al. (2010) and then further divided the second group into IIA and IIB subgroups by Parrella et al. (2011) Later on, the AMV isolates were grouped into four or more different groups based on sequence information of coat protein region by Stanković et al. (2014). Based on CP sequence comparisons, AMV potato isolates under this study were 92 to 99% identical at the nucleotide level (Figure 2) and 95 to 100% identical at the amino acid level.

Parrella et al. (2000) grouped the AMV isolates based on amino acid sequences as subgroups I and II. In this study, the same changes were obtained (Table 2). The amino acid sequence changes were observed at positions for which variability has already been reported (Parrella et al. 2000). Differences in the CP amino acid sequences are shown in Table 2.

Parrella et al. (2000) divided AMV isolates into two groups and suggested that this distinction may either be due to geographical differences or to variations in the amino acid sequence of their CPs, which may be related to the structural features of the virus particles. Later on, AMV isolates have been reported in different groups regardless of regional distribution by Xu and Nie (2006) and Stanković et al. (2014). Abdel Aleem et al. (2018) reported that the Egyptian AMV isolates formed a new group. Recently in a study, Nie et al. (2020) reported that RNA1 and RNA3 segments of AMV have been grouped into three major clades and RNA2 segments have been grouped into two groups. The isolates were divided into 3 groups in the phylogenetic tree formed based on the full genome sequence of the RNA3

segments containing the CP and MP protein regions. In this study, based on the CP region sequences, the AMV Tokat isolates were grouped into three major groups as reported by Nie et al. (2020). In major group 1, the two Turkish AMV isolates were clustered with Serbian and Turkish isolates which was previously reported. Group II has also a common feature in all these groupings. In this study, Turkish AMV isolates showed the same similarity with subgroup I isolate. Group III includes only Eagyptian isolate.

4. Conclusion

AMV was determined in potato-growing areas in the Tokat province. In this study, molecular characterisation of AMV isolates was performed. The Tokat AMV CP isolates (PN3-5 and PN3-6) showed high nt (92-99%) and aa homology (95-100%) with other world AMV isolates. Based on the phylogenetic tree, two isolates from Tokat were clustered in group I, together with isolates from France, Serbia, Saudi Arabia, and England. CP regions of AMV pepper isolate from Tokat were previously studied and were not included in this study because they were 333 bases long. This is the first potato isolate of AMV to be identified at the molecular level in the Tokat province. This information will contribute to further analysis of AMV on potatoes and other host plants in Turkey.

Table 1. Information about reference isolates used in the study

Accession number	Host	Isolate name	Country
KX710198	*Capsicum annuum*	R236	Bosnia and Herzegovina
MT210178	*Medicago sativa*	Alakoy Y1	Turkey
MT210179	*Medicago sativa*	Alakoy Y9	Turkey
MW962976	*Medicago sativa*	Bingol A8	Turkey
MW882261	*Medicago sativa*	Igdir 1	Turkey
KF147805	Tomato	258-11	Serbia
MG600289	*Trifolium pratense* L.	AMV-PV1	Czech Republic
MW882262	*Medicago sativa*	Igdir 9	Turkey
MT669393	*Glycine max*	IA-4-2018	USA
MG922819	*Solanum lycopersicum* (tomato)	219-14"	Serbia
JQ685860	*S. tuberosum*	Ke.Ba.Po	Iran
HQ288892	*S. tuberosum*	-	Egypt
AF294432	*S. tuberosum*	KR1	Korea
AF294433	*S. tuberosum*	KR2	Korea
DQ314755	*S.tuberosum*	Ca518	Canada
DQ314753	*S. tuberosum*	Ca401	Canada
AF015717	*Garden lupin*	VRU	England
AF015716	*Garden lupin*	15/64	England
AJ130708	Carrot	Dac-16	France
L00162	Clover	425 L	USA
KC182568	*Capsicum annuum*	P-27-09	Serbia
MZ221779	*Medicago sativa*	Yuanyang_2/H3	China
MZ221776	*Medicago sativa*	China_Yangling/S	China
MN846751	*Acyrthosiphon pisum*	BJAp1	China
KX535507	Potato	Es.Fa.Po	Iran

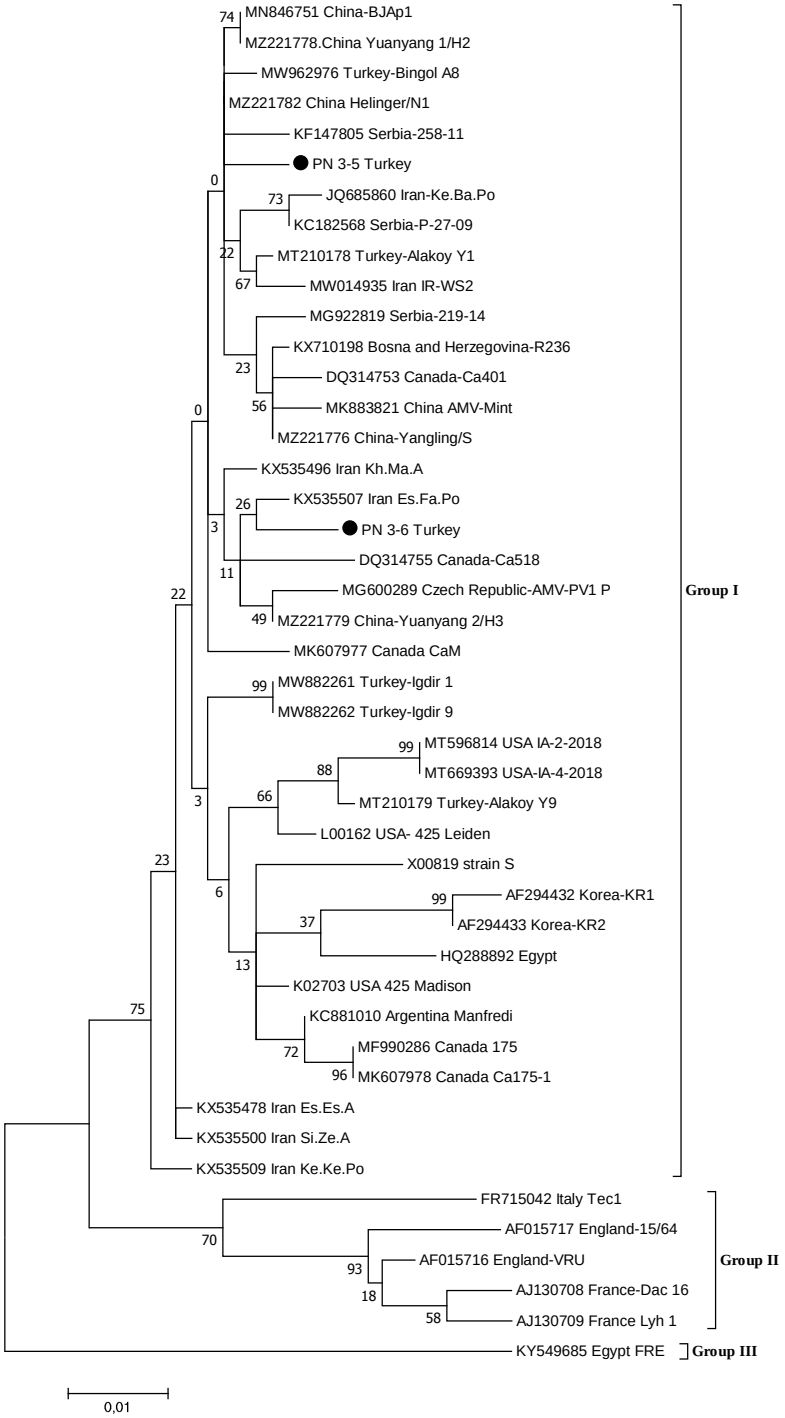

Figure 1. Phylogenetic tree constructed based on the partial nucleotide sequences of the CP gene of two new AMV isolates and references AMV isolates using the maximum likelihood (ML) method of MEGA7 (Kumar et al. 2016). Turkish isolates are indicated using a black-filled circle.

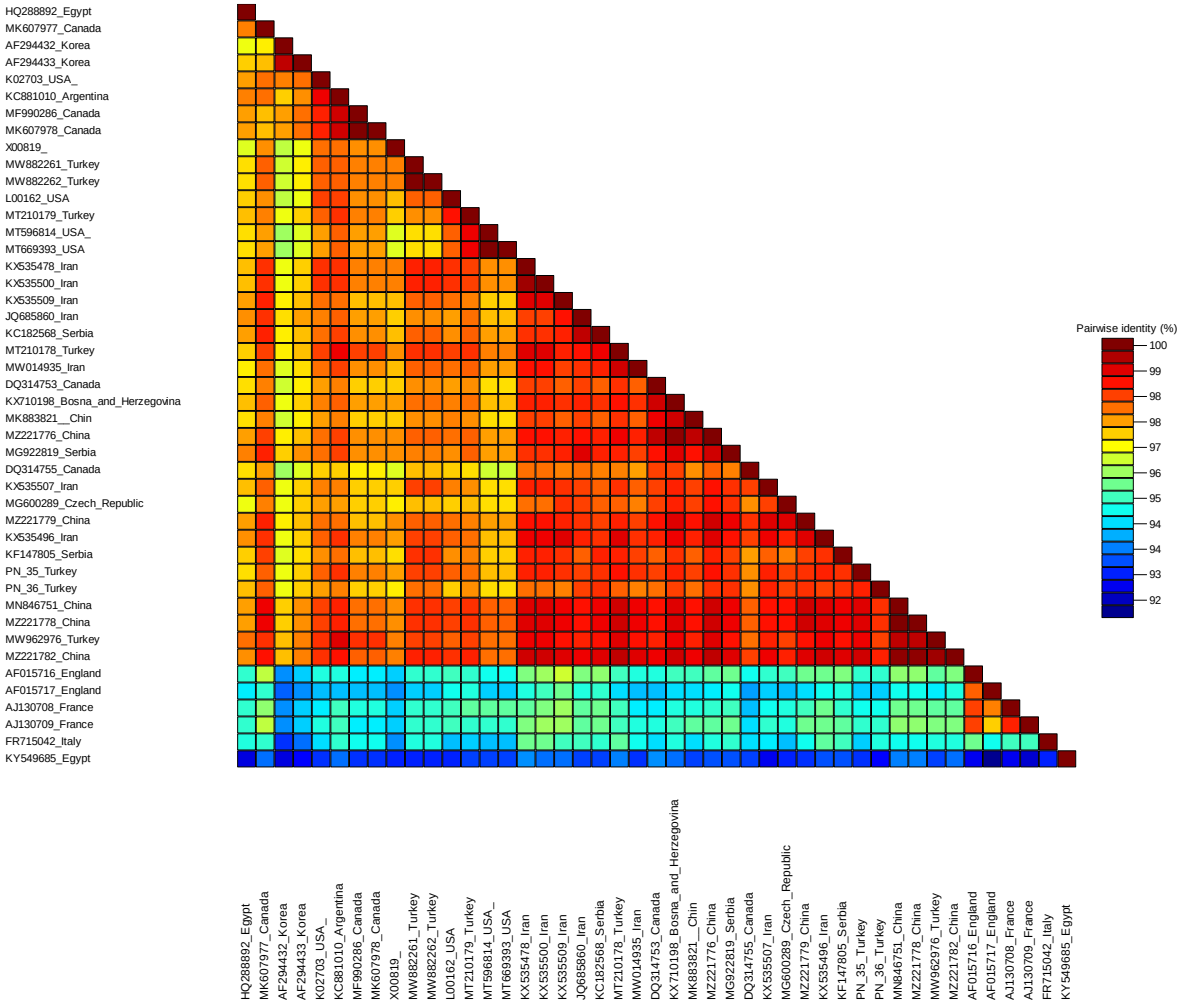

Figure 2. Similarity rates of Turkey AMV isolates with reference isolates.

Table 2. Differences in the CP amino acid sequences

Subgroup I	67	84	94	176	214
KX710198	F	G	Y	Q	E
MT210178	F	G	Y	Q	E
MT210179	F	G	Y	Q	E
MW962976	F	G	Y	Q	E
MW882261	F	G	Y	Q	E
KF147805	F	G	Y	Q	E
MG600289	F	G	Y	Q	E
MW882262	F	G	Y	Q	E
MT669393	F	G	Y	Q	E
MG922819	F	G	Y	Q	E
JQ685860	F	G	Y	Q	E
AF294432	F	G	Y	Q	E
AF294433	F	G	Y	Q	E
DQ314755	F	G	Y	H	E
DQ314753	F	G	Y	H	E
L00162	F	G	Y	Q	E
KC182568	F	G	Y	Q	E
MZ221779	F	G	Y	Q	E
MZ221776	F	G	Y	Q	E
MN846751	F	G	Y	Q	E
HQ288892	F	G	Y	Q	E

Table 2 (continued). Differences in the CP amino acid sequences

Subgroup I	67	84	94	176	214
PN 3-5	F	G	Y	Q	E
PN 3-6	F	G	Y	Q	E
Subgroup II					
AF015716	S	A	F	L	D
AF015717	S	A	F	L	D
AJ130708	S	A	F	L	D

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Investigation of activity of *Tobamovirus* in pepper plants containing *L4* resistance gene

Hakan FIDAN, Ailar GONBADI, Pelin SARIKAYA, Ozer CALIS

Department of Plant Protection, Faculty of Agriculture, Akdeniz University, 07059, The Campus, Konyaalti, Antalya, Türkiye

Corresponding author: A. Gonbadi, e-mail: a.gonbadi@yahoo.com
Author(s) e-mail: hakanfidan@akdeniz.edu.tr, pelinsarikaya75@gmail.com, ozercalis@akdeniz.edu.tr

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ABSTRACT

Pepper mild mottle virus (PMMoV) is a plant virus belonging to the *Virgaviridae* family; it significantly reduces pepper yield production worldwide. The PMMoV is spread by contaminated seeds and there is no chemical treatment available. Therefore, resistant pepper varieties containing the *L4* gene are recommended for the management of PMMoV. A considerable amount of evidence suggests that the *L4* gene confers resistance to PMMoV in pepper. The aim of the project is to confirm the status of the *L4* gene for resistance to PMMoV in pepper varieties, several inoculations were performed on pepper plants containing *L3*, *L4* resistant genes and susceptible pepper plants without the resistance genes. The *L4* resistant plants produced mottling, mosaic, leaf curl, stem necrosis symptoms in the tested pepper plants but there was no amplicon observed with specific primers of PMMoV in RT-PCR analyses. To determine if the *L3* and *L4* genes are controlling resistance to PMMoV, RT-PCR analyzes were conducted using PMMoV and *Tomato brown rugose fruit virus* (ToBRFV) where both viruses belong to the same family. The molecular studies revealed that the *L4* gene controls resistance mechanisms to PMMoV but it is not able to govern *Tobamovirus*, ToBRFV. We showed that pepper plants harboring the *L3* and *L4* gene have the ability to precisely control the mechanism of resistance to PMMoV compared to pepper plants carrying only the *L3* gene. A complete genome sequence of PMMoV was obtained and submitted to Genbank with MW523006 accession number in the NCBI system.

1. Introduction

The *Solanaceae* is a unique family within agronomically important members who are infected with the same or very closely related plant pathogens. Pepper (*Capsicum annuum* L.) is one of the most diverse vegetables in this family (Tsuda et al. 2007). The capsicum plants are roughly infected with 68 viruses belonging to *Potyvirus*, *Carlavirus*, *Potexvirus*, *Tobamovirus*, *Tobravirus*, *Luteovirus*, *Tospovirus*, and *Cucumovirus* genera. Among them, about 20 viruses are reported to cause extensive damage to this valuable vegetable (Moury et al. 2012). One of these viruses, which has been reported from different countries around the world in the last 40 years (Genda et al. 2007; Antignus et al. 2008) and restricts pepper production, is *Pepper mild mottle virus* (PMMoV), which belongs to the *Tobamovirus* genus of the *Virgaviridae* family (Secrist et al. 2018). This virus was first detected in commercial pepper varieties grown in field conditions in Turkey in 1994 (Guldur et al. 1994). The PMMoV is characterized with a typical rod-shaped particle morphology spanning 6357 bp single-stranded RNA (+ssRNA) genome which is encoding four open reading frames (ORFs). The ORF1 and ORF2 are separated by a stop codon and encode non-structural proteins that constructed a replicase complex. The ORF3 is on a large subgenomic RNA producing a non-structural movement protein (MP). The last ORF4 is on the small subgenomic RNA, encodes 17 to 18 kDa coat protein (Tsuda et al. 2007; Rialch et al. 2015). The genus *Tobamovirus* also contains ToBRFV which is another important pathogen causing serious diseases on pepper plants. The ToBRFV transmission is mainly mechanical but it can also be transmitted via contaminated seeds or fruits over long distances likely common to other *Tobamoviruses* (King et al. 2011). The virus is capable of being in direct contact with diseased plants, or infected sap from various surfaces such as harvesting, clothing, pots, packaging which can result in the mechanical transmission of the novel virus within crops (Oladokun et al. 2019). Therefore, in order to control the disease pathogen in pepper production areas, suitable cultural precautions and resistant varieties have to be used (Petrovic et al. 2010). Nowadays, pepper resistance to the viral pathogens is broken except the *L4* resistance gene which still mediates resistance to the viruses in dynamic mechanisms. On the other hand, *Tobamovirus*-tolerant varieties are available in pepper plantations, *L3* resistance breaking isolate and new *Tobamoviruses* like ToBRFV are creating potential problems in the agricultural sector. This study aims to understand the genome organization of *Tobamoviruses* and to determine whether the *L4* gene mediates resistance mechanisms in pepper plants. Therefore, since ToBRFV had not yet been reported in Turkey at the beginning of our study (2018), the route of our research shifted to the activity of the *L4* resistance gene in existing resistant pepper lines (Fidan et al. 2021).

2. Material and Methods

2.1. Preparation of infected PMMoV plants and symptomatological studies

PMMoV isolate was obtained from greenhouses where pepper is grown intensively in the Antalya province and its districts. Intense complaints, especially from the Kumluca region, determined the direction of the study. In the study, it was requested to determine whether the *L4* gene works efficiently or not. With this aim, pepper varieties used as plant material had *L3* and *L4* resistance genes used in pathogenicity tests and the results were observed in greenhouses. *L4* resistance gene source *Capsicum chacoense* pepper genotype and *L4* resistance gene, Koray F1, Mustang, Doğanay, Ozan, Vergase pepper varieties and non-resistant Caltı standard varieties were used. In the experiment established in the greenhouse, the number of plants used per cultivar was ten.

At the beginning of molecular studies, first of all, the resistance status of the cultivars declared *L4* resistance by the companies was determined using the *L4* Locus primers developed by Kim et al. (2008). *Capsicum chacoense*, which is the source of resistance, was obtained from the Alata Horticultural Research Institute (ALATA). Before starting the mechanical inoculation procedures, molecular studies were carried out using 15 different virus-specific primer pairs identified in Table 1 to determine that the source of the inoculum was only infected with PMMoV and free from other viruses. After making sure that our source of inoculum was only infected with PMMoV, mechanical inoculation processes were carried out at regular intervals both on plant materials carrying *L3* and *L4* resistance genes and on sensitive plants lacking these genes. Also, control plants were included in the experiment.

Inoculated plants were kept at 23±3°C for 16 hours during the day and 8 hours at night with appropriate culture management such as irrigation, fertilization, and pest control at 7-day intervals throughout the trial period. The entire experiment was set up in a greenhouse with no artificial lighting or heating used during the studies in 3 replications. While the plant materials were in the true second leaf stage, they were inoculated with PMMoV isolate obtained from the Akdeniz University Virology Laboratory, while in the control plants, distilled sterile water was preferred for inoculation, and finally the complementary Koch's postulates were executed.

Table 1. The 15 viruses were tested for understanding which virus causes disease on pepper plants in RT-PCR analyzes

Virus Name	Primer Name	Primer Sequences (5'→3')	Product Length (Bp)	Reference
AMV	AMV (F)	GTGGTTGGAAAGCTGGTAAA	700	(Buzkan and Yuzer 2009)
	AMV (R)	CCCCCAGTGGAGGTCAGCATT		
ChiVMV	D (F)	GGAAAGGCGATCCCGATCTACTAT	788	
	E (R)	CGCGCTAATGACATATCGGT		
CMV	CMV (F)	TAACCTCCCAGTTCTCACCGT	513	
	CMV (R)	CCATCACCTTAGCTTCCATGT		
PMMoV	P12/3 (F)	ACAGCGTTTGGATCTTAGTAT	836	
	P12/3A (R)	GTGCGGTCTTAATAACCTCA		
PepMoV	P3 (F)	AATGCAAAGCCAACATTC	345	
	M4 (R)	CTAATACGAACACCAAGCAT		
PVMV	D (F)	GGAAAGGCGATCCCGATCTACTAT	737	
	E (R)	CGCGCTAATGACATATCGGT		
PVX	PVX (F)	TAGCACAACACAGGCCACAG	562	
	PVX (R)	GGCAGCATTCATTTCAGCTTC		
PVY	PVY (F)	ACGTCCAAAATAGAGATGCC	480	
	PVY (R)	TGGTGTTCGTGATGTGACCT		
TEV	TEV-CP2-F	CTAAATGGATTTATGGTGGTGGTG	391	
	TEV-CP2-R	CAGTACCCACGTTGCCATCA		
TMV	TMV(F)	GCACATCAGCCGATGCAGC	880	
	TMV(R)	ACCGTTTTCGAACCGAGACT		
ToMV	ToMV(F)	CGAGAGGGGCAACAAACAT	318	
	ToMV(R)	ACCTGTCTCCATCTCTTTG		
TSWV	L1TSWVR	AATTGCCTTGCAACCAATTC	276	
	L2TSWVF	ATCAGTCGAAATGGTCGGCA		
TYLCV	VP2715	ATACTTGGACACCTAATGGCTATTTGG	543	
ToBRFV	ToBRFV1F	CTTCCAAACGTGTACGCACC	475	(Fidan et al. 2021)
	ToBRFV1R	ATGCATCTTCCATTGCGCTG		
General Tobamo virus	R-4718	CAATCCTTGATGTGTTTAGCAC	1052	(Tsuda et al. 2007)
	F-3666	ATGGTACGAACGGCGGCAG		

The development of symptoms was monitored from the initiation of the first symptoms to full appearance, and the inoculated plants were photographed at all stages in a greenhouse located at Akdeniz University (Figure 1). The inoculated samples were collected from fresh leaves and fruits showing typical symptoms such as chlorosis, mild mosaic on the leaf, reduced fruit size, mottling, brown necrotic spots, and streaks on fruit. The collected leaf samples were crushed in an extraction buffer in a mortar, and their total nucleic acids were isolated. In the total nucleic acids extracted, DNA and RNA ratios were measured in a one microliter (1 µl) solution and then their concentrations were optimized for further molecular studies. The mechanical inoculation procedure was repeated 3 times in 2 week intervals, the inoculated leaves were analyzed to confirm PMMoV inoculation with RT-PCR tests. Subsequently, all inoculations described above were carried out with an isolate obtained from plants showing ToBRFV symptom and collected from pepper growing areas. The ToBRFV isolate was identified and used in our trials with precautions to avoid contamination during inoculations as previously described (Davino et al. 2020). Similar mechanical inoculations were repeated using inocula from PMMoV negative, but ToBFRV positive, plant samples in RT-PCR analyses in 2019.

2.2. Verification of the L4 gene and determination of PMMoV infection by RT-PCR

Pepper plants with the *L4* gene were tested with PCR amplification using *L4* gene-specific primers to confirm the presence of the *L4* gene (Kim et al. 2008). After mechanical inoculation, total nucleic acid isolation was performed from plants with typical virus symptoms. The nucleic acid extractions from PMMoV and ToBRFV inoculated pepper plants were conducted using GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, USA). For *L3* and *L4* gene analyzes, GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. All inoculated plant materials were tested for PMMoV infection with PMMoV-specific primers using Verso One-step RT-PCR ReddyMix kit (Thermo Fisher Scientific, USA) in RT-PCR analyzes.

2.3. Screening of plants containing L4 gene in terms of Tobamoviruses

We observed disease symptoms on pepper plants containing the *L4* gene; they were showing typical leaf and fruit symptoms inoculated with the *Tobamovirus* genus. ToBRFV which belongs to the *Tobamoviruses* was first reported on tomato plants (Caglar et al. 2013). The virus is known to infect the *Solanaceae* family's plants, with this information a separate trial was immediately conducted with ToBRFV using all these plants. Resistant plants with both the *L3* and *L4* genes and susceptible pepper plants without any of these genes were mechanically inoculated with the ToBRFV isolate, followed by inoculated plants transferred in a growth chamber. After symptoms developed on the inoculated pepper plants, RT-PCR analyzes were carried out using specific primers to ToBRF (Fidan et al. 2021).

2.4. Designing of PMMoV specific primers

For PMMoV, a complete genome was constructed with specific primers using the Primer-BLAST program from the National Center for Biotechnology Information (NCBI) system. Specific primer pairs (Table 2) were designated and synthesized in a commercial company (Nanogen Medical, Turkey). After minor errors were corrected using the MEGA 7.0 (Stecher et al. 2020) and Chromas (version 2.6) programs, the whole genome of PMMoV was aligned with these specific primer combinations (Table 2). Additionally, to obtain sequences from the 5'-ends, the FirstChoice® RLM-RACE Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. This generated a single sequence overlapping and bidirectional, with the forward and complementary sequences provided 6357 bp length complete genome of PMMoV. The 6313 bp complete genome of the ToBRFV isolate in pepper was obtained using specific primers as previously described (Fidan et al. 2021).

Figure 1. Symptoms of mottling, chlorosis, and curl signs are observed in leaves with mechanical inoculation with *Pepper mild mottle virus* (1, 2, 3, A). A hypersensitive response (HR) is appeared on leaf harboring *L4* resistance gene (B). The pepper plants have typical trunk necrosis on stems (C).

Table 2. Primer pairs are designed within the primer BLAST program at National Center for Biotechnology Information (NCBI) system

Primer pairs	Sequence (5'->3')		Starting point	Ending point	TM	Product length (bp)
Primer 1	Forward	GGGAATAACCCCTTGGTGAA	121	140	57.09	153
	Reverse	CTCAGGGTAGGCCTTAGTTG	273	254	57.01	
Primer 2	Forward	GGGAATAACCCCTTGGTGAA	121	140	57.09	1194
	Reverse	TAAGCGCTTTCGACTGGTAT	1315	1296	57.05	
Primer 3	Forward	CTGTCGCTTTGCACAGTTTA	662	681	56.96	654
	Reverse	TAAGCGCTTTCGACTGGTAT	1315	1296	57.05	
Primer 4	Forward	ACATAGGCGCCTTCTTCTCG	803	822	59.90	1047
	Reverse	TTGCTGCCACCAATGGATCT	1849	1830	59.96	
Primer 5	Forward	TGGGATGAGATTACAGCCGC	1525	1544	59.89	751
	Reverse	TCGCAGCTGTGTCCTTGATT	2275	2256	59.96	
Primer 6	Forward	ATTTAGACAGCCTGGTAGCC	2201	2220	56.99	743
	Reverse	GACCTCGAGTTGACTCACAT	2943	2924	56.98	
Primer 7	Forward	ATGTTACACCCTGGTTGTGT	2800	2819	56.96	729
	Reverse	CGGCAAACACTTGTCGTAAT	3528	3509	57.04	
Primer 8	Forward	GTGTTAACCTTTTCGTCGCA	3452	3471	56.98	622
	Reverse	AGCGCATTGATTTTCTTGCT	4073	4054	56.98	
Primer 9	Forward	CCGTTGATCAATACAGGCAC	3953	3972	56.89	607
	Reverse	CCCTGTTGAATATCGGGGAA	4559	4540	56.98	
Primer 10	Forward	GGTGCGAACCTTCTCTGGAA	4558	4577	59.97	1098
	Reverse	CGACTCCGAGTTCAACCCAA	5655	5636	59.97	
Primer 11	Forward	ATCAGTTCCAATGGCTGACA	5505	5524	57.11	799
	Reverse	CGTTCGCTAATACACGTCAC	6303	6284	57.05	

2.5. RT-PCR amplification, sequencing and phylogenetic studies

RT-PCR amplification was carried out in a total volume of 15 µL containing: 1 µL template RNA, 200 nmol of each primer, 0.25 µL Verso enzyme mix, 0.75 µL RT-Enhancer, 7.5 µL One-Step RT-PCR ReddyMix (Thermo Fisher Scientific, USA), and 3.5 µL nuclease-free water. The amplified products were run on 1.5% agarose gel then amplified fragments were cut from the gel and purified using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, USA). The sequences of the amplified and gel-purified PCR products were obtained from Medsantek Company (Istanbul, Turkey).

The RT-PCR program executed the reverse transcription of RNA at 50 °C for 30 min, and performed PCR step at 95°C for 2 min followed by 35 cycles at 95 °C for 30 s, 52 °C for PMMoV and 59 °C for ToBRFV for 30 s, and 72 °C for 1 min, followed by a final 72 °C extension step for 5 min. The entire PMMoV sequences were deposited on pepper (Ailar3, MW523006) in the GeneBank Database at NCBI. Furthermore, the whole PMMoV sequence was compared with 10 available sequences from different countries in the world in the NCBI database. A phylogenetic tree was constructed to understand the relationships of PMMoV to other PMMoV isolates (Table 3).

3. Results and Discussion

Pepper plants containing the *L4* gene were resistant to PMMoV without any leaf symptoms but the capsicum plants showed brown streaks in fruits during warm periods in the Mediterranean region, Turkey. The fruit symptoms seem strange; they are likely produced by PMMoV creating misconceived situations. Further symptomotological observations revealed that there were no virus symptoms developed until fruiting on which hypersensitive reactions (HR) were observed on the plants harboring the *L4* gene (Figure 1). Two weeks after mechanical inoculations, typical virus disease symptoms such as dwarfing on young plants, puckering, and yellow mottling on leaves appeared (Figure 2).

When inoculated pepper plants reached the fruiting period, their fruits were deformed and their size slightly reduced than older fruits which exhibited brown streaks with undesirable colors. The detection of ToBRFV by RT-PCR confirmed the presence of ToBRFV in tested pepper plants displaying similar symptoms with PMMoV inoculated resistant plants (Figure 3). The experiment was started in spring 2018 under controlled conditions and continued until the first days of summer. With the warming of the weather, the symptoms seen in the material plants began to appear more intensely. This situation was attributed to the fact that both the viruses had enough time to multiply in the plant and that the resistance might have been broken as a result of the increase in temperature.

Although, the inoculated resistant pepper plants were free from PMMoV infection, indicating that the *L4* gene is still conferring resistance to PMMoV, the *L4* gene-mediated resistance was no longer controlling resistance to the ToBRFV isolates either above 32 °C temperatures or repetitive inoculations (Figure 3). In mixed infections, it was very difficult to discriminate the PMMoV symptoms from *Tomato spotted wilt virus* (TSWV) symptoms (Fidan and Sarı 2019) which has been causing epidemics on-field and greenhouse grown pepper plants. Although, pepper seeds in fruits did not darken withTSWV infection, pepper seeds darkened from light to bold brown color as observed in PMMoV infections (Figure 4).

Visually, this is one of the best ways to distinguish the two viral diseases symptomatologically. We ensured that the *L4* gene still mediates resistance to PMMoV but it is not responsible to control resistance to ToBRFV. Producers have problems in mixed infections with TSWV and ToBRFV causes epidemics in all pepper-growing areas in the world. In the study, the *L4* gene

Table 3. Complete genome sequences of *Pepper mild mottle virus* isolates used in phylogenetic analyzis

Isolate Name	Origin	Source	GenBank Accession Number	Identity%
BL14	U.S.A	Pepper	MH063882	94.31
Chaff RNA	Korea	*Achyranthes aspera*	LC538100	94.49
ZJ2	China	Pepper	MN616927	94.65
BR-DF01	Brazil	Pepper	AB550911	94.31
PMMoV-16.9	India	Pepper	MN496154	94.60
VE	Venezuela	Pepper	KU312319	94.34
PMMoV-WW17	Slovenia	Tobacco	MN267900	94.37
IW	Japan	Pepper	AB254821	94.70
Spainish isolate	Spain	Pepper	AJ308228	100
Ailar3	Turkey	Pepper	MW523006	100
TBRFV-Ant-Pep	Turkey	Pepper	MT118666	93.33

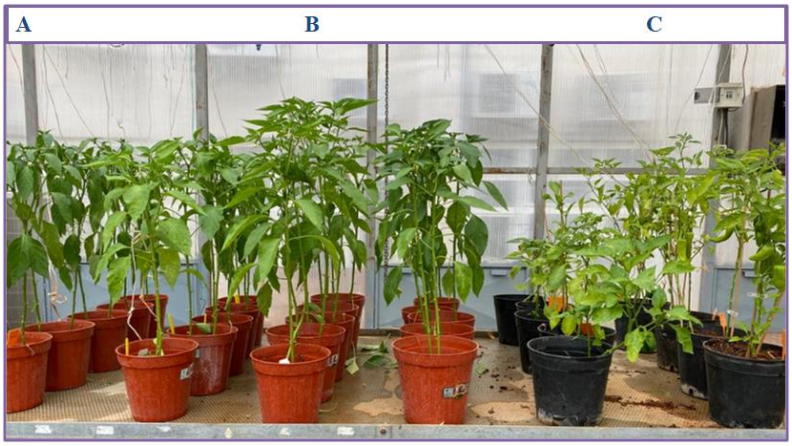

Figure 2. Plants carrying the *L3*, *L4* resistance gene and susceptible pepper plants lacking any of these genes, thet are mechanically inoculated with PMMoV. A) *L3* gene mediated resistant pepper plants, B) *L4* gene mediated resistant capsicums and C) Susceptible pepper plants containing any resistance gene.

Figure 3. Plants carrying the *L3*, *L4* resistance gene and susceptible pepper plants lacking any of these genes. are mechanically inoculated with ToBRFV. Their phenotypic reactions are observed at 30 days post inoculation. a) *L3* gene containing pepper fruits are infected with ToBRFV with typical Kebab appearance; b) *L3* resistance gene containing pepper plants infected with ToBRFV show mosaic symptoms on leaves; c) Healthy control pepper plants are inoculated with distilled water without any symptoms; d) Mottling symptoms in pepper plants infected with ToBRFV carrying the *L3* resistance gene. e) L3 and L4 gene containing pepper plants' stems are showing trunk necrosis, and f) L4 resistance gene containing capsicums are exhibiting HR after ToBRFV inoculations. The inoculated plants are kept below 32 °C in the growth chamber.

still provided resistance to PMMoV but further molecular analyzes revealed that ToBRFV was not able to control the *L4* resistance gene. Molecular studies with RT-PCR-based amplification using PMMoV specific primers showed that PMMoV infection is not detected in *L4* resistant plants, whereas viral infection is confirmed in no gene containing and *L3* gene containing pepper plants with amplifying 836 bp fragment to PMMoV (Figure 4).

Therefore, these results indicate that the *L4* gene mediates resistance against PMMoV infection and the *L4* resistance gene will be able to be used to control PMMoV infection for breeding studies in Turkey. Sequence data analysis revealed that there is no mutation in the genome of the PMMoV isolate (Ailar3) When comparing open reading frame (ORF) regions; no mutation was found in the (Ailar3). There is no mutation seen and the *L4* gene effectively mediates resistance against PMMoV in pepper plants. The sequence of the PMMoV was submitted to the NCBI GenBank with an MW523006 accession number.

A phylogenetic tree was constructed using our PMMoV sequence and other available sequences on the NCBI database, the constructed phylogenetic tree was divided into two main groups as Group 1 and Group 2 (Figure 5). Group 1 is further subdivided into subgroups 1a and 1b, respectively. The PMMoV isolate used in our study was in the same group (Group 2) as the Spanish and Korean isolates. These results indicate that there are close relationships among Turkish, Spanish, and Korean PMMoV isolates.

The results also suggest a divergent group of PMMoV isolates which share specific clustering motifs. When all the obtained ToBRFV (MT118666) and PMMoV (MW523006) genomes were compared, it was determined that they were typical *Tobamovirus* members with 4 ORFs as the genome structure, but when their ORF structures were analyzed on the basis of nucleotides, they were found to be separate viruses. These results also ensured that the two viruses were included in two separate branches in the phylogenetic tree (Figure 5).

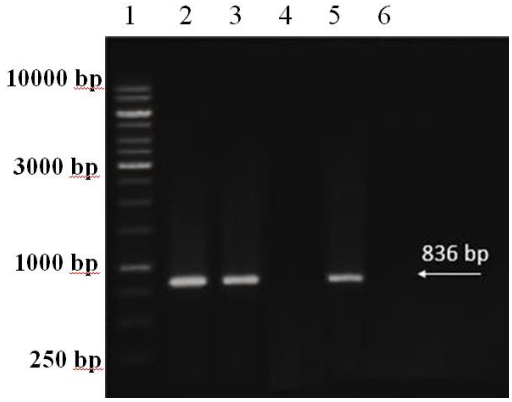

Figure 4. The *L3*, *L4* resistance gene and no gene containing susceptible pepper plants are inoculated with PMMoV and their total nucleic acids are studies in RT-PCR analyzes. 1) 1kb DNA ladder; 2) *L3* gene containing pepper plant; 3) *L4* gene containing pepper plant, 4) None of a gene containing susceptible pepper plant; 5) The PMMoV positive control pepper plant, 6) The PMMoV negative control pepper plant.

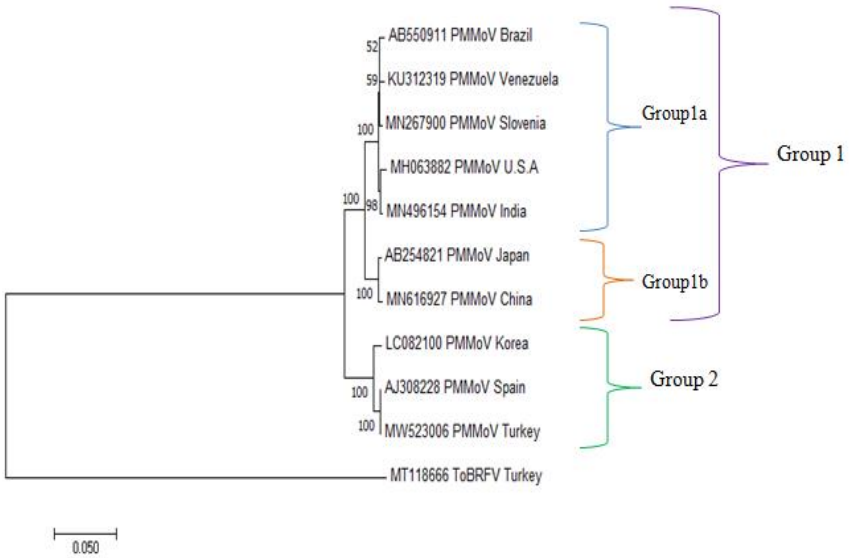

Figure 5. A phylogenetic tree was constructed with known PMMoV and ToBRFV Turkey sequences. It is clear that PMMoV is different from ToBRFV. All sequences were analyzed using MEGA 7.0 software according to the neighbor-joining method.

Although PMMoV and ToBRFV are two separate types of viruses in the *Tobamovirus* genus, it is difficult to distinguish their symptoms on pepper plants. Both viruses can be transmitted viainfected seeds, mechanical inoculations, Bombus bees, and irrigation. These viruses cause morphological changes in host cells resulting in dwarfism (Afaf et al. 2017), chlorosis, mottling, deformations, bleaching. It is known that all viruses are insensitive to certain chemicals; therefore, resistant pepper varieties are the only effective method for viral disease management. For the production of resistant pepper varieties, reliable sources of resistance are needed in the breeding studies of pepper seeds.

4. Conclusion

We observed the presence of PMMoV in the tested pepper plants under different temperature conditions during four seasons. As a result of the typical symptoms similar to the *Tobamovirus* group in peppers in 2018, it was thought that the *L4* gene-mediated resistance was broken in these plants. however, with the report of another virus belonging to the same family (ToBRFV) that caused similardisease symptoms in Turkey in 2019 (Fidan et al. 2021), the course of the study was shifted to this new virus, which has caused epidemics in pepper growing areas around the world. The molecular analysis performed revealed that the *L4* gene is most likely to control resistance to PMMoV (Hamada et al. 2002), but the *L4* resistance gene is not responsible for controlling ToBRFV. In RT-PCR assays utilizing specific primers, ToBRFV was found in plants with *L4* resistance.

This result revealed that the ToBRFV overcame the *L4* mediated resistance and it is likely that the *L3* resistant pepper plants are very susceptible to both PMMoV and ToBRFV infections with severe symptoms. Molecular studies were carried out by giving priority to the *Tobamoviruses* in studies conducted to investigate the source of infection. Accordingly, if there is an *L4* resistance gene in the infected pepper plant and symptoms are seen, it can be said that the cause of this infection is ToBRFV. If the infected pepper plant has *L3* resistance and *L4* resistance, the cause of the infection may be PMMoV and ToBRFV, respectively. The source of infection can easily be detected by the RT-PCR method using PMMoV and ToBRFV specific primers (Fidan et al. 2021). In our study, we aimed to determine the susceptibility or resistance levels of pepper fields against PMMoV infections, scanned samples using PMMoV genome primers in molecular studies, and obtained the complete genome sequence from samples with positive results.

Additionally, it has been found that the infections which cause browning and necrosis around the seed in plants containing the *L4* gene that provides resistance to PMMoV were not caused by PMMoV but by another virus in the *Tobamovirus* group, namely ToBRFV. In Turkey, PMMoV has been identified on pepper several times since 2013 (Caglar et al. 2013). In a study conducted in Antalya, it was reported that genes that provide monogenic resistance to TSWV such as *Tsw* and *Sw-5* become inactive at high temperatures and the state of resistance disappears (Kabas et al. 2021).

As a result of this study, it was revealed that the *L4* gene was broken by the ToBRFV in infections above 32 °C and consecutive infections. In other words, while resistance to the *Tobamovirus* group is effective under 32 °C, it can break at high temperatures (Kabas et al. 2022). The similarity of these symptoms with PMMoV showed that the *L4* gene still retained its activity against PMMoV. In cases, where the temperature limit of 32°C is exceeded, it is of great importance to conduct resistance studies against ToBRFV disease, which causes severe symptoms, and to find a new source of resistance.

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Morphological and physiological variation in drought tolerance of wheat landraces originated from southeast Türkiye

Kubra BUDAK[1], Husnu AKTAS[2], Sertan CEVIK[3]

[1]Akdeniz University, Department of Agricultural Biotechnology, Antalya, Türkiye
[2]Mardin Artuklu University, Kiziltepe Vocational School, Kiziltepe, Mardin, Türkiye
[3]Harran University, Department of Molecular Biology and Genetics, Sanliurfa, Türkiye

Corresponding author: S. Cevik, e-mail: srtncvk@gmail.com
Author(s) e-mail: budakkubra35@gmail.com, husnuaktas@artuklu.edu.tr

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ABSTRACT

Drought stress, which is the most important abiotic stress factor affecting arable land in the world, causes serious crop losses. These crop losses reach up to 70% in some agricultural plants. Understanding the complex drought stress response is very important to develop a strategy against this form of stress. Although some progress has been achieved with the previous studies, the desired targets have not been reached up to now. Therefore, using resistant varieties in environmental conditions has become a widely used strategy in combating drought stress today. In this study, a total of 23 cultivars (16-landraces and 7 modern wheat cultivars) were used. The aim of this study was to reveal the drought tolerance degrees of 16 landraces by comparing them to 7 modern wheat cultivars. For this purpose, 23 cultivars were exposed to drought stress for seven days by withholding watering. After that, stem length, MDA and proline content of cultivars were determined and compared. According to our results, MDA and proline contents of sensitive modern cultivars were found to be high, while tolerant cultivars were found to be low. It has also been determined that some of the landraces exhibit a similar profile to the cultivars known to be tolerant. Among these cultivars, especially 88, 90 and 108 cultivars have low MDA and proline content under stress, which may indicate that these cultivars are potentially drought tolerant.

1. Introduction

The world's population has been dramatically increasing and is expected to hit 9 billion by 2050, food demand is also increasing accordingly. Wheat is one of the most cultivated agricultural products in the world and the demand for wheat is increasing day by day (Dixon et al. 2009). Drought has become one of the major challenges for crop productivity, as a result of altered precipitation patterns and insufficient rainfall (Toker et al. 2007; Mir et al. 2012). In agricultural areas, drought stress is often accompanied by high temperatures, which directly affects the kinetics of photosynthesis. The fact that photosynthesis kinetics are affected by environmental stresses causes significant decrease in crop yield (Tuberosa and Salvi 2006).

Plants have developed different mechanisms to cope with drought stress. Insufficient understanding of the physiological basis of drought stress has made it difficult to improve drought-tolerant crop varieties (Sinclair 2011). The seedling stage is critical to coping with drought stress. Physiological and morphological characteristics such as MDA content, proline, the water content in leaf, root and stem length provide important data to understand the drought tolerance state of wheat during the seedling stage (Polania et al. 2017; Mwenye et al. 2018; Chun et al. 2018; Dien et al. 2019).

Food security will rely on improved resistance cultivars to drought (Borlaug 2007; Tester and Langridge 2010; Chapman et al. 2012). Breeders need large variations in wheat that are responsible for drought resistance traits. Southeast Anatolia, part of the Fertile Crescent is the origin and site of genetic diversity for wheat. Durum and bread wheat landraces have been cultivated since ancient times in southeast Anatolia (Gökgöl 1939; Özkan et al. 2011). Wheat landraces have a trait that is resistant to biotic and abiotic stress (Nevo et al. 2002). Responses of wheat landraces under drought stress provide an opportunity to understand the mechanism of drought tolerance and gene discovery related to drought tolerance. Researchers depicted that landraces cultivated under biotic and abiotic stress conditions have better performance compared to modern wheat varieties in terms of vigor in the seedling stage, for some morphological parameters and grain yield (Aktaş et al. 2017; Aktaş et al. 2018).

Wheat landraces have the ability to adapt to arid and semi-arid areas due to their genetic variation. Domestication of wheat took place in southeast Anatolia and that part of the Fertile Crescent has rich genetic diversity in terms of wheat landraces. Plant responses to environmental stresses can be studied by evaluation of traits at morphological, physiological and molecular levels (Praba et al. 2009). It is important to carry out such studies, as the characterisation of landraces that have a wide variation in terms of phenotypic and physiological defense mechanisms will contribute to the development of model plant varieties for plant breeders. The response of wheat landraces to

drought stress provides unique information because of their huge genetic diversity. We aimed to determine the drought tolerance level of wheat landraces from the Fertile Crescent by comparing them to modern wheat cultivars under drought stress. For this purpose, malondialdehyde (MDA) and proline content, and shoot length were evaluated under drought stress in this study. 16 major landraces which originated from the Fertile Crescent plus 2 sensitive (Atik, Güney Yildizi) and 5 drought tolerant modern wheat cultivars were used. Genotypes used in the study may be useful material candidates for cultivation and for a better understanding of the mechanisms of drought tolerance for the seedling stage in wheat.

2. Materials and Methods

2.1. Plant material and stress treatment

In the present study, 23 different wheat genotypes (Table 1) were used. After the seeds were sterilized, they were planted in pots containing a 5:3:2 soil:peat:sand mixture. The field capacity of the prepared soil mixture was determined before sowing and the seeds were sown in this soil mixture which was irrigated up to the field capacity. Seeds irrigated once a week were grown for three weeks after germination, and half of the plants were exposed to drought stress for 7 days by withholding watering. At the end of this period, all the plants were harvested and placed in liquid nitrogen and stored in a deep freezer until the analysis.

2.2. Measurement of stem length

Stem length was measured from 5 plants of each variety. The main stem length was measured with a ruler. Average values were calculated for each variety.

2.3. Determination of MDA content

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) level according to Ohkawa et al. (1979). Leaf tissue (0.25 g) was homogenised 2 mL (5%) trichloroacetic acid (TCA) solution. The homogenate was centrifuged for 10 min at 8000 rpm. After that, supernatant, thiobarbituric acid and TCA solutions were mixed in equal volumes in tubes and tubes were incubated at 96ºC for 25 minutes. The tubes were placed in an ice bath to terminate the reaction and centrifuged at 6000 rpm for 5 min. The mixture was measured at 532 and 600 nm. The MDA content was calculated by using the extinction coefficient.

2.4. Determination of free proline content

Free proline content was determined according to Bates et al. (1973). Leaf tissue (0.5 g) was homogenised in 3% sulfosalicylic acid. The homogenate was centrifuged for 3 min at 3000 rpm and then the supernatant mixed well with acid ninhydrin and glacial acetic acid in equal volumes and incubated at 100ºC for 1 hour. The reaction was terminated by adding cold toluene (4 mL) to the tubes. The toluene phase was evaporated and analysed by spectrophotometry at 520 nm. The proline level was determined from a standard curve.

2.5. Statistical analysis

Variance analyse was performed by using GENSTAT 12th (GENSTAT 2009) statistical program and the difference between the mean of the data.

Table 1. Wheat cultivars used in the study

Number/Cultivar	Origine	Traits	Drought Tolerance
8	Diyarbakır	High Plant Height	Tolerant
16	Diyarbakır	High Plant Height	Tolerant
25	Diyarbakır	High Plant Height	Tolerant
29	Diyarbakır	High Plant Height	Tolerant
Sorgül	Diyarbakır	High Plant Height	Tolerant
30	Adıyaman	High Plant Height	Tolerant
46	Adıyaman	High Plant Height	Tolerant
58	Adıyaman	High Plant Height	Tolerant
70	Adıyaman	High Plant Height	Tolerant
73	Mardin	High Plant Height	Tolerant
85	Mardin	High Plant Height	Tolerant
87	Mardin	High Plant Height	Tolerant
88	Mardin	High Plant Height	Tolerant
90	Şırnak	High Plant Height	Tolerant
108	Şırnak	High Plant Height	Tolerant
109	Şırnak	High Plant Height	Tolerant
Atik	Private Company	Medium Plant Height	Sensitive
Güney Yıldızı	Research Inst.	Medium Plant Height	Sensitive
Fırat-93	Research Inst.	Short Plant Height	Tolerant
Aydın-93	Research Inst.	High Plant Height	Tolerant
Sümerli	Research Inst.	Medium Plant Height	Medium Sensitive
Sarıçanak	Research Inst.	Medium Plant Height	Medium Sensitive
Svevo	Italy	High Plant Height	Tolerant

3. Results and Discussion

Drought stress increased the Malondialdehyde (MDA) content in all wheat cultivars used in this study. The five cultivars whose MDA content increased the most under drought stress were 73, Atik, 8, 46 and 30, respectively, while those with the least increase were Fırat, Svevo, Sarıçanak, 88 and Sümerli (Figure 1). MDA, the end product of lipid peroxidation, is one of the important indicators of oxidative stress. The MDA content reflect the degree of damage under adverse conditions (Yang and Deng 2015). High MDA content is known to be an indicator of membrane damage caused by oxidative stress (Gawel et al. 2004; Morales and Munné-Bosch, 2019).

Mehmood et al. (2020) determined a good correlation between MDA and H_2O_2 level. This finding may indicate that the increase in ROS that occurs under stress conditions causes membrane damage and that the MDA content increases as an indicator of this situation. In many studies, it has been shown by various researchers that the MDA content increases with drought stress in different plants (Pandey et al. 2010; Yildizli et al. 2018; Khaleghi et al. 2019). However, it has been reported that cultivars with lower MDA content under drought (Ma et al. 2015; Mihaljević et al. 2021) and salt (Kiran et al. 2019) conditions have higher tolerance to these stresses. Based on this information in the literature, it can be said that the Fırat, Svevo, Sarıçanak, 88 and Sümerli varieties used in our study are more tolerant in terms of MDA content under drought conditions. In many studies comparing cultivars with known tolerance under environmental stress conditions, it has been shown that tolerant cultivars have a much stronger antioxidant system and, accordingly, suppress oxidative damage caused by stress more rapidly and more strongly than sensitive cultivars (Sultan et al. 2012; Amoah et al. 2019). This information provides clues that the antioxidant systems of these five cultivars may be stronger than other cultivars. The data obtained may shed light on the molecular studies planned to be conducted on this subject.

Proline content of all cultivars also increased under drought stress. The five cultivars with the highest increase in Proline content were 87, 46, Atik, 25 and 73, while those with the least increase were 70, Sarıçanak, 90, Fırat and 109 under drought stress (Figure 2). When the proline results are examined, it can be seen that the proline contents of Fırat and Sarıçanak varieties, which have low MDA content under drought conditions, are also lower than other varieties. The increase in Proline content under

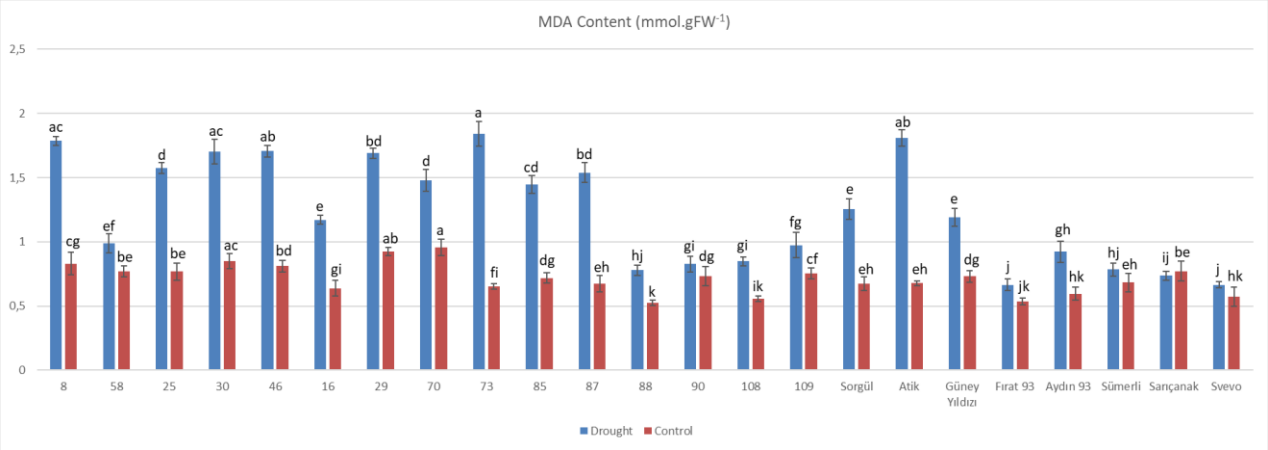

Figure 1. MDA content in leaves of wheat plants under drought and control conditions. Data are showed as mean ± SE of three independent biological replicates. Different letters indicate significant differences between groups $P < 0.05$ (Drought LSD 0.175, Control LSD 0.110).

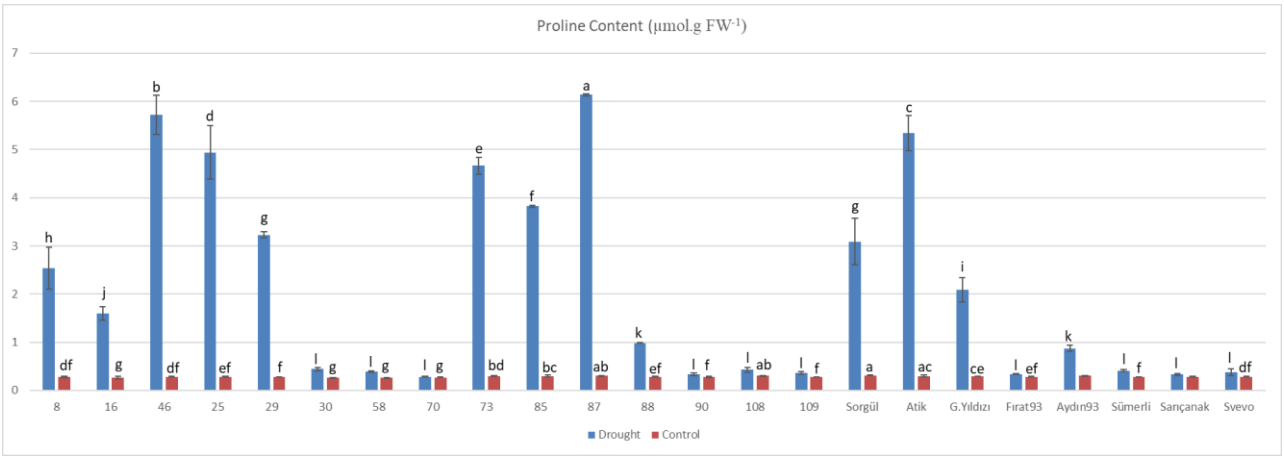

Figure 2. Proline content in leaves of wheat plants under drought and control conditions. Data are showed as mean ± SE of three independent biological replicates. Different letters indicate significant differences between groups $P < 0.05$ (Drought LSD 0.173, Control LSD 0.011).

various environmental stresses has been shown in many studies (Johari-Pireivatlou 2010; Ahmed et al. 2017; Chun et al. 2018). However, when studies using cultivars with different tolerances are examined, it can be seen that there are different results in the literature in terms of proline content under stress. In some of these studies, proline content was found to be higher in tolerant cultivars (Solanki and Sarangi 2014; Mwadzingeni et al. 2016; Dien et al. 2019), while in some others it was found to be higher in sensitive cultivars (Duangpan et al. 2007; Ergen et al. 2009; Marček et al. 2019). This situation may occur depending on the species or varieties. Marček et al. (2019) emphasized that proline should not be shown as a specific drought tolerance indicator for wheat varieties. The best-known feature of proline is that it is a good osmoprotectant. It is thought that the amount of water-related stress increases and contributes to the protection of water under stress. It is also reported to have antioxidant properties (Arteaga et al. 2020). In our study, the high proline content of cultivars with high MDA content (Atik, 46 and 73) may be an indication of high oxidative stress in these cultivars. The low proline content of cultivars with low MDA content (Firat, Sarıçanak, Svevo and Sümerli) may be an indication that these cultivars are exposed to a lower oxidative stress compared to cultivars with high proline content. However, it is thought that this stress response can be better understood by investigating the changes in the activities and amounts of antioxidant system elements under drought conditions. Isoenzyme analysis may be the best indicator to show antioxidant responses of these cultivars under drought stress.

Drought stress decreased stem lengths (Figure 3) in all cultivars. However, these reductions were not statistically significant between cultivars in this study. When the literature is examined, it is seen that there are many studies showing that drought stress reduces stem lengths (Rauf et al. 2007; Polania et al. 2017; Mwenye et al. 2018). Studies show that different results can be obtained depending on the onset time of stress and the application period.

4. Conclusion

When all the results were evaluated together, it was seen that both MDA and proline content of the Atik variety, which is known to be sensitive, were high, and stem length was also suppressed by drought stress. On the contrary, other cultivars known to be tolerant to drought were found to have particularly low MDA and proline content. However, it has been determined that there are local cultivars that exhibit a profile similar to the cultivars known to be tolerant. Among these cultivars, especially 88, 90 and 108 cultivars have low MDA and proline content under stress, which may indicate that these cultivars are potentially drought tolerant.

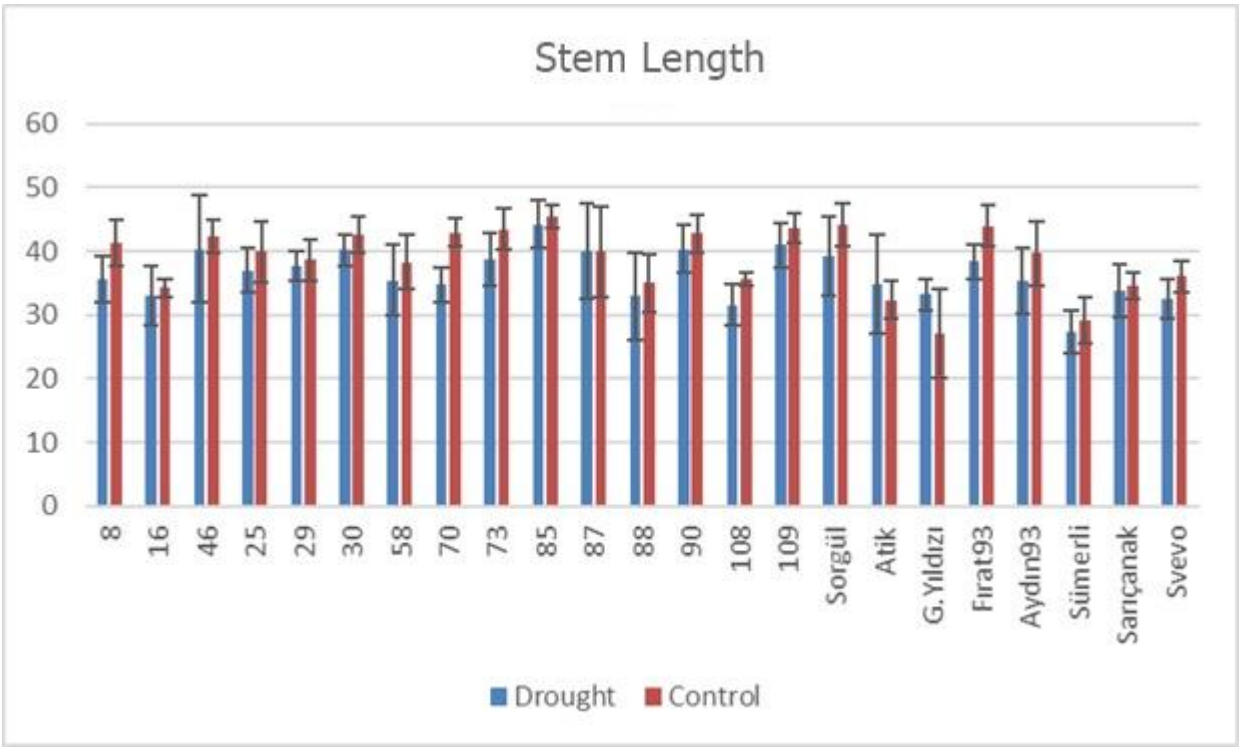

Figure 3. Shoot length of wheat plants under drought stress.

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Assessment of irrigation water salinity effects on red beet under Mediterranean conditions

Ahmet KURUNC[1], Kivanc Hayri DOGANAY[2]

[1]Faculty of Agriculture, Department of Farm Structures and Irrigation, Akdeniz University, Antalya, Türkiye
[2]Faculty of Agriculture, Department of Farm Structures and Irrigation, Dicle University, Diyarbakir, Türkiye

Corresponding author: A. Kurunc, e-mail: akurunc@akdeniz.edu.tr
Author(s) e-mail: hayri.doganay@dicle.edu.tr

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ABSTRACT

Plant tolerance to salinity stress is vital for irrigation scheduling, decision-making, planning and operation, and most critically, water resource management. Although there are numerous scientific data on the response of various plants to salinity stress, there are few studies on red beet in the literature, and specifically under Mediterranean conditions. This study aimed to investigate the effects of water salinity stress on water use, growth, yield parameters, and salinity threshold and slope values of red beet in Mediterranean conditions. In addition to control (0.6 dS m^{-1}), five irrigation water salinity levels including low (1.5 dS m^{-1}), medium-low (3.0 dS m^{-1}), medium (4.5 dS m^{-1}), medium-high (6.0 dS m^{-1}) and high salinity (8.0 dS m^{-1}) stresses were used as treatments. Increased water salinities caused increases in electrical conductivity and pH values of saturated soil paste extracts and drainage waters, while decreases in water use affected plant height storage root yield and water use efficiency. The salinity threshold and slope values of red beet were determined as 3.10 dS m^{-1} and 4.42% per dS m^{-1}.

1. Introduction

Salinity limits plant productivity, particularly in arid and semi-arid climates and is seen to be one of the most significant environmental challenges (Ashraf and Harris 2004). Soil and/or irrigation water salinity is one of the major abiotic stress factors on agriculture worldwide, and the situation has worsened over the last 20 years due to the increase in irrigation requirements in arid and semi-arid regions such as those found in the Mediterranean region (Munns and Gilliham 2015). It is estimated that about 20% of total cultivated and 33% of irrigated agricultural lands are affected by high salinity in the world. Furthermore, the salinized areas are increasing at a rate of 10% annually for various reasons such as low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices (Jamil et al. 2011). Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al. 2003; Jamil et al. 2011).

Even though, most of the salinity and all of the sodicity is natural, a significant proportion of recently cultivated land has become saline because of land clearing, shallow saline water tables and saline irrigation water especially coupled with poor irrigation management. Crops grown on saline soils suffer on an account of high osmotic stress (physiological drought), ion toxicities, nutritional disorders (ionic stress), poor soil physical conditions and reduced crop productivity (Shrivastava and Kumar 2015). However, with proper scheduling, saline water available in different regions of the world has been used successfully for irrigation purposes (Rhoades et al. 1992). Theiveyanathan et al. (2004) claimed that accurate scheduling of irrigation, essential for maximizing crop production, requires a good knowledge of water demand and salinity tolerance of the crop in addition to soil water characteristics.

Soil salinity response and tolerance of plants vary widely among crop species and varieties. Although salinity threshold and slope values of more than 130 crop species have been determined under experimental conditions, there is an obvious need for research since little or no useful information exists on crop salt tolerance for a great number of species (Shannon and Grieve 1999). The purpose of this study was to generate realistic data on red beet (*Beta vulgaris* var. *Conditiva* Alef.) under irrigation water salinity levels up to 8.0 dS m^{-1} to fill this gap in the literature.

2. Materials and methods

2.1. Experimental site

The experiment was carried out at the Akdeniz University's Agricultural Research and Implementation Area in Antalya, Turkey, under a polyethene-covered rain-out shelter with uncovered sides. The experimental area is located at 36° 53' 15" north latitude and 30° 38' 53" east longitude, with an average altitude of 54 meters. The Mediterranean climate prevails in this 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 mm, 538 mm falling between January and April, 61 mm between May and September, and 460 mm between October and December (Anonymous 2021).

2.2. Plant material

The plant material used was the red beet cultivar of *Beta vulgaris* var. *Conditiva* Alef.. As a cool climate vegetable, it grows best at 15-18ºC in well-drained loam, sandy or clayey loam soils. The tap root of the plant can reach a depth of 30-40 cm. The plant has the highest water consumption during the period when the storage roots begin to develop. Compared to storage roots, K, Mg, Na, P and vitamin A and C are richer in fresh leaves. Although the fresh beet leaves are used as a filling ingredient of the pasteries, the main part of the plant which is consumed is the storage roots which are pickled or canned (Şalk et al. 2008).

2.3. Experimental design and treatments

The experiment was conducted as a randomized complete block design with four replications per treatment. There were six irrigation water salinity levels (S) with different electrical conductivities including S_0= 0.6 dS m^{-1} (control), S_1= 1.5 dS m^{-1} (low), S_2= 3.0 dS m^{-1} (medium-low stress), S_3= 4.5 dS m^{-1} (medium), S_4= 6.0 dS m^{-1} (medium-high) and S_5= 8.0 dS m^{-1} (high). The experimental soil was sieved with a 4 mm screen to remove large particles and 33 kg of air-dried soil was placed in each lysimeter pot 36 dm^3 in volume. Properties of the soil used in the experiment are presented in Table 1.

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

Physical Properties			
Particle size distribution		Soil water contents	
Sand (%)	57.8	Saturation (%)	31.5
Silt (%)	20.4	Field capacity (%)	17.0
Clay (%)	21.8	Wilting point (%)	9.5
Bulk density (g cm^{-3})	1.4		
Chemical Properties			
Electrical cond. (paste) (dS m^{-1})	0.4		
pHe (paste)	7.7		

Saline waters were prepared by using $CaCl_2$, $MgSO_4$ and NaCl salts. For all salinity treatments, the sodium adsorption ratio (SAR) was kept as close as possible to the SAR value of the tap water source in order to prevent the dominant effect of a particular ion, eliminate the effect of the SAR on the results and therefore only examine the effects of the total salinity. To achieve the desired electrical conductivity values in irrigation waters (EC_i) with a SAR value of less than 5 and a Ca/Mg ratio of 1/1, the required amounts of salts were calculated and EC_i values of the treatments were checked in the laboratory (Duzdemir et al. 2009a, 2009b; Ünlükara et al. 2010; Kurunc et al. 2011; Hancioglu et al. 2019).

All irrigation water salinity treatments were irrigated when 45 to 55% of available water was consumed in the control treatment. To control the soil water status, lysimeters belonging to the control treatment were weighed every other day. The amount of applied irrigation water (AIW) was determined by weighing each lysimeter pots just before irrigation application and calculated by using Equation (1) (Duzdemir et al. 2009a, 2009b; Ünlükara et al. 2010; Kurunc et al. 2011; Hancioglu et al. 2019):

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

Where: W_{fc} and W_a are the weights of the lysimeter at field capacity and just before irrigation practice (kg); ρ_w is bulk density of water (1 kg l^{-1}); and LF is leaching fraction, which was set to a target of 0.15 as suggested by (Ayers and Westcot 1985). A drainage container underneath each lysimeter pot was used to collect drainage water due to the leaching practices. The volume of the drainage water collected in the containers was measured after the drainage ceased in order to control the targeted leaching fraction of 0.15 and adjust field capacity changes of the lysimeters due to plant growth. Also, in situ EC and pH analyses of the leachate water (EC_{dw} and pH_{dw}) were measured with an EC-pH meter after each irrigation (Hancioglu et al. 2019).

Three red beet seeds were directly sown in each lysimeter pot at the end of October. One month after sowing, only one seedling was left in each pot and the saline water application was started. During the experimental period, 5 irrigation practices were realised, except for the life water. Irrigation practices were performed at 11 to 21-day intervals. In order to meet the plant nutrition needs, 3.45 g potassium nitrate and 2.9 g of MKP (mono potassium phosphate) at the beginning of the experiment and 0.7 g of ammonium nitrate at 1.5 months after starting the experiment were applied to each lysimeter (Şalk et al. 2008).

2.4. Data collection and analysis

The volume of crop evapotranspiration (ET_v) between two-sequenced irrigation applications was calculated by using 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 the 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 (ET_d) was calculated from ET_v volume divided by the surface area of soil in the lysimeter and the number of days between the two-sequenced irrigation applications. Then the seasonal ET (ET_s) was calculated from ET_d and the length of the growing season.

Plant heights were measured weekly, in addition certain physical and physiological changes were recorded. At the end of February, the harvested plants were cleaned, leaves and storage roots were weighed and the tap root lengths were measured in the laboratory. Water use efficiency was obtained by using Equation (3):

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

Where: Y_{sr} is storage root yield (g) and ET_s is seasonal evapotranspiration (mm season^{-1}).

Immediately after the harvest, soil samples were obtained from the lysimeters. These samples were air-dried and sieved with a 2 mm screen. Then electrical conductivities of the saturated extracts (EC_e) and pH values (pH_e) were measured by using an EC and pH meter (Richards 1954; Carter et al. 2007).

The threshold soil salinity and slope values for the storage root yield of red beet were obtained by using the salt tolerance model suggested by Maas and Hoffman (1977) (Equation 4):

$$\frac{Y_a}{Y_m} = 1 - \frac{b}{100} \times \left(EC_e - EC_{e(threshold)} \right) \quad (4)$$

Where: Y_m and Y_a are the maximum and actual yields (g) from the control (non-saline) and the saline treatments, respectively, b is the slope value (% per dS m^{-1}), $EC_{e\ threshold}$ and EC_e are threshold soil salinity and soil salinity beyond the threshold value (dS m^{-1}).

2.5. Statistical analysis

SPSS statistical analysis software (IBM SPSS Inc. 2012) was used to analyze the obtained data at a significance level of 1%. Where appropriate, mean separations of the data were attained by the Duncan test at a $P<0.05$ level of significance. Correlation coefficient (r) values were used to determine the strength of the linear relationships between the investigated parameters as strong ($r \geq 0.8$ or $r \leq -0.8$), moderate ($0.5<r<0.8$ or $-0.8<r<-0.5$) and weak ($-0.5 \leq r \leq 0.5$) (Peck and Devore 2012).

3. Results

Statistical analysis results for the investigated parameters including electrical conductivity and pH values of the soil and drainage water, evapotranspiration, plant height, tap root length, fresh leaf weight, storage root yield and water use efficiency are given in Table 2. If evaluated in general; tap root length and fresh leaf weight were not affected by irrigation water salinity levels, however, plant height and water usage efficiency at 5% and EC_e, pH_e, EC_{dw}, pH_{dw}, evapotranspiration and storage root yield at 1% probability level showed significant differences among the treatments.

3.1. Effect on soil and drainage water

In the experiment, attained leaching fractions ranged from 15% (for S_0, S_3 and S_4) to 16% (for S_1, S_2 and S_5) with no significant difference among treatments, indicating that a constant leaching fraction was maintained as aimed for. Since the same leaching fraction with different salt concentrations were applied to the plants during the growing period, significant differences among treatments were observed for EC_e, pH_e, EC_{dw} and pH_{dw} values ($P<0.01$). In general, increasing salinities caused increases in both soil and drainage water EC but decreases in pH values (Table 2).

The changes in average EC_{dw} values throughout the growing season are presented in Figure 1. Differences in average EC_{dw} values among the treatments started to form at the beginning of the experiment. In general, EC_{dw} values throughout the growing season presented relatively stable trends for control and low salinity treatments, while it shows a moderate increase for medium-low salinity and high increased trends for medium, medium-high and high salinity treatments (Figure 1).

The Duncan's test results showed that the lowest soil and drainage water EC value was determined for the control treatment (0.76 and 0.87 dS m^{-1}, respectively), whereas the highest value was observed for high salinity treatment (11.13 and 12.28 dS m^{-1}, respectively) (Table 2). Unlike EC_e and EC_{dw} values, the highest pH_e value was observed for the control (7.75), low (7.75) and medium-low (7.59) salinity treatments whereas the highest pH_{dw} value for the control treatment (8.04). The lowest both pH_e and pH_{dw} value were obtained for the high salinity treatment but they were not significantly different from those of medium and medium-high treatments (Table 2).

3.2. Effect on crop evapotranspiration

Throughout the experiment, changes in daily ET values (mm day^{-1}) of each treatment were recorded and are presented in Figure 1. Differences in daily water consumption among treatments began to assume a pattern at the beginning of the experiment. The highest daily ET value in all treatments, except medium-high and high water salinity, occurred during the third irrigation period. However, seasonal ET (175-261 mm) and daily ET (2.3-3.4 mm) showed statistically significant but relatively low change among treatments. The biggest variation in daily plant water consumption was observed for control, low and medium-low salinities while the lowest change occurred under high water salinity treatment (Figure 2).

The highest ET value was determined as 261 mm for control but this value did not differ statistically from those of low and medium-low salinity treatments. As expected, the lowest water consumption was measured for medium-high (189 mm) and high salinity treatments (Table 2). Compared to the control, decreases in water consumption ranged from 16% (medium salinity) to 33% (high salinity).

3.3. Effects on growth and yield parameters

Throughout the growing season, changes in plant heights under different irrigation water salinity levels were recorded and are presented in Figure 3. In general, it is seen that plant heights increased rapidly at the beginning of the experiment, and then slowed down during the 4-5 weeks before harvest. By the end of the growing period, the average plant lengths ranged from 42.3 cm for low and medium-low salinities, which were not significantly different from those of control, medium and medium-high salinities, to 34.8 cm for high water salinity, which was statistically different from all other treatments (Table 2).

Even though tap root lengths and fresh leaf yields of red beet plants ranged from 15.8 to 18.5 cm and from 142 to 153 g $plant^{-1}$, respectively, statistical analyses showed that these parameters were not affected by increasing irrigation water salinities. On the other hand, average storage root yields of red beet plant showed statistically significant changes due to increasing irrigation water and hence soil salinity levels ($P<0.01$). The highest storage root yield was observed for control (244 g $plant^{-1}$) but it was not significantly different from that of low salinity treatment (237 g $plant^{-1}$) whereas the lowest storage root yield (140 g $plant^{-1}$) was recorded for high salinity treatment (Table 2). Compared to control, calculated decreases in storage root yields were 6, 10, 24 and 42% for medium-low, medium, medium-high and high water salinity treatments, respectively.

3.4. Effect on plant water use efficiency

Statistical analysis results show that the WUE values of red beet plant were significantly affected by the irrigation water salinity levels ($P<0.05$). According to the results, while the highest water use efficiency was obtained from medium salinity with 1.01 g mm^{-1}, this value was found to be significantly different from high water salinity treatment (0.80 g mm^{-1}) which has the lowest water use efficiency (Table 2).

Table 2. Effect of irrigation water salinity on soil, drainage water and water use, growth, and yield parameters of red beet

Analysis	Irrigation water salinity (dS m^{-1}) treatments						P>F
	S_0 (0.6)	S_1 (1.5)	S_2 (3.0)	S_3 (4.5)	S_4 (6.0)	S_5 (8.0)	
Leaching fraction	0.15[#]	0.16	0.16	0.15	0.15	0.16	ns
Saturated paste extract EC_e (dS m^{-1})	0.76f[†]	2.31e	5.14d	7.50c	8.62b	11.13a	**
Saturated paste extract pH_e	7.75a	7.75a	7.59a	7.49b	7.52b	7.38b	**
Drainage water EC_{dw} (dS m^{-1})	0.87f	1.97e	5.16d	7.87c	9.83b	12.28a	**
Drainage water pH_{dw}	8.04a	7.94b	7.71c	7.64cd	7.63d	7.62d	**
ET (mm $season^{-1}$)	261a	253a	235ab	220b	189c	175c	**
Plant height (cm)	41.8a	42.3a	42.3a	39.3ab	38.0ab	34.8b	*
Tap root length (cm)	15.8	16.0	17.5	18.3	18.5	18.5	ns
Fresh leaf yield (g $plant^{-1}$)	153	156	144	143	142	143	ns
Storage root yield ((g $plant^{-1}$)	244a	237ab	229bc	220c	185d	140e	**
Water use efficiency (g mm^{-1})	0.94a	0.95a	0.98a	1.01a	0.99a	0.80b	*

#: 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.

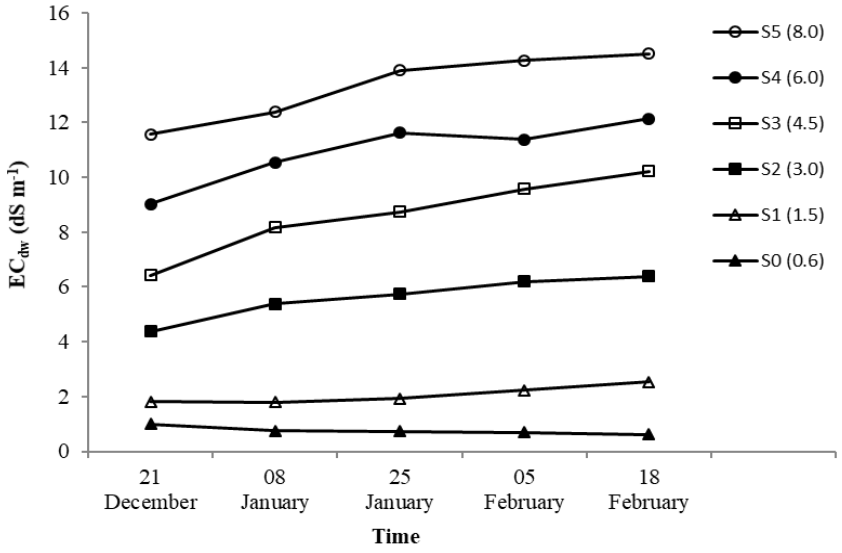

Figure 1. Changes on drainage water throughout the growing season.

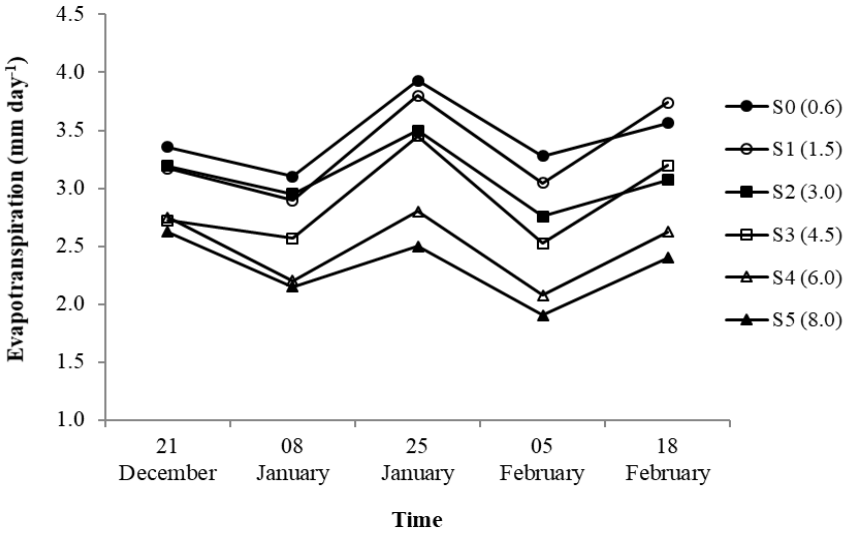

Figure 2. Changes on daily ET of red beet throughout the growing season.

The salinity-response model was created and threshold salinity and slope values were determined for red beet storage root yield. The salt tolerance model for red beet plant is presented in Figure 4. As shown, threshold salinity and slope values of red beet plant were determined as 3.10 dS m^{-1} and 4.42%, respectively.

3.5. Relationship between parameters

The correlation coefficients (r) and significance levels of the relationships between all the parameters obtained from the experiment are given in Table 3. There were significantly important (P<0.01) strong-positive linear correlations between EC_e vs EC_{dw}; ET vs storage root yield, whereas strong-negative linear correlations between EC_e vs pH_{dw}, ET and storage root yield; EC_{dw} vs pH_{dw}, ET and storage root yield. Similarly, significantly important (P<0.01) moderate-positive linear correlations between pH_e vs pH_{dw}, ET and storage root yield; pH_{dw} vs ET, plant height and storage root yield; tap root length vs fresh leaf yield; storage root yield vs plant height and water use efficiency whereas moderate-negative linear correlations between EC_e vs pH_e and plant height; EC_{dw} vs pH_e and plant height were observed. There were also significantly important (P<0.05) weak-positive linear correlations between plant height vs ET and water use efficiency (Table 3).

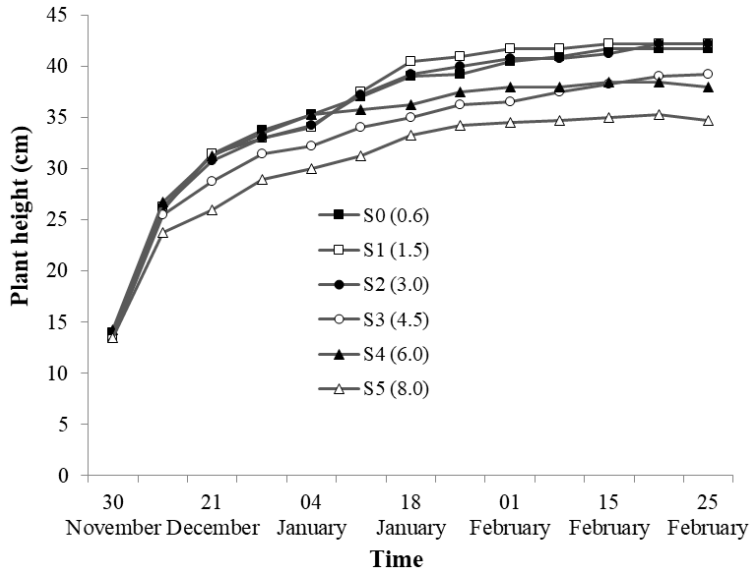

Figure 3. Changes on red beet plant heights throughout the growing season.

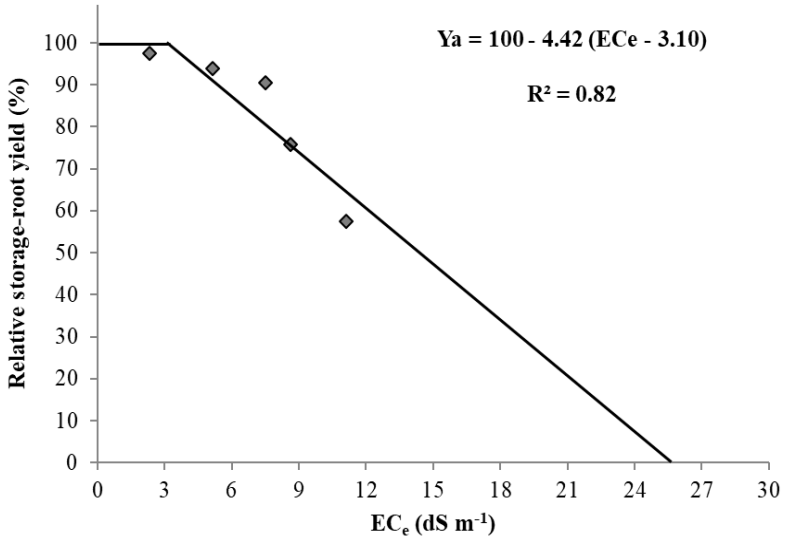

Figure 4. Yield response factors for storage root and fresh leaf yields of red beet.

Table 3. Relationship between investigated parameters

	EC_e	pH_e	EC_{dw}	pH_{dw}	ET	PH	TRL	FLY	SRY
pH_e	-0.73 **								
EC_{dw}	0.99 **	-0.74 **							
pH_{dw}	-0.89 **	0.65 **	-0.89 **						
ET	-0.86 **	0.67 **	-0.89 **	0.72 **					
PH	-0.61 **	0.37 ns	-0.60 **	0.52 **	0.42 *				
TRL	0.29 ns	-0.26 ns	0.31 ns	-0.35 ns	-0.40 ns	-0.28 ns			
FLY	-0.23 ns	0.14 ns	-0.24 ns	0.23 ns	0.17 ns	-0.08 ns	0.63 **		
SRY	-0.87 **	0.60 **	-0.90 **	0.68 **	0.83 **	0.66 **	-0.29 ns	0.12 ns	
WUE	-0.24 ns	0.06 ns	-0.24 ns	0.08 ns	-0. 04 ns	0. 50 *	0.08 ns	-0.06 ns	0.53 **

EC_e: Electrical conductivity of soil saturated paste extract, pH_e: pH of soil saturated paste extract, EC_{dw}: Electrical conductivity of drainage water, pH_{dw}: pH of drainage water, ET: evapotranspiration, PH: plant height, TRL: tap root length, FLY: fresh leaf yield, SRY: storage root yield, WUE: water use efficiency, **: significant at $P<0.01$, *: significant at $P<0.05$, ns: non-significant.

4. Discussion

Ayers and Westcot (1985) declared that assuming ECe= 0.5×ECsw, (EC of soil water), the expected EC_e/EC_w ratio is 1.6 under a leaching fraction of 0.15 i.e. the ECe value will be about 1.6 times of the EC_w. The EC_e/EC_w ratios were calculated as 1.26, 1.54, 1.71, 1.67, 1.44, and 1.39 for the control, low, medium-low, medium, medium-high, and high water salinity levels, respectively. In general, EC_e/EC_w ratios of all treatments were close to the specified value except for the control treatment which had a relatively lower ratio. Similarly, EC_w and EC_e values under low, medium-low, medium, medium-high and high water salinity levels were 2.5, 5.0, 7.5, 10.0, and 13.3 times and 3.0, 6.8, 9.9, 11.4, and 14.7 times, respectively, higher than that of the control treatment. According to these results, compared to the control, the EC_w ratios of all treatments were less than the EC_e ratios of the same treatments.

EC_{dw} values can also be calculated from the EC_w/LF relationship (Ayers and Westcot 1985). Using the actual LF values given in Table 2, EC_{dw} values were calculated as 3.90, 9.23, 19.27, 31.01, 40.05, and 51.31 dS m^{-1} for the control, low, medium-low, medium, medium-high, and high water salinity levels, respectively. However, the actual EC_{dw} values were 4.50, 4.70, 3.73, 3.94, 4.08, and 4.18 times, respectively, which was less than those of the calculated EC_{dw}'s. All these results may indicate that the plant removes some salt from the soil and/or the number of irrigations applied during the growing period was not sufficient to stabilize soil and thus drainage salinity. The ongoing increases in the EC_{dw} values shown in Figure 1, especially for medium, medium-high, and high salinity levels, indicate that the equilibrium conditions in terms of salinity had not occurred in the soil and drainage water. As a general approach, soil and drainage salinities might become stable, by at least 4-6 irrigation applications and in some cases after a few growing periods depending on management practices, climate and soil characteristics, and the irrigation water salinity level (Ayers and Westcot 1985).

Daily mean ET values were calculated as 3.4, 3.3, 3.1, 2.9, 2.5, and 2.3 mm under the control, low, medium-low, medium, medium-high, and high water salinity levels, respectively. Similarly, many researchers reported decreased water consumption under salinity conditions for plants i.e. pepper (Ünlükara et al. 2015), oregano (Hancioglu et al. 2019), bell pepper (Kurunc et al. 2011), pea (Duzdemir et al. 2009a), cowpea (Duzdemir et al. 2009b), and eggplant (Ünlükara et al. 2010).

Storage root yields declined significantly, especially with an irrigation water salinity lever higher than 3.0 dS m^{-1}. Rhoades et al. (1992) concluded that yield is reduced due to excessive salinity, because plants divert their energy to making the biochemical adjustments necessary to survive under stress conditions, instead of plant growth and yield. Pessarakli (1991) reported that the use of nutrients taken by the plant under the salinity stress was greatly reduced and thus the growth and yield decreased significantly.

In Maas and Hoffman (1977), a threshold salinity of 4.0 dS m^{-1} and a slope value of 9.0% for red beet plant was reported. When compared, the threshold salinity and slope values determined in our study were found to be lower than those reported in Maas and Hoffman (1977). The disparities in these values are thought to be caused by the variety of plant used in the experiments and the differences in the environmental conditions in which the investigations were conducted.

5. Conclusions

In this study, the effects of irrigation water salinity on growth (plant height and tap root length), yield parameters (fresh leaf and storage root yields), evapotranspiration and water use efficiency of red beet were investigated. Under the same leaching fraction, different salt concentrations were applied to the plants throughout the growing season. In general, increasing irrigation water salinities caused increases in both soil and drainage water EC as expected but also decreases in pH values. Although the differences in EC_{dw} values among the treatments started to form at the beginning of the experiment, the ongoing increases in the EC_{dw} values under the application of saline irrigation water with greater than 3 dS m^{-1} indicate that the equilibrium conditions did not occur in soil and drainage water. Daily water consumption among treatments began to differentiate at the beginning of the experiment. Throughout the growing season, the smallest variation in daily plant water consumption was observed for irrigation waters having greater than 3.0 dS m^{-1} salinities. Even though tap root length and fresh leaf weight were not affected by irrigation water salinity levels, plant height and storage root yield

have significantly declined especially with irrigation water salinity greater than 3.0 dS m^{-1}. Red beet plant water use efficiencies showed an increasing pattern with increased irrigation water salinity up to 6.0 dS m^{-1} and then decreased. In general, significantly important positive or negative linear correlations were observed among EC_e, pH_e, EC_{dw}, pH_{dw}, ET, plant height and storage root yield values. The salinity threshold and slope value of red beet were determined as 3.10 dS m^{-1} and 4.42% per dS m^{-1} for red beet plant under Mediterranean climate conditions.

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Effect of fertigation with different pH and EC levels on selected physical soil properties

Mustafa KAPLAN[1], Erdem YILMAZ[1], Ahmet Safak MALTAS[2], Seda TORUN[3], Emine Ruya NAMAL[4]

[1]Akdeniz University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, 07058, Antalya, Türkiye
[2]Akdeniz University, Finike Vocational School, 07740, Finike, Antalya, Türkiye
[3]Ministry of Agriculture and Forestry, 15900, Çavdır, Burdur, Türkiye
[4]INOVA Seed Company, 7112, Aksu, Antalya, Türkiye

Corresponding author: E. Yilmaz, e-mail: erdemyilmaz@akdeniz.edu.tr
Author(s) e-mail: mkaplan@akdeniz.edu.tr, ahmetsafak@akdeniz.edu.tr, torun.seda07@gmail.com, ruyanamal@gmail.com

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ABSTRACT

In this study, the effects of fertigation with different pH and EC level on soil physical properties such as aggregate formation (AF), aggregate stability (AS) and available water content (AWC) of soil were investigated. In the study, single crop tomato (*Solanum lycopersicum*, Anıt F1) was grown under cover for two consecutive years. A total of six fertigation applications (F_1: pH 7.2/EC 2.0; F_2: pH 7.2/EC 3.5; F_3: pH 6.5/EC 2.0; F_4: pH 6.5/EC 3.5; F_5: pH 5.0/EC 2.0 and F_6: pH 5.0/EC 3.5) were created, two different EC levels and three different pH levels. Fertigation applications were applied to the soil in three replications and the study was carried out in 18 plots in total. Based on our results, the effect of fertigation applications on the AF of the soil and the AWC during the year was not significant. On the contrary, the effect of fertigation on AS has occurred at different levels and degrees of importance in terms of the effect between years. Fertigation F_5, which has a pH 5.0/EC 2.0 levels, caused a significant increase in the stability of 2-1 mm aggregates.

1. Introduction

The most important condition for obtaining high yield from the soil is to know the soil properties well and to utilize it according to its capabilities and sustainability. A fertile soil has high organic matter and biological activity, friable stable aggregates, and a porous medium in which plant roots and water can move easily (Lewandowski and Zumwinkle 1999). On the other hand, the ability of the plant to develop well in the soil is significantly related to the physical properties of the soil environment in which it grows. Physical soil quality reflects the compatibility of the physical properties of the soil with plant productivity and environmental quality (Lal 1998). The most important physical soil quality parameters are the percentage of aggregation, the mean weighted diameter of the aggregates, the pore size distribution, and the water–holding characteristics of the soil (Subbian et al. 2000). Effective fertilizer management is important in improving the physical quality of the soil (Lal 1997).

Soil aggregates are generally examined in two categories as macro (>250 µm) and micro (<250 µm). Macroaggregates are formed by the combination of microaggregates (Golchin et al. 1994). Microaggregates are more resistant to external disruptive forces than macro aggregates (Christensen 2001).

The formation of aggregates in the soil and their size distribution are very important in terms of the movement of water and air in the soil, the development of plant roots and the balance of air and water in the soil. With the dispersion of aggregates, the disappearance of the pores in the soil, a decrease in the amount of aeration and infiltration capacity, an increase in the level of surface flow and erosion, and an increase in exposure to plant water stress and its frequency occur. It has been reported in various studies that the crop production system and fertilizer applications affect aggregation and mean weighted diameters of aggregates (Tripathi et al. 2014; Peng et al. 2015; Guo et al. 2019). As a result of the significant increases in root biomass provided by farm manure and inorganic fertilizer applications, high organic matter formation occurs in the soil. Thanks to the cementing effect, soil organic matter provides significant increases in the mean weighted diameters of aggregates (Benbi and Senapati 2009).

Aggregate stability is an expression of the resistance of the soil to the mechanical forces disrupting the soils and the degree of aggregate stability of the soils is accepted as an indicator of soil quality (Six et al. 2000). Aggregate stability often depends on soil properties such as organic matter, clay and oxide content (Zhang and Horn 2001; Prěvost 2004). Organic carbon and sesquioxides have a very important role in the aggregate formation of red soils (Yao et al. 1990). According to Mahimairaja et al. (1986) aggregate stability in humid regimes differs depending on fertilization and nutrient management. Many studies have been conducted on the effectiveness of fertilization on aggregate stability and different opinions have been reported (Bronick and Lal 2005; Yin et al. 2016; Xin et al. 2016).

For a given soil, soil aggregation can be altered by fertilization and management strategies which can impact on the biotic and abiotic cementing agents (Noellemeyer et al. 2008; Sodhi et al. 2009). On the other hand, soil aggregate stability is predominately influenced by the following factors: soil organic carbon (SOC) content, texture, temperature, water content, freeze–thaw conditions, wetting–drying cycles, differences in soil management (e.g., tillage and crop rotation and residue management), acidity levels and calcium carbonate (CaCO3) concentrations, root mass, root length and microbial richness (Are et al. 2018).

Soil moisture is the most important factor that directly affects both soil formation and development and the growth and development of plants. Global climate changes cause significant drought problems in the world and this situation makes the methods to be applied in the protection of soil moisture important. The soil moisture regime affects the nutrient status of the soil under different agricultural production systems, as well as the distribution of plant roots to the soil and water use efficiency (Lata et al. 2020). Soil water retention is seen as a function of plant production systems and fertilization levels as well as the basic properties of the soil (Subbian et al. 2000). The physical, chemical and biological properties of the soil, the differences in meteorology and the pattern of the grown crops, and the changes in soil moisture in the surface and root zone are defined temporally and spatially (Monti and Zatta 2009).

Thanks to the aggregating and stabilizing functions of the materials applied in order to improve the aggregate formation in the surface soil, the rate of water entry into the soil and the amount of water retained in the soil are affected. In many studies, it is reported that balanced inorganic or organic fertilizer applications improve the physical properties of the soil by increasing the nutrient content and increase the productivity of the soil (Chen et al. 2009; Sun and Huang 2011). It has been reported by different researchers that crop production systems and fertilizer applications affect the water–holding capacity of the soil (Walsh et al. 1996; Bassouny and Chen 2016). The water–holding capacity of the soil also largely determines the mechanical resistance to root penetration. The penetration resistance of the soil can control plant growth by reducing the rate of root growth (Fasinmirin and Reichert 2011). The water–holding capacity of the soil in the plant production season is a basic feature that affects plant development, transport and transformation of plant nutrients, and the water and energy budget in the soil–plant system (Kahlon et al. 2013).

The aim of this study is to determine the effects of fertigation with different pH and EC values on physical soil properties such as aggregate formation, aggregate stability and available water content.

2. Materials and Methods

2.1. Study area and experimental methods

This study was carried out on *Lithic Rhodoxeralf* (Soil Survey Staff 2014) soil with a high lime content and clay loam texture. The study area (36° 53' N, 30° 38' E) is located in the Akdeniz University Faculty of Agriculture Research and Application area (Antalya, Turkey). The research was carried out as two–season single–crop tomato cultivation under greenhouse conditions. The trials were designed and conducted in a factorial experiment with 3 repetitions according to the randomized blocks experimental design.

Fertigation applications applied in the research include F_1: pH 7.2 / EC 2.0 dSm^{-1}, F_2: pH 7.2 / EC 3.5 dSm^{-1}, F_3: pH 6.5 / EC 2.0 dSm^{-1}, F_4: pH 6.5 / EC 3.5 dSm^{-1}, F_5: pH 5.0 / EC 2.0 dSm^{-1} and F_6: pH 5.0 / EC 3.5 dSm^{-1}. During the production season, in order to create 2.0 dSm^{-1} and 3.5 dSm^{-1} salinity levels, Ammonium Nitrate (NH_4NO_3), Mono Ammonium Phosphate (MAP), Mono Potassium Phosphate (MKP), Potassium Nitrate (KNO_3), Calcium Nitrate ($CaNO_3$), Magnesium Nitrate ($MgNO_3$) and Magnesium Sulphate ($MgSO_4$) were applied. Also micro element fertilizer containing iron, manganese, zinc and copper was used. In order to establish the salinity levels determined during the production season, the fertilization programme was carried out by using the pure substance amounts given in Table 1.

In the study, tomato plant (Solanum lycopersicum) was grown and Anıt F1 variety was used as a tomato variety in order to determine the effects of applications on yield and quality parameters in plant production. Tomato seedlings were planted in a double row (40 x 90 cm planting distance) in plots with a length of 10 m. A total of 50 seedlings were used, 25 tomato seedlings in each plot. Seedling planting was carried out on 17.10.2015 in the first year of the study and on 20.10.2016 in the second year (Fig. 1). Fertigation and other cultural processes (hoeing, tying, plant protection measures, etc.) after planting the seedlings were carried out regularly in the trials, which were carried out for approximately 8 months in both years. Fertilizer applications were made with drip irrigation. During the growing season, considering the climate and plant needs, irrigation was done at least 3 to 8 days apart.

2.2. Soil analysis methods

Soil samples were taken from 0–30 cm depth in order to determine the soil properties before and after fertigation. After the soil samples were air–dried, they were sieved through a 2 mm sieve and some physicochemical soil properties were determined.

Table 1. The amounts of nutrients used to reach the determined EC values in fertigation

EC ($dS\ m^{-1}$)	Fertigation components ($kg\ da^{-1}$)					
	First season					
	N	P_2O_5	K_2O	CaO	MgO	Micro-nutrients (Fe, Mn, Zn and Cu)
2.0	48.92	42.36	60.18	12.32	6.59	0.40
3.5	85.56	74.13	105.31	21.55	11.53	0.70
	Second season					
2.0	46.68	40.49	57.19	11.66	6.31	0.40
3.5	81.69	70.86	100.01	20.41	11.04	0.70

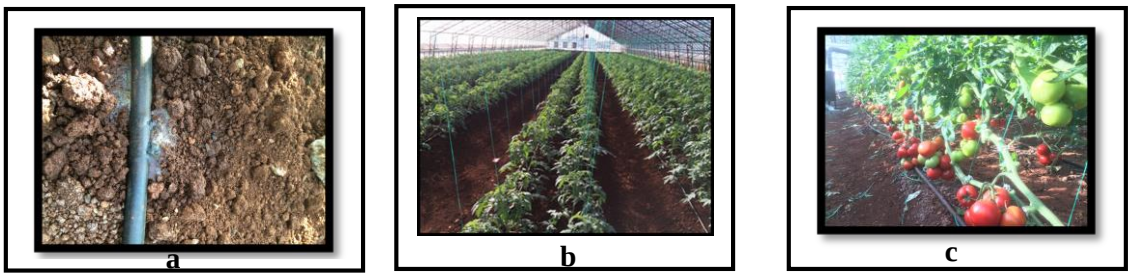

Figure 1. Fertigation (a) and tomato production (b, c) in the study area.

The texture was determined using the hydrometer method (Bouyoucos 1953). Soil pH values (Jackson 1967) and electrical conductivity (EC) were measured in a mixture of soil and water (ratio of soil to water 1: 2.5) by a digital pH meter and conductivity meter (Rhoades 1982). The carbonate ($CaCO_3$) content of soil was measured with a Scheibler calcimeter (Allison and Moodie 1965). The soil's total organic carbon content was determined using the modified Walkey–Black method (Black 1965). The organic matter content of soil was calculated by multiplying the organic carbon value by Van Bemmelen factor (1.724) (Nelson and Sommer 1982). Total nitrogen was determined using the modified Kjeldahl method (Kacar 1995). Available P (with $NaHCO_3$) was determined using the Olsen method (Olsen and Sommer 1982). The concentrations of DTPA-extractable Fe^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} of soil were measured according to Lindsay and Norwell (1978). The exchangeable K^+, Ca^{2+}, Mg^{2+} and Na^+ of soil samples were extracted by 1 N ammonium acetate (CH_3COONH_4), and determined by using an ICP–OES (PE–Optima7000DV) device (U.S. Salinity Laboratory Staff 1954).

Aggregate size distribution was determined by sieving 750 g of soil through sieves of <0.05, 0.05–0.25, 0.25–0.5, 0.5–1.0, 1–2, 2–4 and >4mm with a 75-stroke frequency/for 5 min in the rotary sieve machine (Chepil 1962). Macro– and micro–aggregate stability was determined by wet sieving each aggregate fraction (0.25 mm and 1–2 mm), which was obtained by dry sieving, for 5 min at 1.3 cm stroke length and 34 cycle/min (Yoder 1936). Aggregate stability percentage was calculated with Kemper's aggregate stability formula* (Kemper and Koch 1966). A sieve with 100 µm mesh aperture was used to correct the sand fraction weights.

*: Aggregate Stability (%) = $100 \times [(P1 - P2) / (P - P2)]$. (1)

P: Oven dry weight of soil (g)
P1: Stable aggregate + sand fraction weight (g)
P2: Sand fraction weight (g).

The water–holding characteristics of soil were calculated with a pressure plate extractor, corresponding to the field capacity (%) and the permanent wilting point (%), respectively. The soil's field capacity was determined using the undisturbed soil samples taken by a steel cylinder which the stainless-steel cores were 50 mm in height and 50 mm in diameter (98.125 cm^3 inner volumes), and the wilting point of soil was determined using disturbed soil samples (Richards 1947). The principal physical and chemical properties of the soils are represented in Table 2.

Table 2. Some physical and chemical analysis results of the research soil

Soil properties	Value
Sand (%)	22.12
Silt (%)	40.00
Clay (%)	37.88
Texture	Clay loam
pH (1: 2.5)	7.42
$CaCO_3$ (%)	17.20
Electrical conductivity-EC (dS m^{-1})	0.42
Organic matter (%)	2.43
Total N (%)	0.15
Available P (mg kg^{-1})	236
Exchangeable K (cmol kg^{-1})	0.67
Exchangeable Mg (cmol kg^{-1})	3.88
Exchangeable Ca (cmol kg^{-1})	31.36
Available Fe (mg kg^{-1})	4.73
Available Mn (mg kg^{-1})	10.70
Available Zn (mg kg^{-1})	9.24
Available Cu (mg kg^{-1})	6.80

2.3. Statistical analysis methods

All data were analyzed by the DUNCAN multiple comparison test ($P \leq 0.05$). All results presented in the text are expressed as mean values (n= 3). Statistical analyses were performed using MINITAP 16.1.1 (Minitab 2010).

3. Results and Discussion

3.1. Aggregate formation

The effect of six different fertigation cycles carried out in both years of the study on aggregate formation (AF) was not found to be statistically significant in any aggregate size. However, when the difference of the effect of fertigation on AF between years was examined, there were statistically significant differences in some aggregate sizes. F6 provided a significant increase ($P<0.05$) in the amount of aggregates with 2–1 mm size in the second year of the study compared to the first year. On the contrary, all fertigation applications except F_3 and F_4 in aggregate size of 0.5–0.25 mm, and F_1 in size <0.050 mm caused a decrease in the amount of aggregate in the soil in the second year of the study (Table 3). In particular, the increase in the amount of 2–1 mm aggregate obtained with low pH and high EC fertigation may be related to the increase in the amount of free Ca^{+2} ions. In other words, it is thought that both the dissociation of calcium

Table 3. The effect of fertigation with different pH and EC levels on aggregate formation (%)[1]

Fertigation	Aggregate Size (mm)								
	>4		LSD_Y[3] (%5)	4–2		LSD_Y[3] (%5)	2–1		LSD_Y[3] (%5)
	1. Year	2. Year		1. Year	2. Year		1. Year	2. Year	
F_1	26.60	23.41	n.s	18.44	18.94	n.s	17.52	19.95	n.s
F_2	25.56	19.96	n.s	18.73	19.77	n.s	18.21	20.39	n.s
F_3	24.77	24.45	n.s	18.33	22.45	n.s	17.84	20.81	n.s
F_4	20.87	23.03	n.s	16.12	18.68	n.s	18.50	20.92	n.s
F_5	22.90	19.94	n.s	19.73	17.93	n.s	18.81	21.05	n.s
F_6	26.07	19.42	n.s	16.61	17.88	n.s	17.25**B**[2]	21.49**A**	*
Mean	**24.46**	**21.70**		**17.99**	**19.28**		**18.02**	**20.77**	
LSD_F (%5)[3]	n.s	n.s		n.s	n.s		n.s	n.s	

Fertigation	Aggregate Size (mm)											
	1–0.5		LSD_y[3] (%5)	0.5–0.25		LSD_y[3] (%5)	0.25–0.050		LSD_y[3] (%5)	<0.050		LSD_y[3] (%5)
	1. Year	2. Year		1. Year	2. Year		1. Year	2. Year		1. Year	2. Year	
F_1	14.79	17.44	n.s	11.18	10.38	n.s	9.40	8.39	n.s	2.00	1.19	n.s
F_2	14.77	17.70	n.s	10.85	11.57	n.s	9.61	9.05	n.s	2.16**A**	1.26**B**	**
F_3	15.07	15.93	n.s	11.53**A**	8.46**B**	*	10.02	6.61	n.s	2.25**A**	1.00**B**	*
F_4	17.62	17.97	n.s	13.84**A**	10.34**B**	*	10.85	7.77	n.s	2.04**A**	1.04**B**	*
F_5	15.52	18.82	n.s	11.35	11.59	n.s	9.50	9.08	n.s	2.02**A**	1.27**B**	**
F_6	15.45	19.25	n.s	12.12	11.46	n.s	10.23	8.84	n.s	2.11**A**	1.36**B**	**
Mean	**15.54**	**17.85**		**11.81**	**10.63**		**9.94**	**8.29**		**2.10**	**1.19**	
LSD_F (%5)	n.s	n.s		n.s	n.s		n.s	n.s		n.s	n.s	

1: Values of n= 3, 2: The difference between values not shown with the same letter are significant at $P<0.05$ level. Capital letters indicate the differences between the years, 3: Significance: *significant at $P<0.05$; **significant at $P<0.01$; n.s: not significant.

carbonate in the soil by fertigation with low pH and the Ca^{+2} ion originating from the CaO used in fertigation play a role in this event. Soil aggregation results from the rearrangement, flocculation and cementation of particles. It is mediated by soil organic carbon, biota, ionic bridging, and clay and carbonates (Bronick and Lal 2005). The increases in Ca^{+2} cations from the dissociation of $CaCO_3$ lead to coagulation of organic and mineral colloids from soil, promoting their flocculation (Gliński et al. 2011). Muneer and Oades (1989) report that the predominance of Ca^{2+} in the soil exchange complex acts as a physical stabilizer of soil organic matter as it allows better particle aggregation. Ca^{+2} acts as a binding agent between the organic and mineral fraction of soil, favoring the association and strengthening the links between mineral and organic particles, favoring the aggregates formation (Gliński et al. 2011; Briedis et al. 2012). The increase in Ca^{+2} and Mg^{+2} in the soil as a result of fertilization play an important role in forming aggregates through flocculation of clay particles (Rengasamy and Marchuk 2011).

Fertigation and crop rotation regulate C cycle dynamics and C storage, as they increase the biological activity in the soil and affect the amount and quality of residues returned to the soil (Aune and Lal 1997). The balanced use of organic and inorganic fertilisers is the most accepted strategy for maintaining agricultural productivity and increasing soil fertility (Sharma and Subehia 2003; Manna et al. 2007). In various studies, it has been reported that the total mean weighted diameter (MWD) of the soil significantly increased with different NPK levels and farm manure applications (Brar et al. 2015; Zhang et al. 2016). It has been reported by some researchers that the MWD of the aggregates increase, especially with nitrogen fertilizer applications (Subbian et al. 2000).

3.2. Aggregate stability

The effect of fertigation with different pH and EC levels on the stability of 2–1 mm aggregate in both years of the study was found to be statistically significant at $P<0.001$ and $P<0.01$ levels, respectively (Table 4). F4, F5 and F6 provided an increase in aggregate stability (AS) and the highest increase in stability was obtained from F5 application in both years (17.04% and 21.26%). This effect reveals the effect of both a decrease in pH and an increase in EC level on stability. In addition, fertigation shows that the decrease in pH level is more effective on stability than the increase in EC level. Tang at al. (2020) stated that the ratio of water–stable macro aggregates (0.25–2 mm) in silt loam and silty clay textured soils increased depending on the increase in soil EC level. It is thought that fertigation with a low pH level may cause the dissociation of $CaCO_3$ in the research soil with high lime content and thus increase the amount of free Ca^{+2} ions. In fact, it has been reported by researchers that calcium ion is an important cementing agent in many soils and increases aggregate stability. On the other hand, it is stated in various studies that with the increase in the EC level of the soil, the cation concentration of the soil increases and that there also may be significant increases in stability due to cation bridges. The role of carbonates, as a source of Ca^{+2}, in promoting mineral bonds and mineral-SOM interactions mediated by cation bridges has been described as being responsible for microaggregate formation and stability in several studies (Muneer and Oades 1989; Baldock and Skjemstad 2000).

The effect of fertigation on the stability of 0.25–0.050 mm aggregates was not found to be significant in both years of the study. However, considering the difference between years, there was no significant difference in the stability of 2–1 mm aggregate with fertigation, but F2 created a significant ($P<0.01$) difference in 0.25–0.050 mm aggregates. The effect of F2 on AS was greater in the second year than in the first year of the study (Table 4). Especially with fertilizer applications made in the greenhouse

production system, significant increases in aggregate stability are obtained, but the effect of inorganic applications are less than organic applications (Herencia et al. 2011). On the contrary, it has been reported in some studies that especially nitrogen fertilizer applications disrupt the soil aggregate system and cause a decrease in stability (Fonte et al. 2009; Brtnicky et al. 2017).

3.3. Available water content

The effect of fertigation with different pH and EC levels on the available water content (AWC) of the soil was not found to be significant in both of the research years (Table 5). However, considering the difference between years, the effect of F4 application on soil AWC was found to be significant (P<0.05) and it provided a higher increase in soil AWC in the second year (8.23%) compared to the first year (6.23%) of the study (Table 5).In the second year of the study, fertilization with low pH and high EC levels increased the amount of aggregates, especially in 2–1 and 1–0.5 mm sizes. This is an indication that the macro and medium dimensional pore volume of the soil has increased. It can be said that due to the positive development provided in the pore structure of the soil, the amount of available water in the soil has also been improved. Guber et al. (2003) reported that aggregate size distribution parameters can be useful in estimating soil water retention parameters especially that the content of medium–sized aggregates affects the water content at -33 and -1500 kPa.

Bassouny and Chen (2016) reported that after 14 years of organic and inorganic (NPK) fertilizer applications, inorganic fertilizer applications increased the amount of water content at 0–10 and 10–20 cm soil depth at all tensions in the 0–1500 kPa range. On the other hand, Herencia et al. (2011) reported that there is no significant difference between organic and inorganic fertilization in terms of the available water capacity of the soil in greenhouse or open field production. Lata et al. (2020) stated that three different nitrogen fertilizer applications in four different production systems did not make a significant difference in the moisture characteristics of the soil, and the water–holding characteristics of the soil were strongly affected by texture and physical conditions.

The ideal soil EC value in plant production is 2-4 dS m^{-1}. Soils with an EC value above 4 dS m^{-1} are considered saline soils (Qadir et al. 2007). Above this value, many plants are adversely affected. The EC levels of the majority of greenhouse soils in Antalya, where greenhouse production is carried out intensively, are above 4 dS m^{-1} (Sönmez et al. 2004). High EC increases the osmotic pressure of the soil environment, making it difficult for the plant to absorb water and nutrients (Ding et al. 2018). In our study, the EC level, which is the upper limit for plant production, was not exceeded. In addition, although it was statistically insignificant, especially in the second year of the study, an increase in the amount of aggregates with a size of 2–1 mm was achieved also with other applications other than F6. This effect shows the importance of the effect of fertigation on the AWC due to the improvement in soil structure.

Table 4. The effect of fertigation with different pH and EC levels on aggregate stability (%) [1]

Fertigation	Aggregate Size (mm)					
	2–1			0.25–0.050		
	1. Year	2. Year	LSD_Y (%5)[3]	1. Year	2. Year	LSD_Y (%5)[3]
F_1	2.97**c**[2]	3.48**d**	n.s	97.23	96.18	n.s
F_2	3.64**c**	3.84**d**	n.s	96.29**B**	98.41**A**	**
F_3	3.25**c**	3.41**d**	n.s	96.56	97.06	n.s
F_4	8.96**b**	9.37**c**	n.s	97.39	95.68	n.s
F_5	17.04**a**	21.26**a**	n.s	96.72	95.44	n.s
F_6	9.57**b**	10.42**b**	n.s	96.99	97.12	n.s
Mean	**7.57**	**8.63**		**96.86**	**96.64**	
LSD_F (%5)[3]	***	**		n.s	n.s	

1: Values of n= 3, 2: The difference between values not shown with the same letter are significant at P<0.05 level. Small letters indicate the differences within the year, and capital letters indicate the differences between the years, 3: Significance: **significant at P<0.01; ***significant at P<0.001; n.s: not significant.

Table 5. The effect of fertigation with different pH and EC levels on available water content (AWC) of soil (%) [1]

Fertigation	Available Water Content (AWC) (%)		LSD_Y (%5)[3]
	1. Year	2. Year	
F_1	6.73	6.29	n.s
F_2	6.30	6.49	n.s
F_3	6.47	5.88	n.s
F_4	6.23**B**[2]	8.23**A**	*
F_5	6.73	5.97	n.s
F_6	6.20	7.91	n.s
Mean	**6.44**	**6.79**	
LSD_F (%5)[3]	n.s	n.s	

1: Values of n= 3, 2: The difference between values not shown with the same letter are significant at P<0.05 level. Capital letters indicate the differences between the years, 3: Significance: *significant at P<0.05; n.s: not significant.

4. Conclusions

In our study, the effect of fertigation with different pH and EC values on selected physical properties of the soil occurred at different levels and directions. It can be seen that fertigation with high EC and low pH levels can be important especially in macro–scale aggregation. With high EC value fertigation, cation increase will be provided in the soil, and due to the cation bridges that will be formed between the colloids as a result of this increase, an improvement in aggregation will be achieved. In addition, with low pH level fertigation, the lime in the trial soil with high lime content will be partially dissolved and a significant amount of free Ca^{2+} ions will be released. In this way, it is thought that promoting cation bridges between colloidal surfaces may be important.

The highest aggregate stability values were obtained especially at low pH levels. It is thought that the possible high Ca^{2+} concentration in the soil solution due to low pH fertigation and $CaNO_3$ fertilizer application s are important in this effect. In addition, as a result of fertigation with low pH and high EC values, there was an increase in the amount of available water in the soil, especially with the increase in the amount and stability of aggregates with macro size. As a result, it is understood that fertigation with high EC and low pH values will make important contributions to the improvement of the physical properties of calcareous soils with high pH values. However, considering the negative effects of high EC level in plant production, it was predicted that fertigation with low pH and medium EC level would be more suitable in terms of productivity, especially in soils with high lime content.

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Erratum

MEDITERRANEAN AGRICULTURAL SCIENCES (2022) 35(2): 113

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There is an error in corresponding author designation of the article, “Obtaining humic acid from stabilized and dried domestic sewage sludge and its utilization in grass (*Lolium perenne* L.) growth” by Şule ORMAN, Hüseyin OK, Moilim FAHAD, Aylin ÖZGÜR (MEDITERRANEAN AGRICULTURAL SCIENCES (2020) 33(3): 411-416 DOI: 10.29136/mediterranean.781464). Corresponding author of this article is "Moilim FAHAD". The authors regret this error.

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Examples:

Journal:

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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Address:

Faculty of Agriculture
Akdeniz University
07058 Antalya, Türkiye

Phone: +90 242 310 2412
Fax: +90 242 310 2479
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