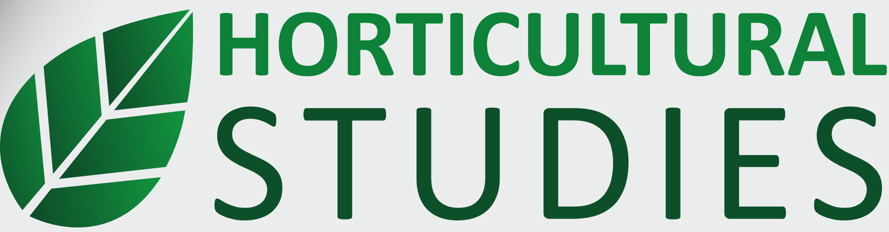

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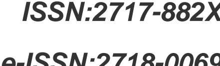

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Volume:40 Number:3 December 2023

CONTENTS **Page**

Research Articles

Improvement of Citrus Rootstock Hybrids Derived by 2x × 2x Intra Crosses with the Aid of Embryo Rescue and Ploidy Detection 72-78
Şenay KURT Fatma KOYUNCU

Determination of Insecticide Resistance in Western Flower Thrips [*Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)] Causing Problems in Carnation Cultivation 79-85
Musa KIRIŞIK Tuba KAHRAMAN

Micropropagation of Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] Leaf Explants and Single Nodes in Response to Plant Growth Regulators 86-97
Hakan AKTAŞ Gizem Gökçe AKKAYA Ebru AKYÜZ ÇAĞDAŞ Gülden HASPOLAT Şeküre Şebnem ELLİALTIOĞLU

Nematode Diversity Associated with Grapevines in İzmir, Manisa, Çanakkale, Balıkesir, and Bilecik Provinces in Türkiye 98-106
Lerzan ÖZTÜRK

Determination of the Efficiency of Moringa Leaf Extract Treatments on Seedling Vigor in Marigold 107-112
Kübra ÖZMEN Fulya UZUNOĞLU Emine ERĞAN Kazım MAVİ

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RESEARCH PAPER

Improvement of Citrus Rootstock Hybrids Derived by 2x × 2x Intra Crosses with the Aid of Embryo Rescue and Ploidy Detection

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Abstract

Diploid *Citrus* × *Poncirus* hybrids have significantly contributed to citrus rootstock evaluation. In Türkiye, common sour orange rootstock is used in many different climates and soil conditions at almost 85% of citrus plantations, but this rootstock is susceptible to Citrus tristeza virus disease. This study was conducted to improve new rootstock genotypes by traditional hybridization. Common sour orange (CSO) and Common mandarin (*Citrus deliciosa* Ten.) (CM) were crossed with Troyer citrange (TC) while King mandarin (KM) was crossed with Carrizo citrange (CC). Embryos obtained from crosses were taken on 110, 120 and 130 days after artificial pollination (DAP), and were germinated on MT culture media. The embryos of 120 DAP of combinations had the highest germination rate within CSO×TC, 95.15%; CM×TC, 96.25%; KM×CC, 95.23%. The trifoliate rates for each combination at subculture (CSO×TC, 17.40%; CM×TC, 11.11%; KM×CC; 6.17%) were obtained from 110 DAP embryos. Survival rates of the genotypes were ranged between 72.13% and 90.28% in subculture and varied from 40.17% and 64.71% in the greenhouse. As a result of the ploidy analysis by flow cytometry, the nuclear DNA content of diploid genotypes were found between 0.78 pg/2C and 0.93 pg/2C. One of the genotypes derived from CM×TC hybridization on 120 DAP was determinated as a triploid plant.

1. Introduction

The rootstocks are the vital spot for the adaptation to different environments of citrus trees. In the Mediterranean basin, citrus tristeza virus (CTV) poses a great threat due to the widespread use of sour orange (*Citrus aurantium* L.) rootstock (Pestana et al., 2005). Sour orange rootstock has no incompatibility, yield, and quality problems with many other species and varieties. However, its susceptibility to CTV and its occurrence in some growing areas in Türkiye necessitate the improvement of this disease tolerant rootstocks that can be used instead of citrus rootstock (Tuzcu, 1978).

In order to achieve a more successful breeding program biotechnological methods have great importance in addition to classical breeding methods. *Poncirus* and its hybrids are the most important genetic resources to obtain new rootstocks, especially by combining with different oranges and mandarins (Castle, 2010).

In terms of nursery production, citrus rootstocks should have a high polyembryony level and high germination rate (Broadbent and Gollnow, 1993). The citrus are vegetatively propagated with generative material, and homogeneous genetically identical plants are obtained from their parents. Therefore, citrus breeders generally use at least one polyembryonic parent to receive polyembryonic hybrids. In polyembryonic citrus, the zygotic embryo has to compete with strong embryos derived from nucellar tissues for nutrient and expansion space (Soost and Roose, 1996). Since nucellar embryos

confine the formation of hybrid seeds by suppressing the development of zygotic embryos any variability in the population cannot be achieved (Pena et al., 2007). Most citrus cultivars are polyembryonic, and when used as the female parent in crosses, usually immature zygotic embryos are suppressed by nucellar embryos. Embryo rescue technique is carried out to capture the zygotic embryo and also to obtain hybrid plants from them after crosses (Cameron and Frost, 1968). The success of embryo culture depends on the plant growth regulators, nutrients, the embryo excision technique, and often the developmental stage of the extracted embryos (Raghavan, 1980; Xie et al., 2019). Most citrus varieties are diploid. However, polyploid plants such as triploid and tetraploid can be found among diploid populations (Aleza et al., 2011). Natural tetraploidization by folding the nucellar tissue as a result of mutation is rarely seen in citrus species. There is no natural or artificial tetraploid genotype that can be used commercial or in breeding programs in Türkiye even in plantations where preserved citrus genetic resources. In the present study, we have studied 2x × 2x intra spesific hybridizations applied between citrus species to obtain new hybrid rootstock genotypes. We have obtained different genotypes at different developmental stage embryos. We analysed the performance of *in vitro* embryo rescue conditions and then the ploidy levels of obtained genotypes by flow cytometry.

2. Materials and Methods

2.1. Hybridizations

Plant materials used in hybridizations studies were located at the genetic resource collection of Bati Akdeniz Agricultural Research Institute (BATEM), in Antalya (Türkiye). A total of 1215-controlled crosses were performed in all three hybridization combinations. The controlled hybridization studies were performed according to Batchelor (1943). Hybridization combinations for breeding new citrus rootstocks are showed in Table 1.

2.2. Embryo rescue and hybrids obtaining

Immature fruits obtained from articifical pollinations in specified combinations were harvested 110, 120 and 130 days after pollination (DAP). Fruits surface sterilization were performed by keeping them in 70% ethanol for 5 minutes and then immersed in 20% sodium hypochlorite for 30 minutes. Fruits were rinsed three times in a sterile flow cabinet. The fruits were cut horizontal using bistoury without damaging the embryo and the immature seeds were extracted with forceps. Immature embryos were removed under binocular microscope by carefully cutting the micropile end of the seeds taken at different developmental stages (Figure 1).

Table 1. Parents used in 2x × 2x crosses.

Female parent	Male parent
Common sour orange (CSO) (*Citrus aurantium* L.)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
Common mandarin (CM) (*Citrus deliciosa* Ten.)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
King mandarin (KM) (*Citrus nobilis* L.)	Carrizo citrange (CC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)

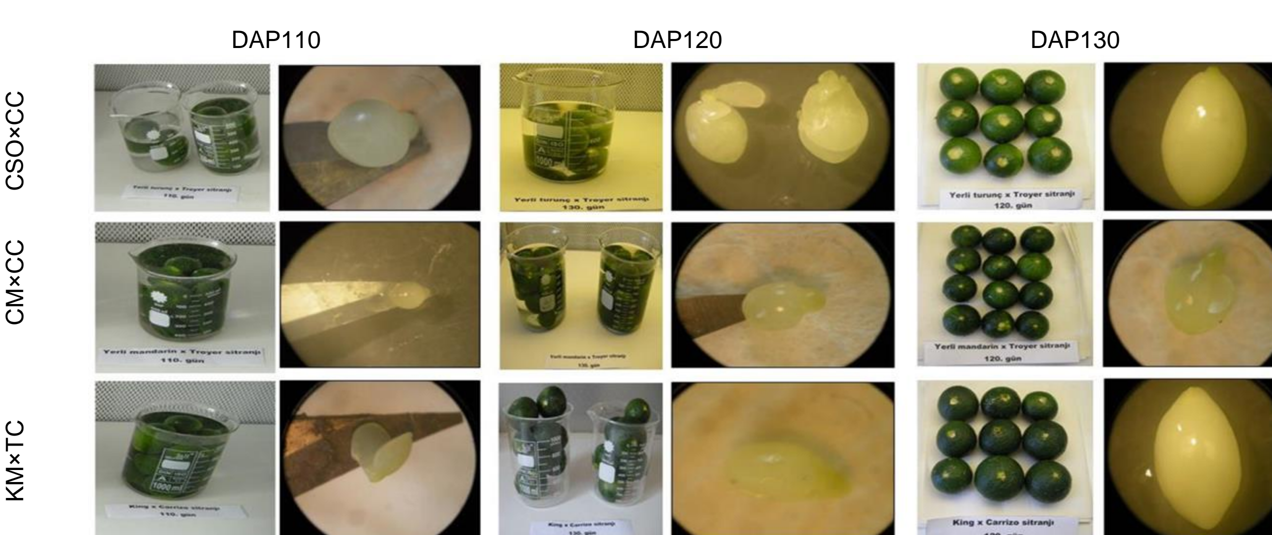

Figure 1. Harvested immature fruits at 110, 120, 130 DAP and extracted embryos under a binocular microscope (DAP: Days after artificial pollination, CSO: Common sour orange, CM: Common mandarin, TC: Troyer citrange, KM: King mandarin, CC: Carrizo citrange).

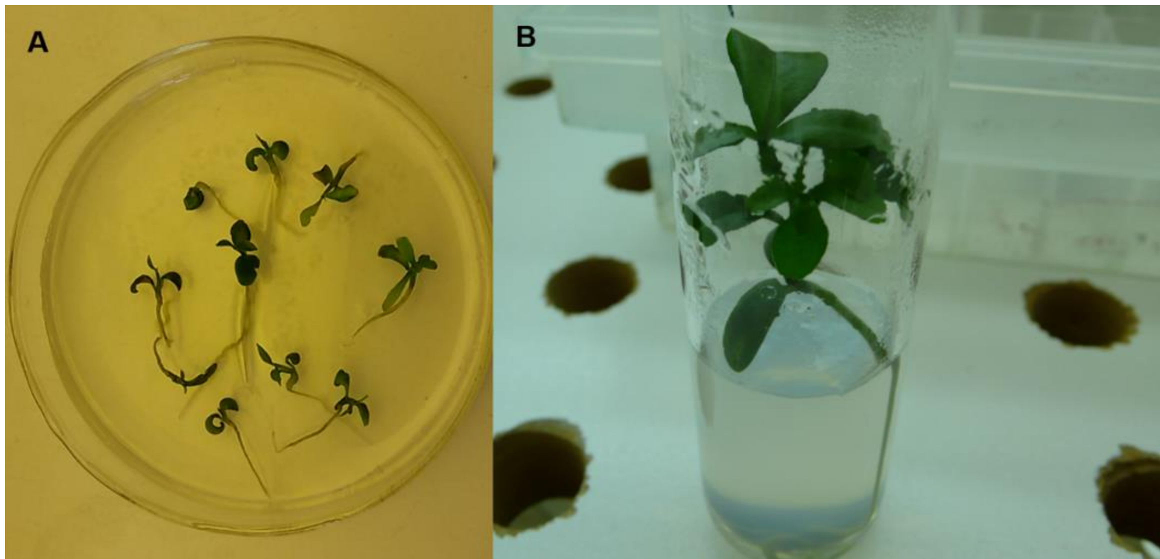

Figure 2. Germinated embryos (A) and subcultured trifoliate hybrids (B).

Modified Murashige and Tucker (1969) (MT) culture medium supplemented with 50 g l^{-1} sucrose, 25 mg l^{-1} adenine sulfate, 500 mg l^{-1} malt extract, 1.5 mg l^{-1} GA_3, and 8 g l^{-1} agar at pH 5.7 were used for embryo germination. Embryos excised at different developmental stages were placed with 10 embryos in each petri plate in a modified MT culture medium for germination and then petri plates were covered with parafilm. The embryos were kept in an incubation room at 26°C and in 16 h/8 h of photoperiod with 1000 lux light intensity.

According to their germination and development stage, embryos were transferred to culture tubes containing Murashige and Skoog (MS) (1962) culture media supplemented with 0.02 mg l^{-1} NAA, 20 g l^{-1} sucrose, and 8 g l^{-1} agar at pH 5.7 in order to increase plantlets development (Perez-Tornero and Porras, 2008) (Figure 2). At this stage, three different hybridization combinations were appointed for their effect on embryo rescue at three different embryo developmental stages and subcultured plantlets were determined trifoliate ratio and survival ratio.

After 60-80 days of subculture, in order to transfer the developed plantlets to *in vivo* conditions, polystyrene pots (250 ml) were filled with peat:perlite (3:1) mixture. To prevent water loss, they were covered with polyethylene and incubated in the growth rooms at 25-26°C temperature and 80-85% humidity for the high survival rates. Later, the seedlings were transferred to a greenhouse for acclimatization to *in vivo* conditions. Survival percentage of seedlings was determined after transferred to the mixture and also in the greenhouse.

Surviving seedlings in greenhouse were observed agro-morphologically such as stem diameter and height, leaf characteristics and thorn status. Morphological characteristics were examined by International Plant Genetic Resources Institute (IPGRI, 1999) for citrus features. Data on morphological characteristics were not considered in this study.

2.3. Ploidy analysis with flow cytometry

Ploidy levels of the genotypes were determined using a flow cytometer (Partec®). Ploidy analysis was carried out in 5 parents and 194 hybrid plants. Leaves were collected from each plants were kept between two layers of filter paper soaked in petri dishes and were kept cold during transportation. Samples were prepared using the ready kit of Partec and protocol of Partec®. The suspension of nuclei was filtered with 50 µl Partec® CellTrics filter and was transferred into the eppendorf tube, and was added 2 ml of 4.6 diamidino-2-phenylindole (DAPI, Partec®) staining solution (Aleza et al., 2009).

Histograms were analyzed using the Partec software, which determines peak position, coefficient of variation (CV), and the relative peak index of the samples. Nuclear DNA content was calculated according to the formula (Dolezel et al., 2007) below:

Sample 2C DNA content (pg/2C) = [Fluorescent density of hybrid sample (The value of the G1 peak) / Fluorescence intensity of the standard (The value of the G1 peak)] x Standard 2C DNA content.

2.4. Data analysis

The research was established in a randomized plot design. All data were subjected to analysis of variance with mean separation by Duncan's multiple range tests. Cultured embryos were observed and embryo germination ratio (%) was designated by rating the germinated embryo number to the total number of cultured embryos. Before comparing the percentage ratio with variance analyses, square root transformation was performed on the data.

3. Results and Discussion

3.1. Fruit set of controlled pollinations and seed formation

The number of pollinations (total 1215 crosess), number of obtained fruits, and fruit set ratio were presented in Table 2. In hybridization studies carried out with different citrus species and varieties until today, it has been shown that the effect of different pollinators on fruit set rate, fruit size, and number of seeds per fruit has shown a wide variation. Fruit setting in citrus can be affected by environmental conditions such as rootstock, climatic conditions and parents (Soost and Cameron, 1975). Aleza et al. (2012) reported that the fruit set rate was 36-39% in the crossbreeding study performed with different combinations in mandarins.

In the present study, the fruit set ratio in combinations in which mandarins are used as the female parent is close to the literature reports. It has been reported that the fruit set ratio of the crossbreeds in which the common sour orange was used as the female parent, the volkamer lemon, orange, lime, and mandarins as the male parents varied between 1.10% and 4.30% (Al-Naggar et al., 2009). In the combination in which we used the common sour orange as the female parent and Troyer citrange as the male parent, although the intergeneric hybridization, the fruit set was determined to be quite high, consistent with the literature reports. This might have been caused by the difference in pollen genotype, but the exact reason requires further study. The number of seeds may vary depending on the type of pollinator. However, there are studies reporting that the number of seeds is higher in more developed fruits (Yun et al., 2007).

The number of seeds obtained from fruits taken at different days after pollination (DAP) are presented in Table 3. The highest seed number was present in fruits harvested at 130 DAP from the CM×TC combination. Kim et al. (2020) indicated that the correlation coefficient between the number of seeds in the fruit and the average number of embryos per seed fluctuates from year to year, tree to tree, branch to branch and is not regular.

3.2. Germination rate of the embryos

Germination percentage of immature hybrid embryos under *in vitro* conditions was significantly affected by the embryo development stage, except for the KM×CC combination (Table 4). In embryo recovery studies, it is reported that different embryo development stages are appropriate depending on the species and variety. Raghavan (1980) stated that success in embryo rescue depends largely on the developmental stage of the isolated embryos, and Viloria et al. (2005) reported that the germination capacity of citrus embryos is affected by the genetic structure of the embryo and the embryo development stage. Regarding the time of embryo rescue in hybrids obtained from citrus; successful results were reported in studies conducted 50th (Wang et al., 1999), 80th (Tan et al., 2007) and 85th (Xie et al., 2019), 100th (Deng et al., 1996) days after pollination. Carimi et al. (1998), Tusa et al. (2002), and Ferrante et al. (2010) suggested that 105 days after pollination is suitable for embryo rescue time. Similar to our results, some researchers marked that immature embryos were removed 120 days after pollination for embryo culture in various citrus species and cultivars (Perez-Tornero et al., 2011; Kurt and Ulger, 2014). In addition, other studies showed that embryo rescue time was 118th (Chagas et al., 2005), 130-140th (Soni et al., 2019) and 145th (Kim et al., 2020) days after pollination for *in vitro* embryo culture.

The results of the study were consistent with the reports of the researchers and the highest germination rates were found on the 120 days after pollination in embryos obtained from both CSO×TC and CM×TC crosses. Although there was no statistically significant difference at KM×CC combination in terms of embryo germination rates, high germination was achieved with an average of 94.14% in all three stages. Our results clearly showed the species and varieties used in the

Table 2. Number of pollinations, number of fruits and fruit set ratio in 2x × 2x crosses.

Hybridizations	Number of pollinations	Number of fruits	Fruit set ratio (%)
CSO×TC	390	41	10.51
CM×TC	422	135	31.99
KM×CC	403	162	40.20

(CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

Table 3. The number of fruits, total seeds number and seed number per fruit taken at different developmental stages after pollinations.

Parameters	Number of fruits			Total seeds number			Seed number per fruit		
DAP	110	120	130	110	120	130	110	120	130
CSO×TC	8	11	4	123	210	55	15.4	19.1	13.8
CM×TC	15	22	20	233	382	417	15.5	17.3	20.8
KM×CC	20	25	30	110	208	275	5.5	8.3	9.2

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

Table 4. The effect of embryo recovery phase on germination in crosses and trifoliate plantlet ratio (%) of seedlings.

Parameters	DAP	Crosses		
		CSO×TC	CM×TC	KM×CC
Number of embryos cultured	110	350	350	320
	120	330	400	440
	130	230	250	320
Germination rate (%)	110	85.43 ± 1.76 b*	92.86 ± 1.27 a	94.38 ± 1.18[Ns]
	120	95.15 ± 1.08 a	96.25 ± 1.06 a	95.23 ± 0.83
	130	93.91 ± 1.37 a	84.80 ± 1.31 b	92.81 ± 1.29
Number of seedlings	110	115	72	81
	120	292	122	218
	130	96	193	163
Trifoliate plantlet ratio (%)	110	17.40 ± 0.37 a	11.11 ± 0.37 a	6.17 ± 0.37[Ns]
	120	13.70 ± 0.37 c	6.56 ± 0.37 b	5.50 ± 0.37
	130	15.63 ± 0.37 b	5.70 ± 1.38 b	6.13 ± 0.22

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).
* Significant at the $p < 0.05$ level, Ns: Not significant.
Mean followed by different letters within columns differ significantly ($p < 0.05$).

Table 5. Survival percentage of seedlings in subcultured (SPSS), in transplanted to mixture (SPSTM) and in transferred to the greenhouse (SPSTG).

Crosses	DAP	SPSS (%)	SPSTM (%)	SPSTG (%)
CSO×TC	110	80.00 ± 1.49[Ns]	85.87 ± 1.49 a*	59.49 ± 1.49 a*
	120	84.60 ± 1.49	78.14 ± 1.49 b	49.20 ± 1.49 b
	130	80.21 ± 1.49	77.92 ± 1.49 b	58.30 ± 1.49 a
CM×TC	110	90.28 ± 1.49 a*	78.46 ± 1.49 [Ns]	64.71 ± 1.49 a*
	120	72.13 ± 1.49 b	81.82 ± 1.49	62.50 ± 1.49 a
	130	87.57 ± 1.49 a	79.88 ± 1.49	47.41 ± 1.49 b
KM×CC	110	85.19 ± 1.49 ab*	73.91 ± 1.49 a*	52.94 ± 1.49 a*
	120	89.90 ± 1.49 a	57.14 ± 1.49 b	40.17 ± 1.49 b
	130	84.05 ± 1.49 b	76.64 ± 1.49 a	41.90 ± 1.49 b

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).
* Significant at the $p < 0.05$ level, Ns: Not significant.
Mean followed by different letters within columns differ significantly ($p < 0.05$).

embryo recovery significantly affected the embryo development stage.

3.3. Trifoliate plant ratio

The trifoliate feature in citrus is controlled by two dominant genes (Cameron and Frost, 1968). For this reason, zygotic seedlings are obtained from controlled hybridization between *Citrus* and *Poncirus* have trifoliate characteristics (Ozsan and Cameron, 1963). In this study, based on this principle, among the individuals obtained from each combination, those showing trifoliate features were determined. Our results displayed that the embryo development stage has a significant effect on the ratio of trifoliate plantlets. The highest trifoliate ratio was observed in the seedlings obtained on the 110th day in CSO×TC and CM×TC combinations as 17.40% and 11.11%, respectively (Table 4).

Generally, the identification of hybrid embryos requires some additional analysis such as cytological, flow cytometry, isoenzyme or molecular analysis (Tusa et al., 2002). Since the trifoliate characteristic of the *Poncirus* genus is a dominant character, it is a powerful determinant used to define crossbred individuals morphologically and this is a useful tool that can be used to identify zygotic individuals (Rodríguez et al., 2004).

3.4. Survival percentage of seedlings

Survival percentage of seedlings was calculated over the total number of individuals transferred at each stage. The survival rates of seedlings were quantified for subcultured between 72.13% and 90.28%, for transplanted to mixture between 57.14% and 85.87%, for transferred to greenhouse between 40.17% and 64.71% (Table 5).

Regarding survival rates of seedlings after embryo rescue; successful results were reported in studies conducted 62.56% (Kurt, 2010) and 89.7% (Aleza et al., 2012) for subcultured; 59.0% - 89.3% (Jaskani et al., 2005) for transplanted to mixture; between 40.17% and 64.71 % (Jaskani et al., 2005), 68% (Singh et al., 2006) and 90% (Aleza et al., 2010) for transferred to greenhouse. When the study is evaluated together with the literature reports, survival rates in the study were close as well as higher or lower survival rates. These results indicated that combination selection as well as soil mix and environmental conditions had an impact on survival.

3.5. Ploidy analysis in hybrids

Nuclear DNA contents in parents and hybrids varied between 0.78 pg/2C and 0.93 pg/2C and

Table 6. Ploidy analysis results of hybrids with flow cytometry.

Parameters	DAP	Crosses		
		CSO×TC	CM×TC	KM×CC
Number of hybrids analyzed	110	22	21	17
	120	34	20	22
	130	17	20	21
Nuclear DNA content (pg/2C)	110	0.79 - 0.85	0.79 - 0.93	0.79 - 0.86
	120	0.78 - 0.85	0.79 - 1.16	0.79 - 0.87
	130	0.79 - 0.84	0.78 - 0.83	0.79 - 0.89
Ploidy level	110	Diploid	Diploid	Diploid
	120	Diploid	1 Triploid, Diploid	Diploid
	130	Diploid	Diploid	Diploid

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

were determined as diploid (Table 6). The nuclear DNA content was found 1.16 pg/2C in a hybrid of CM×TC (DAP 120) combination and this individual was evaluated to be triploid. In addition, this triploid hybrid in phenotypic observations had long thorns compared to the other hybrids. The results were compatible with the data of some researchers (Şeker et al., 2003; Ali et al., 2013). Small differences in values of nuclear DNA content obtained by flow cytometry may have resulted from sample preparation, staining, and analysis procedures.

4. Conclusion

In conclusion, we obtained 335 genotypes with the aid of embryo rescue technique from 1215 artificial pollinations of three different crosses. It was determined that parent combinations and embryo development stages have important effects on the germination rates of embryos. It can be said that this embryo development stage will be more appropriate in embryo culture studies, since germination rates are found to be the highest on the 120th days after pollination in hybridization combinations. However, it was found that 110th days embryos gave better results for all combinations in terms of trifoliate rate in hybrids. The survival rates after transfer to the greenhouse varied from 40.17% to 64.71%, and the effect of embryo development stages in all three combinations significantly differed. As a result of ploidy analysis, with the exception of the hybrid found as triploid, the nuclear DNA contents of the hybrids were found between 0.78 pg/2C and 0.93 pg/2C and it was determined that they were diploid. A genotype in CM×TC combination was determined to be triploid. It was also determined the triploid genotype had much longer thorns compared to the all hybrids based on morphological observations. Hybrids are now kept in the greenhouse to be studied for disease tolerance and rootstock properties.

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RESEARCH PAPER

Determination of Insecticide Resistance in Western Flower Thrips [*Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)] Causing Problems in Carnation Cultivation

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Abstract

The fact that *Frankliniella occidentalis* (Thysanoptera: Thripidae) completes its life cycle in a short time reveals the need for continuous pest control. Therefore, pest resistance may occur with the intensive use of chemicals by the growers. In this context, the current sensitivity of *F. occidentalis* to registered insecticides for carnation (methiocarb, formetanate hydrochloride), registered insecticides for vegetables (azadirachtin, malathion, and spinosad), and unapproved insecticides used extensively by growers needs to be determined. Moreover, it is important to test the chemicals (for example, pyridalyl) used against other pests whose spray period is the same as *F. occidentalis*. In this study, it was aimed to determine the sensitivity levels of *F. occidentalis* populations taken from the sites of intensive carnation production in Antalya province in 2018-2020 to these chemicals by the leaf dipping method. As a result of the study, resistance against spinosad (11.00-28.60 times), methiocarb (2.10-2.70 times), malathion (2.05-4.21 times), azadirachtin (3.00-7.00 times), formetanate hydrochloride (1.50-2.00 times) and pyridalyl (2.75-3.89 times) were determined. Given the high resistance to spinosad observed in the study, trials involving the combination of spinosad and formetanate hydrochloride were initiated as a strategy for managing resistance. The resistance against spinosad + formetanate hydrochloride was determined between 4.35 and 9.09 times. Our results suggest that resistance level can be reduced by using resistance management methods such as the use of mixed chemicals, although resistance was detected in all five locations against all active substances.

1. Introduction

Türkiye has a significant advantage in the cultivation of ornamental plants due to its convenient location in terms of both climatic and geographical conditions. Carnation, rose, and gerbera are the leading species in cut flower production. Their share in the total cut flower production areas is 63%, 15%, and 10%, respectively. The greenhouse area constitutes two-thirds of the total production area. Furthermore, most of the production in greenhouses is for export (Anonymous, 2016).

Spider mite, thrips, and cotton bollworm are among the main pests in carnation, however, the most important among them in Antalya province is the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera, Thripidae) (Keçecioğlu and Madanlar, 2002). This pest, whose origin is North America, was first detected in Türkiye in 1993 in carnation greenhouses in Antalya and its host range is very wide (Tunç and Göçmen, 1995). The western flower thrips, and the onion thrips *Thrips tabaci* (Lindeman) (Thysanoptera, Thripidae) are economically important pests in many cultivated plants, including ornamental plants. These two

thrips species are vectors of Tomato spotted wilt virus (TSWV) and Impatiens Necrotic Spot Virus (INSV) virus, which are also a problem in ornamental plants and cause significant problems in production areas (Daughtrey et al., 1997; Ulmann et al., 1997). Dağlı and Tunç (2006) stated in their study that pesticides are widely used against *F. occidentalis* in Antalya province, that growers do not consider chemical control to be sufficient, and that this may be due to resistance developed by *F. occidentalis*. The pest is protected from insecticide applications because it lays its eggs into plant tissue, the adults and larvae are in the inner parts of the flowers, and the pupa is in the parts that are protected from pesticides on the soil or plant (Robb and Parella, 1995). In addition to these biological properties of *F. occidentalis*, it has been demonstrated in many studies that resistance to most of the different insecticide groups makes chemical control increasingly difficult (Immaraju et al. 1992; Brodsgaard, 1994; Zhao et al. 1995; Karadjova, 1998; Kontsedalov et al. 1998; Jensen, 2000; Espinosa et al., 2002; Herron and James, 2005; Bielza et al., 2007).

In this study, the current resistance status of methiocarb and formetanate hydrochloride licensed to *F. occidentalis* in carnation against populations obtained from Antalya carnation areas was determined. Moreover, as it is known in Antalya, greenhouse vegetable production is carried out intensively. Since vegetable production areas and ornamental plant production areas are very close to each other, pests can easily be transmitted from one production area to another. Furthermore, *F. occidentalis* is a pest that can be found in vegetable and ornamental plant production areas and can easily pass from one greenhouse to another. Although malathion, azadirachtin, and spinosad used in this experiment are not licensed for *F. occidentalis* in carnation, they were selected because they are licensed for thrips species in vegetables. The other selected active ingredient, pyridalyl, is licensed against *Helicoverpa armigera* and *Tuta absoluta* in vegetable greenhouses, and the application time coincides with the thrips control time. The determination of the resistance status of *F. occidentalis* against pyridalyl was also included in the experiment.

The aim of the study was to determine the current resistance status of *F. occidentalis* against malathion, methiocarb, azadirachtin, formetanate hydrochloride, pyridalyl, and spinosad and to conduct studies on resistance management if resistance is detected.

2. Materials and Methods

2.1. Materials

In the study, malathion, methiocarb, formetanate hydrochloride, and spinosad, which are commonly used in the control of *F. occidentalis*, as well as pyridalyl and azadirachtin that are licensed for carnation in other countries were discussed. During the bioassay (chemical tests) studies, laboratory materials such as micropipette, disposable plastic Petri dishes, disposable plastic cups, cotton, thin and soft-tipped brush, soft-tipped forceps, 100 ml measuring cylinder, 10 ml pipette, and gloves were used.

2.2. Collection of *Frankliniella occidentalis* populations

Samples of *F. occidentalis* were taken from Aksu, Kepez (Altınova), and Serik districts, where greenhouse carnation cultivation is most common in Antalya (Table 1). In the collection of samples, attention was paid to take samples from the production areas of private sector companies that produce carnations for export. The sensitive population was obtained from Assoc. Prof. Fatih Dağlı (Akdeniz University, Faculty of Agriculture, Department of Plant Protection, Antalya, Türkiye). The samples taken were produced on green bean pods at the climate room of Plant Protection Department of BATEM (Batı Akdeniz Agricultural Research Institute, Antalya, Türkiye) with a temperature of 24±1ºC and a day length of 16:8 h (light: dark).

2.3. Insecticides used in the study

The active ingredients, trade names, formulations and IRAC mode of action classification of the insecticides used in the study are given in Table 2.

2.4. Thrips diagnosis and production

Species identification of populations was made by PhD. Emine Topuz using morphological diagnostic criteria. Green bean pods were used to provide nutrition and egg laying environment for thrips. Plastic containers with lids covered with filter paper were used as production cages and 2-3 layers of paper towels were laid on the bottom of the containers for thrips to pass the pupal stage. Fresh bean pods, which were disinfected with bleach,

Table 1. Places and times of collection of samples.

Cooperated carnation exporter companies	Sample collection date
Antalya Tarım Company Limited (Aksu)	September 19, 2017
Flash Tarım Company Limited (Kepez)	September 19, 2017
Tempo Tarım Company Limited (Kepez)	October 24, 2017
Erkut Tarım Company Limited (Kepez)	October 24, 2017
Ada Tarım Company Limited (Serik)	October 24, 2017

Table 2. Insecticides used in the study, their trade names and mode of action.

Active ingredient	Trade name, formulation, *recommended dose	**IRAC mode of action classification
Methiocarb	Mesurol WP 50% Bayer 1000 ml ha^{-1} (100 L water)	Carbamate (1A), acetylcholinesterase inhibitor in the nervous system
Formetanate hydrochloride	Dicarzol 50 SP 500 g kg^{-1} AMC –TR 100 g 100 L^{-1} water	Carbamate (1A), acetylcholinesterase inhibitor in the nervous system
Spinosad	Laser SC 480 g L^{-1} Dow Agro Sciences, 200 ml ha^{-1} (100 L water)	Spinosyn (5), activating effect on nicotinic acetylcholine receptors in the nervous system
Malathion	Malathion EC 650 g L^{-1} Sefa, 100 ml 100 L^{-1} water	Organophosphate (1B), acetylcholinesterase inhibitor in the nervous system
Pyridalyl	Sumipleo 50 EC 500 g L^{-1} Sumiagro 200 ml ha^{-1}	Unknown
Azadirachtin	Nimbecidine EC 0.3 g L^{-1} Agrobest 500 ml 100 L^{-1} water	Unknown

* Recommended dosage according to label information, **IRAC: Insecticide Resistance Action Committee.

dipped in sugar and amino acid solutions and dried, were left in the production cages together with the thrips desired to be produced, and they were replaced with new ones at 3-4 day intervals.

2.5. Insecticide tests

For insecticide tests, the method of Contreas et al. (2008) was adapted and the leaf dipping method was used (IRAC Test Method: 1068). To determine the lethal concentration (LC) values for *F. occidentalis* population, at least six different dose series were prepared to create a death distribution between 0% and 100%. In addition, only water (+Tx-100) was used in the controls. Cowpea leaf discs with a diameter of 3 cm were immersed in the prepared insecticide concentrations for 5 seconds and dried, and then placed in Petri dishes on which agar was poured. Adult female thrips collected from production containers with a mouth aspirator were transferred to the Petri dishes by temporarily stunned with carbon dioxide, and finally, the top of the stretch film was punctured with an insect needle to allow air to breathe. After the treated thrips were exposed to insecticide residues for two days in the Petri dishes, mortality counts were made.

Obtained mortality rates were subjected to probit analysis and LC_{50}, LC_{90}, and LC_{99} values were determined. In this test, at least four replications for each different concentration and at least 20-25 female thrips adults were used for each replication. Resistivity multiples were determined by comparing sensitive population LC values with all populations.

2.6. Location of the project

The fieldwork in the experiment was carried out in the regions where carnation is produced intensively in Antalya, which is indicated in Table 1. Laboratory studies were carried out in the entomology laboratories of the Batı Akdeniz Agricultural Research Institute (BATEM), Antalya, Türkiye.

2.7. Statistical analysis

In all populations, LC_{50}, LC_{90} and LC_{99} values, slopes and 95% confidence intervals of the populations were obtained by using the probit analysis method in the POLO computer package program (LeOraSoftware, 2008) by using the determined mortality rates.

3. Results and Discussion

The LC values of active substances for *F. occidentalis* populations obtained from Antalya Tarım, Tempo Tarım, Flaş Tarım, Erkut Tarım, and Ada Tarım populations as a result of the bioassay studies are given in Table 3.

The highest resistance rates were determined in the Ada Tarım population with 28.6 times to spinosad, 2.7 times to methiocarb, 4.2 times to malathion, 7.0 times to azadirachtin, 2.0 times to formetanate hydrochloride, and 3.89-times to pyridalyl. The dose level that kills 99% of the resistant population (LC_{99}) in laboratory tests was still higher than the practically recommended dose of spinosad and methiocarb. The results show that applications of spinosad and methiocarb against this pest may fail in the locations where these populations are sampled. With additional research, the prevalence of spinosad and methiocarb resistance throughout the country should be revealed, and the effective life of these insecticides should be tried to be extended by allowing them to be applied for limited times in locations without resistance problems.

However, in this study, it was determined that there was no resistance problem in the sample locations by looking at the LC_{99} values of azadirachtin and formetanate hydrochloride (except Ada Tarım). The reason for identifying pyridalyl-resistant populations is thought to be that it is used to control cotton bollworm in carnations, thus indirectly affecting the thrips populations. The

Table 3. The resistance level of *Frankliniella occidentalis* populations to active substances.

Population	Active ingredients	n	slope±se	LC_{50} mg(a.i.)/l (95% confidence limit)	* Sensitivity differences according to LC_{50} values (=resistance multiples)	LC_{90} mg(a.i.)/l (95% confidence limit)	LC_{99} mg(a.i.)/l (95% confidence limit)	Field recommended dose mg (a.i)/l
Sensitive	Spinosad	401	1.5±0.3	1.4 (0.6-3.1)	-	2.6 (1.4-4.3)	3.1 (1.8-6.8)	96
	Methiocarb	401	1.4 ±0.3	32.8 (19.9-41.1)	-	81.6 (62.4-95.4)	115.1 (96.5-136.5)	500
	Malathion	406	2.0±0.3	99.8 (81.4-115.3)	-	397 (366.4-465.3)	615 (591.4-665.3)	650
	Azadirachtin	401	1.6±0.2	0.1 (0.04-0.3)	-	0.9 (0.4-1.4)	1.2 (0.04-0.3)	1.5
	Formetanate hydrochloride	401	1.4±0.1	40.3 (29.6-59.8)	-	120.5 (102.9-144.3)	180.6 (162.3-204.7)	500
	Pyridalyl	401	1.5±0.2	15.4 (8.5-24.0)	-	32.1 (11.5-53.5)	41.8 (22.6-69.9)	100
	Spinosad+Formetanate hydrochloride	399	1.7±0.3	1.2 (0.5-2.4)	-	2.4 (1.4-3.5)	2.8 (1.3-4.4)	96+500
Antalya Tarım	Spinosad	413	1.4±0.2	32.4 (21.4-65.3)	23.10	125.6 (110.4-295.7)	175.2 (152.2-301.6)	96
	Methiocarb	409	1.5±0.2	70.5 (49.3-89.8)	2.10	513.9 (425.5-998.2)	632.4 (532.4-1024.8)	500
	Malathion	402	1.8±0.2	205.4 (181.4-225.3)	2.05	525.6 (500.4-695.7)	645.2 (632.2-701.6)	650
	Azadirachtin	399	1.5±0.2	0.6 (0.2-1.0)	6.00	1.0 (0.8-1.3)	1.1 (0.9-1.4)	1.5
	Formetanate hydrochloride	408	1.3±0.2	60.4 (39.3-89.8)	1.50	323.9 (305.6-348.2)	436.6 (405.4-464.8)	500
	Pyridalyl	398	1.6±0.3	42.4 (31.4-63.3)	2.75	75.6 (60.4-95.3)	95.6 (90.4-112.7)	100
	Spinosad+Formetanate hydrochloride	403	1.8±0.3	12.4 (8.6-17.2)	10.33	30.4 (22.3-38.4)	41.8 (28.3-66.3)	96+500
Flash Tarım	Spinosad	401	1.3±0.1	26.8 (16.5-52.8)	19.10	132.9 (72.4-183.7)	176.1 (132.8-298.5)	96
	Methiocarb	401	1.4±0.2	86.3 (53.2-96.5)	2.60	550.7 (433.2-957.6)	701.6 (648.6-1357.3)	500
	Malathion	400	1.5±0.1	260.8 (235.5-292.8)	2.61	632.9 (572.4-683.7)	698.1 (638.8-728.5)	650
	Azadirachtin	412	1.3±0.1	0.5 (0.2-0.9)	5.00	0.9 (0.7-1.4)	1.1 (0.8-1.4)	1.5
	Formetanate hydrochloride	399	1.4±0.3	76.3 (55.6-96.9)	1.90	350.7 (333.2-397.3)	455.6 (406.3-487.2)	500
	Pyridalyl	411	1.9±0.4	46.8 (36.5-72.8)	3.03	82.9 (67.2-93.4)	92.9 (72.5-123.7)	100
	Spinosad+Formetanate hydrochloride	415	1.9±0.3	16.7 (12.6-26.8)	13.91	56.5 (48.3-71.4)	72.5 (50.6-95.4)	96+500
Tempo Tarım	Spinosad	395	1.4±0.1	15.4 (8.5-24.0)	11.00	112.4 (86.8-221.3)	148.5 (113.7-242.6)	96
	Methiocarb	395	1.3±0.1	85.6 (52.3-100.2)	2.60	541.3 (312.5-967.6)	687.1 (624.7-1101.6)	500
	Malathion	398	1.3±0.1	346.8 (308.5-424.0)	3.47	612.4 (586.8-641.3)	648.5 (599.7-675.6)	650
	Azadirachtin	395	1.4±0.2	0.5 (0.1-0.9)	5.00	1.0 (0.6-1.3)	1.2 (0.5-1.5)	1.5
	Formetanate hydrochloride	396	1.8±0.4	65.6 (52.3-79.2)	1.60	381.3 (354.3-412.1)	486.1 (422.7-500.6)	500
	Pyridalyl	405	1.8±0.4	49.3 (42.0-75.5)	3.20	105.4 (76.2-121.5)	112.4 (96.8-131.3)	100
	Spinosad+Formetanate hydrochloride	404	2.0±0.5	12.3 (8.1-19.2)	10.25	34.3 (25.2-51.6)	48.6 (30.8-72.2)	96+500
Erkut Tarım	Spinosad	408	2.0±0.3	17.3 (12.0-25.5)	12.40	102.3 (76.3-210.8)	135.6 (101.2-220.5)	96
	Methiocarb	411	1.4±0.1	78.7 (51.0-93.7)	2.40	516.8 (432.6-1012.3)	650.8 (554.2-1142.7)	500
	Malathion	405	1.7±0.2	273.3 (212.0-298.5)	2.73	502.3 (476.3-530.8)	635.6 (601.2-670.5)	650

*: LC_{50} value of populations / LC_{50} value of the most susceptible (lowest LC_{50}) population.
a.i.: active ingredient, n: number of pests used in the test.

Table 3. The resistance level of Frankliniella occidentalis populations to active substances (contin.).

Population	Active ingredients	n	slope±se	LC_{50} mg(a.i.)/l (95% confidence limit)	* Sensitivity differences according to LC_{50} values (=resistance multiples)	LC_{90} mg(a.i.)/l (95% confidence limit)	LC_{99} mg(a.i.)/l (95% confidence limit)	Field recommended dose mg (a.i)/l
Erkut Tarım	Azadirachtin	402	2.2±0.3	0.3 (0.2-0.6)	3.00	0.8 (0.4-1.1)	1.1 (0.8-1.5)	1.5
	Formetanate hydrochloride	400	1.5±0.3	78.7 (62.1-88.6)	1.90	366.8 (332.3-402.1)	474.8 (439.1-498.7)	500
	Pyridalyl	406	2.1±0.3	57.3 (42.0-75.5)	3.72	112.3 (86.4-132.5)	132.3 (96.3-150.8)	100
	Spinosad+Formetanate hydrochloride	398	1.6±0.2	14.2 (10.3-21.2)	11.83	55.1 (46.4-68.4)	72.3 (57.3-96.2)	96+500
Ada Tarım	Spinosad	411	2.1±0.1	40.0 (25.4-72.8)	28.60	140.8 (88.5-261.7)	195.9 (147.6-320.2)	96
	Methiocarb	406	2.0±0.3	90.5 (59.3-139.8)	2.70	620.6 (389.5-1212.3)	745.6 (701.5-1443.2)	500
	Malathion	403	2.1±0.1	420.2 (375.4-472.8)	4.21	640.8 (588.5-661.7)	715.9 (647.6-745.2)	650
	Azadirachtin	400	2.3±0.4	0.7 (0.3-0.9)	7.00	1.3 (0.9-1.4)	1.5 (1.0-1.8.)	1.5
	Formetanate hydrochloride	403	2.1±0.4	80.5 (62.9-96.3)	2.00	389.6 (338.5-415.3)	506.9 (471.5-543.6)	500
	Pyridalyl	402	2.3±0.2	60.0 (45.4-82.8)	3.89	120.8 (98.6-144.3)	140.8 (121.5-161.7)	100
	Spinosad+Formetanate hydrochloride	403	1.9±0.3	31.1 (20.2-40.6)	25.91	80.7 (71.3-98.6)	102.4 (86.5-132.5)	96+500

*: LC50 value of populations / LC50 value of the most susceptible (lowest LC50) population.
a.i.: active ingredient, n: number of pests used in the test.

reason for malathion resistance can be interpreted as the fact that the active ingredients has been used in the control of many pests, including thrips, for many years.

As a result of the study, the highest resistance ratios were observed in spinosad active ingredient. In spinosad's screening for resistance in populations, the lowest resistance rate was determined to be 11.0 times and the highest was 28.6 times. Furthermore, the lowest resistance ratio was observed in formetanate hydrochloride active ingredient with 1.5 times and the highest with 2 times. In the last year of the study, a lower resistance rate was obtained in the spinosad+formetanate hydrochloride mixture test compared to the spinosad application alone test. As a result of this experiment, it was determined that two insecticide mixtures with different modes of action caused a decrease in the resistance of spinosad-resistant populations. It is predicted that this application can be recommended for resistance management in locations with spinosad resistance.

The results obtained from the resistance studies should also give an idea about the level of negative impact on the success of pesticide applications in the field. Considering this situation, the resistance of the populations collected from the field was compared to the susceptible population in our study. Besides, the dose values (LC_{99}) that killed 99% of the field populations were compared with the recommended dose values of the insecticides in question in the field. Although the laboratory results do not reflect the practice exactly, the insecticide test method we used in the study shows a close resemblance to the practice. In our tests, thrips are exposed to the chemical under the most severe conditions. The pests are forced to feed for two days in the chemically treated area on a real leaf disc (without the possibility of escape). Chemicals that are ineffective under these conditions may have great difficulty succeeding under field conditions. From this point of view, if the LC_{99} dose value of an insecticide obtained in laboratory tests against a population is higher than the field-recommended dose of this chemical, it should be considered that the pesticide applications to be made in the field may fail.

Flower thrips have the potential to develop significant resistance to insecticides used for the control of *F. occidentalis*. Studies conducted in America, Australia, European countries, and Israel have reported that the species in question develop varying levels of resistance to the following chemical classes: endosulfan in the cyclodiene group; acephate, chlorpyrifos, malathion, dichlorvos, dimethoate, monocrotophos, and methamidophos in the organophosphorus group; methiocarb, methomyl, carbosulfan, and formetanate in the carbamate group; acrinathrin, bifenthrin, and cypermethrin in the pyrethroid group, abamectin in the macrocyclic lactone group, spinosad in the spinosyn group (Immaraju et al., 1992; Brodsgaard, 1994; Zhao et al., 1995; Karadjova, 1998; Kontsedalov et al., 1998; Jensen, 2000; Espinosa et al., 2002; Herron and James, 2005; Bielza et al., 2007; Dağlı and Tunç, 2007).

Bielza et al. (2007) indicated that the LC_{50} values of *F. occidentalis* samples collected from areas in Murcia where no spinosad application had been made before, were between 0.005-0.077 mg l^{-1} and were sensitive. Moreover, the samples collected from Almeria were resistant to spinosad when compared with the sensitive population of Bielza. In addition, it was determined in the study that spinosad did not have cross-resistance with methiocarb, acrinathrin and formetanate. Besides, Dağlı et al. (2010) determined methiocarb resistance in populations taken from Antalya and its districts between 1.4-15.3 times according to LC_{50} values. It was stated that applications with methiocarb may fail at all points where the populations have been taken. Dağlı et al. (2010) determined resistance to spinosad in populations taken from Antalya and its districts between 1.0-141 times according to LC_{50} values. It was stated that applications with spinosad may fail at all points where the populations have been taken. Furthermore, Dağlı (2018) investigated spinosad resistance in *F. occidentalis* population from Antalya and determined a 235-times loss of sensitivity in the population compared to LC_{50} values. However, in this study, spinosad resistance of populations was determined as 11.0-28.6 times. The reason for the resistance multiples difference between the present study and Dağlı (2018) is thought to be the presence of vegetable fields around the carnation greenhouses and the transition of possible sensitive populations to the carnation greenhouses.

Espinosa et al. (2002) reported 0.5-23.0-times loss of sensitivity to Formetanate and 1.3-22.3 times to methiocarb in *F. occidentalis* populations collected from Spain. However, in this study, a 1.5-2 times loss of sensitivity to Formetanate and a 2.1-2.7 times loss of sensitivity to methiocarb was detected. In another study, Jensen (1998) reported 9.5 times resistance to Methiocarb in the *F. occidentalis* population. Espinosa et al. (2002) determined an approximate 10 times reduction between Formetanate and methiocarb compared to this study. The fact that there are different vegetable areas around and the populations are crossing each other is considered to be the reason for the loss of sensitivity in the populations. Another important result of our study was that applications with spinosad+formetanate hydrochloride against this pest in locations known to have spinosad resistance could reduce the resistance.

4. Conclusion

Economic and environmental losses can be prevented to some extent by avoiding excessive and unnecessary insecticide applications against resistant populations. Studies yet to be conducted should regularly monitor sensitivity to any chemicals that may be used alternatively to control *F. occidentalis* field populations. Moreover, with comprehensive research on populations, cross or multiple resistance spectra for current insecticides, resistance mechanisms and the inheritance pattern of resistance (genetics) should be revealed and basic resistance management programs should be established. Both in this study and other studies on resistance screening, it has been proven that the chemical-based control method becomes ineffective after a while due to resistance. At this point, it is imperative to expand the practical use of other control methods (such as cultural, biological, and biotechnical control) that minimize the selection pressure on pests.

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RESEARCH PAPER

Micropropagation of Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] Leaf Explants and Single Nodes in Response to Plant Growth Regulators

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Abstract

Lisianthus has long-lasting, white, blue-purple, velvet or pink flowers. Varieties that can be used in pots, outdoors, or cut flowers are widely used and it is essential to develop new varieties to increase their commercial value. Breeding studies with classical methods can take a long time in lisianthus. Techniques to create variation in these plants and facilitate their reproduction always contribute to shortening the breeding period and one of the best known of these methods is plant tissue culture. This study aimed to investigate the micropropagation possibilities in lisianthus and examine the effects of auxin and cytokinin applications and doses on shoot reproduction. Supplemented with 10 different plant growth regulators in Murashige and Skoog (MS) medium, leaf explants from lisianthus plants that were germinated under *in vitro* conditions (seedlings) were cultivated. 6-benzyl aminopurine (BAP) was utilized in 5 doses (0.5, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}) alone in the first 5 groups. Naphthalene acetic acid (NAA) was also added to the BAP mediums at a concentration of 0.5 mg L^{-1}. Shoot formation, shoot lengths, the number of shoots longer and shorter than 1 cm, and regeneration of new shoots after subcultures were determined. According to the results, MS mediums containing 3.0 and 4.0 mg L^{-1} BAP could be good starting mediums for regenerating new lisianthus shoots from leaf explants. These treatments got the highest values both in shoot formation and the number of shoots longer than 1 cm. It has been determined that 3.0 mg L^{-1} BAP alone can be used for both organogenesis from leaf explants and shoot proliferation in single node culture. *In vitro* shoots of the lisianthus species were easily rooted and both PGR-free MS or ½ MS mediums. Lisianthus shoots propagated under *in vitro* conditions can be rooted under *ex vitro* conditions both in the float hydroculture and in the soil mixture. The acclimatization stage was optimized also successfully.

1. Introduction

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn.) is an ornamental plant, belongs to the Gentianaceae family, has silky-looking but very durable flowers in bright and pastel colors. It is among the top 10 cut flowers in the ornamental plants trade with long post-harvest life of flowers (Harbaugh, 2007; Hutchinson, 2011; Bertoldo et al., 2015; Ozkan, 2017). Tall types are preferred for cut flower production in the field and greenhouse, and multi-branched dwarf varieties come to the fore when it was planned to be used as potted plants (Harbaugh and Zhanao, 2006; Hanks, 2014; Menge, 2019).

Lisianthus is usually a seed-propagated plant. Root cuttings or tissue culture methods can also be used for propagation. Although the most commonly

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used method of propagation is seed propagation, the very small size of the seeds (10,000 seeds in 1 g) causes the need for seed pelleting (coating) for commercial production. Inbreeding depression is an important limiting feature for lisianthus breeding. Self-depression causes poor seed formation or poor plant growth. However, strong inbred lines need to be used in the generation of F_1 hybrids from strongly developing homozygous parents (Harbaugh, 2007).

However, seed propagation is provided by seedling cultivation, obtaining homogeneous seedlings in double-colored and double-flowered types can sometimes be a problem. There may be differences in flowering time, plant height, and number of flowers in plants grown from seeds in varieties with high heterogeneity (Furukawa, 1993). Micropropagation of the plant is also an issue of interest in lisianthus, as it allows obtaining very high numbers of plants in a short time with a homogeneous structure. Propagation by tissue culture is important for large-scale propagation of high-quality selected or breeding plants (Semeniuk and Griesbach, 1987; Miri et al., 2016; Rahaman et al., 2018). Success in effective micropropagation of lisianthus depends on several factors such as genotype, culture medium, plant growth regulators (PGR), and explant type (Ordogh et al., 2006; Uddin et al., 2017). The importance of micro-propagation in the reproduction of elite clones is indisputable. *In vitro* propagation of *Eustoma*; either by direct organogenesis using explants such as shoot tip, lateral buds, internode stem segments, and leaf fragments (Semeniuk and Griesbach, 1987; Mousavi et al., 2012), organogenesis via callus (Rezaee et al., 2012; Akbari et al., 2014) can also be obtained. Duong et al. (2006) developed a protocol for optimal somatic embryo formation from leaf explants and made it available for plant breeding programs (Yumbla-Orbes et al., 2020).

The explant/shoot tip consists of the shoot apical meristem, non-elongated leaves of various stages of development, and a series of leaf primordia approximately 1 cm long. Cytokinin-rich medium suppresses the apical dominance of shoot-tip explants and promotes the formation of highly branched shoot systems (Saeed et al., 1997). Moreover, *in vitro* plants (shoots, seedlings) containing single nodes can be used as explants, because the nodes contain axillary buds and treatment with cytokinins under *in vitro* conditions can induce branch formation (Barna and Wakhlu 1995). In the shoot multiplication stage of propagation by tissue culture, single-node explants are successfully used to establish subcultures to obtain a sufficient number of micro shoots. Martini et al. (2022) reported that the single node explant of the *Salvia tomentosa* plant in the subculture stage gave high shoot numbers. Single node explants are obtained by separating the shoots grown in tissue culture into micro cuttings. Cuttings are a natural method of vegetatively propagating plants in both *in vivo* and *in vitro* conditions. Leaf axillary buds are comparable to stem tips in terms of mass propagation capacity (Bhatia and Sharma, 2015). This method has been used successfully in the micropropagation of many plants such as *Mentha* sp. (Tepe et al., 2002), *Artemisia* sp. (Türközü et al., 2014), *Centauria* sp. (Okay et al., 2014), *Elatostema* sp. (Mizushima, 2017).

Micropropagation is currently the easiest way to produce healthy, disease-free *Eustoma* (Jafari et al., 2017). The purpose of this research is to determine the optimum *in vitro* propagation method using single node culture from leaf explants of *in vitro* seedlings of lisianthus. In light of the literature, the major goals of the research are to identify a technique that can enable quick plant reproduction, identify developing issues, and obtain knowledge that will provide the foundation for additional studies. After the completion of this study, it is aimed to breed local varieties that will be commercially propagated by vegetative propagation method using *in vitro* mutation methods, using biotechnological accelerator techniques.

2. Materials and Methods

The research was carried out at Isparta University of Applied Sciences, Faculty of Agriculture, Department of Horticulture, and in the tissue culture laboratory and greenhouses of Has Biotech Company in Antalya between 2021 and 2022. Plant material from lisianthus plants with certificate number 552640 and supplied by the Ege Plantek Company's Türkiye representative was used in the study. Commercial varieties of the *Eustoma grandiflorum* (Raf.) Shinners species with purple and white flowers were used to test *in vitro* germination and regeneration. Due to the usage of the leaves of *in vitro* seedlings, appropriate PGR tests were conducted.

2.1. General tissue culture techniques applications

All tissue culture applications were carried out under aseptic conditions, and a sterile cabinet (laminar flow cabinet) was used. The scalpel blade and forceps used were pre-sterilized and during the culturing process, a bead sterilizer was used to keep the forceps and scalpels sterile during sewing. MS (Murashige and Skoog, 1962) basic medium composition was used as the nutrient medium. Commercially available premixed powder of MS was used in the preparation of plant nutrient medium (Sigma). These products, whose macro and micronutrients and vitamins have been adjusted at the dose of use, provide a significant convenience in the functioning of the study. Plant growth regulators (PGR) were added to the basic MS medium, which differed according to the treatments. A few drops of 1 N NaOH or HCl solutions were used as needed to adjust the pH.

Finally, 3.0% sucrose and 0.7% agar were added to the nutrient medium. All components were dissolved on a hot plate magnetic stirrer. After the nutrient mediums were autoclaved in 250 or 500 mL autoclave bottles with caps, they were distributed in the previously sterilized Petri dishes as 10 mL each in the sterile cabinet and 40 mL in the jars. Sterilization of the medium in the autoclave was carried out under 1.2 atm of pressure, at 121°C for 20 minutes. After being removed from the autoclave, the bottles containing the sterile nutrient medium were shaken well to ensure that the agar was completely mixed with the medium, and then the medium was distributed in Petri dishes or jars in the cabinet. Following the solidification of the agar at room temperature, the medium was prepared for planting the explants.

2.2. Sterilization of seeds

Lisianthus seeds of two different varieties were brought to the laboratory to germinate on nutrient medium under *in vitro* conditions after being kept at refrigerator temperature for 10 days. The seeds were first placed in small packets prepared from filter paper and these packets were attached with paper clips. Due to the leakage of very small seeds through the wire strainers, it is necessary to immerse them in a disinfectant solution in cheesecloth bags or paper packages. Paper packets containing seeds were dipped in 20% commercial sodium hypochlorite (bleach) for 15 minutes for surface sterilization.

Meanwhile, 3 drops of Tween-20 (0.2% dose) were dropped into the disinfectant solution. After that, it was rinsed with sterile distilled water 3 times for 5 minutes each. After the final rinse water in the glass jar, where sterilization was made, was filtered, the paper packets containing the seeds were taken on sterile blotting paper and the excess water was absorbed into the blotting paper. The paper clips were opened and the seeds stuck on the paper were transferred to the surface of the nutrient medium in February 2021. In this way, the seed planting process has been completed. All these processes were performed in a laminar flow cabinet under aseptic conditions.

2.3. Preparation, planting, and incubation of explants on nutrient medium

After germination of sterile lisianthus plants leaf explants were cultured in different PGR experiments. Under aseptic conditions, the material in the form of rectangular leaf explants was prepared with a scalpel on sterilized filter papers in the cabinet, and the underside of the leaf was placed on the nutrient medium in Petri dishes (Figure 1). After placing 5-6 explants in each Petri dish, they were wrapped with stretch film strips for cutting off their contact with the atmosphere. After planting the explants in MS medium in Petri dishes, the cultures were incubated at 25±2°C and in photoperiodically arranged climate chambers with 16 hours light/8 hours dark.

2.4. Establishment stage

Leaf explants taken from *in vitro* germinated plants (seedlings) of lisianthus were cultured in MS medium with 10 different plant growth regulators. In the first 5 groups, 5 doses (0.5, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}) of BAP (6-benzylaminopurine) were used alone. In the second group, 0.5 mg L^{-1} NAA (Naphthalene acetic acid) was also added to the BAP mediums in the experiment. An average of 4-6 leaf explants were planted in each Petri dish and 10 Petri dishes were used for each treatment. PGR (plant growth regulators) doses and combinations used in the experiment are shown in Table 1. The regenerated shoots from leaf explants about 4 cm in length were cultured by micro-cutting method and single nodes were planted in the mediums.

When the 8th week of culture was completed, observations, counting of the number of shoots, and evaluations were made. In the 12th week, the shoot

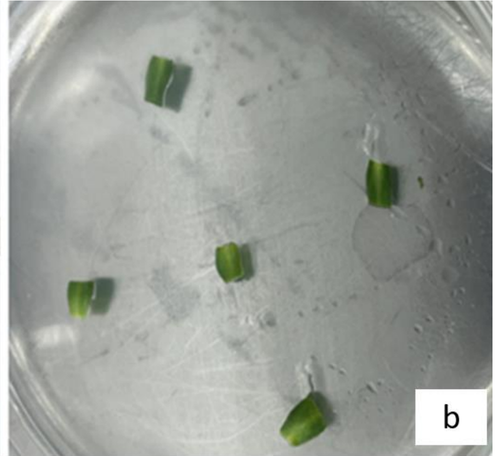

Figure 1. *In vitro* seedlings (a) and leaf explants (b).

Table 1. Plant growth regulators.

Plant growth regulators	Concentration (mg L^{-1})	Plant growth regulators	Concentration (mg L^{-1})
BAP	0.5	BAP+NAA	0.5+0.5
	1.0		1.0+0.5
	2.0		2.0+0.5
	3.0		3.0+0.5
	4.0		4.0+0.5

BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

Figure 2. Planting the plantlets in vials (a) and placing in covered plastic boxes and keeping them in the climate room (b).

lengths and shoot numbers shorter and longer than 1 cm of the regenerating shoots were estimated and tables were created. After this stage, the explants longer than 1 cm were sub-cultured and incubated for 4 weeks in a 16/8-hour photoperiod in a climate chamber at 25°C. At the end of this period, the number of plants formed after subcultures and the number of plants shorter and longer than 1 cm were also recorded.

2.5. Rooting stage

Rooting experiments were established using shoots longer than 1 cm in subcultures, and 20 shoots representing each growth medium (10 different compositions) were transferred to the PGR-free MS and ½ MS mediums. Thus, it was also investigated whether the propagation medium composition would have an effect on rooting. After 4 weeks, the rooting was observed and the rooted shoots were transferred to the acclimatization stage. On the other hand, after 100 shoots were placed in styrofoam viols, they were taken into floating water culture in plastic containers, where both rooting and acclimatization stages were aimed together. Afterward, 100 shoots were placed directly in a mixture of peat and perlite filled in pots with a diameter of 10 cm, watered, and put into plastic boxes. The lids of the boxes were first kept closed and gradually opened within a week from the second day.

2.6 Adaptation to external conditions

In acclimatization of rooted lisianthus plantlets to external conditions, the plantlets were taken out of glass jars and their roots were washed under tap water and purified from the agar-based nutrient medium. The plantlets (200 pieces in total), which were planted in 5 cm diameter vials in which filled peat: perlite (2:1) mixture, were placed in deep and covered plastic boxes and kept in the climate room. Before the plastic cover was covered, the plants were irrigated and the leaves were sprayed with a mini hand sprayer. Water spraying was continued by opening the cover of plastic boxes (mini-greenhouse) twice a day for 4 days, after which the cover was gradually opened slightly and completely removed at the end of a week. These processes were carried out in the tissue culture climate chamber (25°C) with daily illumination of 16 hours (Figure 2).

The plants were transported to the greenhouse in boxes with the covers completely opened, then transferred to new pots filled with peat (Clasmann) one by one. They were placed on styrofoam under a black net in a shaded area in the greenhouse. Spraying water on the leaves twice a day with a hand sprayer was done for 5 days, after this stage, water was sprayed once a day with an interval of two days, and after the 10th day, the plants were allowed to develop normally.

2.7 Analysis of data

The data of the experiment were statistically evaluated in the Completely Randomized Design, 3 replications and a total of 5 explants were used in each replication (3 replications × 10 combinations of PGR's). Statistical analyses were performed in the SAS-JMP pro13 program (SAS Institute Inc., Cary, North Carolina, USA). Differences between data were tested by analysis of variance, moreover,

means determined to be statistically different were compared with the LSD test.

3. Results and Discussion

3.1. Seed germination studies

Lisianthus seeds belonging to two different genotypes were kept in a closed glass jar in the refrigerator at +4°C for 10 days, and then they were sterilized and planted under sterile conditions in the prepared PGR-free MS medium. The first germination occurred after 17 days. Although the germination of the white-flowered variety, 99.8% germination was observed from 200 seeds sown, and 23.6% heterogeneous germination was observed in the variety with purple-white variegated flowers. This situation was also mentioned by Furukawa (1993) and Haspolat et al. (2020) that there is substantial genetic heterogeneity in seed germination and that physiological requirements might vary significantly between genotypes. As a result of this finding, the study was continued on the white-flowered variety, in which germinated and sterile seedlings were obtained.

3.2. Shoot formation of leaf explants

Leaf explants produced shoot differentiation in all treatments. It was demonstrated that leaf explants can be successfully used in lisianthus tissue culture for organogenesis. Previous studies have shown that different explants can be used in *in vitro* propagation of lisianthus. For example, Paek and Hahn (2000), Ordogh et al. (2006), Nhut et al. (2006), Esizad et al. (2012), and Yumbla-Orbes et al. (2020) were able to provide shoot organogenesis when they used different explants such as internodes, roots, and petals in lisianthus as starting material. However, Rezaee et al. (2012), proved in their study that leaf explants had higher replication capacity than all other explants. Deroles et al. (1993), Semeria et al. (1996), and Handa and Deroles (2001) reported that leaf explants were successful for *in vitro* shoot regeneration and could be the most suitable expander for genetic transformation studies in lisianthus.

3.2.1. Number of shoots

The shoot numbers were measured after the 8th week of culture. In terms of the average maximum number of shoots, all the treatments were not statistically significant. The maximum number of shoots was observed at the mediums with 3.0 and 4.0 mg L^{-1} BAP (14.9 and 14.5). In the 12th week, shoot numbers of shorter and longer than 1 cm were estimated (Figure 3). The average maximum number of shoots measured over 1 cm in length was observed in the medium containing 3.0 mg L^{-1} BAP (9.4 shoots/explant) followed by the 4.0 mg L^{-1} BAP medium. However, since the total number of shoots formed was different, it is clear that these values alone may not be sufficient for medium selection. Because of this, different BAP doses (1.0, 2.0, 3.0, and 4.0 mg L^{-1}) appear to be leading in this regard when the ratio value between shoots longer than 1 cm/shoots shorter than 1 cm was analyzed in terms of providing an idea. In other words, the formation of long shoots was observed more than the number of short and rosette-like shoots in the mediums with BAP concentrations. Within the scope of all these evaluations, it is possible to say that 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} BAP mediums are preferable in terms of both total shoot formation and shoot-forming properties suitable for sub-culturing longer than 1 cm (Table 2).

3.2.2. Shoot lengths

In addition to the number of shoots obtained, it is important that they have reached a size that can be sub-cultured. For this reason, to determine the composition of the nutrient medium to be used, the lengths of the shoots formed in the 12th week were also measured. Different medium treatments were determined to be statistically significant in terms of shoot length values. The average shoot length was measured as the longest in 0.5 mg L^{-1} BAP medium and followed by other BAP treatments which were located in the same statistical group. The maximum

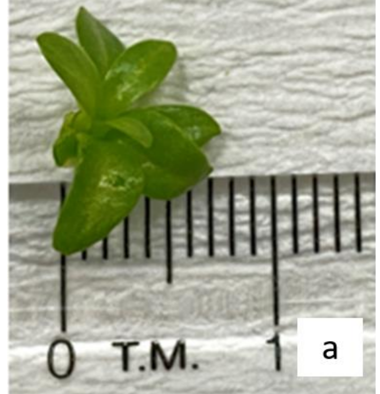

Figure 3. Twelve weeks after planting the leaf explants, the shoots shorter than 1 cm (a), and longer 1 cm (b).

Table 2. The average shoot number, number of shoots less than 1 cm in length, and number of shoots higher than 1 cm.

Plant growth regulators	Concentration (mg L^{-1})	The average shoot number	Number of shoots <1cm (A)	Number of shoots ≥1cm (B)	B/A ratio
BAP	0.5	9.5* ± 3.11	4.0 ± 1.71	5.5 ± 0.23	1.4
	1.0	8.7 ± 3.11	4.0 ± 1.71	4.7 ± 1.56	1.2
	2.0	7.2 ± 0.17	3.0 ± 0.33	4.2 ± 0.17	1.4
	3.0	14.9 ± 5.09	5.5 ± 3.92	9.4 ± 1.54	1.7
	4.0	14.5 ± 4.79	4.0 ± 3.19	7.5 ± 1.91	1.9
BAP+NAA	0.5+0.5	9.7 ± 1.01	4.5 ± 1.03	5.2 ± 1.01	1.2
	1.0+0.5	7.7 ± 0.47	7.2 ± 5.70	6.5 ± 5.69	0.9
	2.0+0.5	11.7 ± 0.39	6.4 ± 0.40	5.3 ± 0.00	0.8
	3.0+0.5	8.9 ± 4.46	6.3 ± 4.25	2.6 ± 0.75	0.4
	4.0+0.5	12.6 ± 5.03	5.5 ± 2.68	7.1 ± 2.54	1.3

*All mediums are non-significant.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

Table 3. The average, maximum, and minimum shoot lengths.

Plant growth regulators	Concentration (mg L^{-1})	The average shoot length (cm)	Minimum shoot length (cm)	Maximum shoot lengths (cm)
BAP	0.5	0.63 a* ± 0.06	0.3	1.4
	1.0	0.58 ab ± 0.16	0.2	1.0
	2.0	0.56 ab ± 0.21	0.2	1.3
	3.0	0.51 ab ± 0.20	0.2	1.4
	4.0	0.59 ab ± 0.18	0.3	0.9
BAP+NAA	0.5+0.5	0.43 bc ± 0.15	0.2	0.8
	1.0+0.5	0.55 ab ± 0.09	0.2	0.8
	2.0+0.5	0.41 bc ±0.09	0.1	0.7
	3.0+0.5	0.30 c ± 0.10	0.2	0.8
	4.0+0.5	0.46 abc ± 0.12	0.2	0.9

* The average shoot lengths, Medium $P<0.05$, LSD: 0.2; CV: % 20.49.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

shoot length was determined as 1.4 cm both in 0.5 mg L^{-1} BAP and 3.0 mg L^{-1} BAP mediums. The shortest average shoot length was measured in 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA medium and took place in a different statistical group (Table 3).

When the BAP cytokine was used in the study alone, it was able to successfully perform vascular meristematic tissue induction and subsequent shoot regeneration. The addition of a single dose of NAA (0.5 mg L^{-1}), used as an auxin source, was not observed to be a significant promoter of organogenesis, and even it was observed that it had some inhibitory effect. It was determined that there was some vitrification in the medium containing NAA. Therefore, the use of BAP alone was determined to be preferable for shoot regeneration and propagation from leaf explants. It can be observed from the literature that different types and doses of growth regulators are used for *in vitro* somatic embryogenesis or organogenesis in lisianthus (Kaviani, 2014; Pop et al., 2016; Yumbla-Orbes et al., 2020).

3.2.3. Sub-culture

Single node explants prepared from shoots longer than 1 cm formed the material for subculture. Images showing the development of single node explants in 5 different doses of BAP and the second 5 mediums combined with NAA are given in Figure 4. The results of average shoot number, number of shoots less than 1 cm in length, and number of shoots higher than were evaluated four weeks after the transfer of single node explants to 10 MS mediums with the same content as the one used at the beginning are given in Table 4.

The most effective medium in terms of the number of plants formed after subcultures were 3.0 mg L^{-1} BAP and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and were in the same statistical group in terms of the highest number of plants after subcultures (respectively 152 and 151 regenerated plants/10 plants).

For the number of plants formed under 1 cm in height, the highest value was observed in 1.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA medium, while the lowest number of plants obtained under 1 cm in the treatment of 1.0 mg L^{-1} BAP medium. The number of plants below 1 cm was 80 with the use of 1.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA, whereas the height of 71 plants was recorded above 1 cm. In terms of the number of plants over 1 cm in height, 3.0 mg L^{-1} BAP medium had the highest value, and 1.0 mg L^{-1} BAP medium followed this medium in a different statistical group (Table 4).

An average of 11.5 shoots/explants were obtained from the explants prepared as single node explants and sub-cultured. This number is above the previous studies. Uddin et al. (2017) determined that up to 3.23 new shoots developed from single shoots sub-cultured at 0.1-1.5 mg L^{-1} BAP and GA_3 combinations. It was thought that keeping the doses used lower as well as the genotype difference might have caused this effect. Yumbla-Orbes et al. (2020) reported that 10 µM 2,4-D (2,4-D dimethylamine salt) doses were optimal for somatic embryogenesis

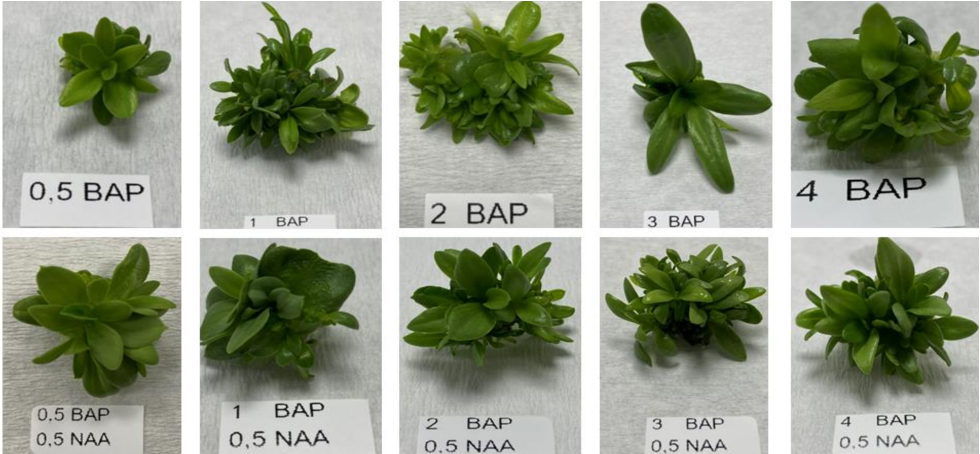

Figure 4. The appearance of 5 doses of BAP (6-benzyl aminopurine), and their second 5 series with 0.1 mg L^{-1} NAA (Naphthalene acetic acid.added) in lisianthus single node explants, 8 weeks after culturing.

Table 4. The average shoot number, number of shoots longer than 1 cm in length, and number of shoots shorter than 1 cm.

Plant growth regulators	Concentration (mg L^{-1})	Number of subcultured plants	Number of plants formed after subcultures	Number of plants <1cm	Number of plants ≥1cm
BAP	0.5	10	84 g* ± 2.52	39 e ±1.15	45 f ± 1.73
	1.0	10	110 d ± 1.53	23 f ± 1.15	87 b ± 1.00
	2.0	10	83 g ± 0.58	39 e ± 1.15	43 f ± 1.53
	3.0	10	152 a ± 1.53	56 c ± 5.51	95 a ± 4.16
	4.0	10	119 c ± 2.31	45 d ± 1.53	74 c ± 3.61
BAP+NAA	0.5+0.5	10	105 e ± 0.58	45 d ± 1.53	61 d ± 2.08
	1.0+0.5	10	151 a ± 2.00	80 a ± 0.58	71 c ± 2.08
	2.0+0.5	10	117 c ± 1.00	65 b ± 1.53	52 e ± 2.52
	3.0+0.5	10	94 f ± 0.58	68 b ± 1.53	26 g ± 1.00
	4.0+0.5	10	134 b ± 1.53	60 c ± 1.53	74 c ± 1.00

*Number of total plants formed after subcultures, Medium $P<0.01$. LSD: 2.6; CV: 1%; Number of plants <1cm, Medium $P<0.01$. LSD: 3.7; CV: 4%; Number of plants ≥1cm, Medium $P<0.01$. LSD: 4.1; CV: 4%.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

induction from leaf explants and (2 µM BA; 2 or 4 µM mT (meta-Topolin)) doses for maturation of the resulting Embryos. Mousavi et al. (2012), applied BAP and GA_3 to the B5 Gamborg medium instead of the MS medium, and determined that the most appropriate dose was 1.0 mg L^1 BAP. Ghanati et al. (2012), compared LS, B5, and MS mediums, and reported that the most shoot regeneration was obtained in B5 medium containing 1.0 mg L^1 BAP and GA_3.

Kaviani (2014), used kinetin as a cytokinin in treatments with combinations with NAA, and stated that the best shoot regeneration was obtained from MS medium containing 0.5 mg L^1 kinetin. In a different study, they obtained callus and shoot organogenesis in MS medium containing a combination of 0.1 mg L^1 BA and 0.2 mg L^1 NAA (Kaviani et al., 2014). In our study, single node explants prepared from *in vitro* regenerated shoots were used in the sub-culturing stage and new axillary shoot development per explant could be achieved up to 15.2 shoots in the most suitable medium composition. Pop et al. (2016), successfully obtained axillary shoots from single node cultures. The low doses of PGR used in their study, resulted in a limited number of axillary shoots obtained. While in our study, like the regeneration numbers, the axillary shoot-forming capacity was also higher due to the relatively high doses used.

It is possible to multiply examples of shoot regeneration studies in lisianthus, but two points come to the fore here: The first is the basic nutrient medium composition in all studies; the lisianthus plant gives shoot regeneration even in different types and doses with PGRs, and the studies suggest a successful PGR combination of nutrient medium. This indicates that lisianthus has a high tissue culture regeneration potential and can form plants without being too selective in terms of nutrient medium and PGRs. Secondly, the reason why different results were obtained from different studies and the optimum composition came out as different suggestions is that it can be considered as genotype x application interaction. As a matter of fact, in our study, genotype reactions were different while the seeds germinated. In general, cytokinin (BAP) doses between 0.1-4.0 mg L^1 were applied in treatments and the best results of the study were obtained in environments with 3.0 mg L^{-1} BAP doses. Although there was an increase in the number of shoots shorter than 1 cm as the cytokinin doses increased with the combination auxins, it was observed that they also grew when the culture period was extended. The results obtained in our study also contributed to the literature in this respect.

The addition of GA_3 to a nutrient medium promotes the elongation of small shoots and it is

frequently used in *in vitro* propagation studies (Ozcelik, 2000). Mousavi et al. (2012) and Uddin et al. (2017) preferred the addition of Gibberellic acid for regeneration of lisianthus. It may be useful to conduct new trials by adding GA_3 to 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} BAP treatments, which are approved by us. Thus, both the number of shoots will be increased and the number of developed shoots that can be separated more easily during the subculture of the shoots.

3.2.4 Rooting stage

The rooting experiment was started since a sufficient number of shoots were obtained in the subculture. For this purpose, 20 shoots longer than 1 cm were taken from each medium and transferred to MS and ½ MS mediums without adding any PGRs. After 4 weeks, it was determined that all the transplanted shoots were rooted. The rooting rate was 100% on both mediums. In fact, it was observed that rooting already occurred in some subculture mediums (especially in mediums containing NAA). There was no difference in rooting rates as a result of transferring lisianthus shoots to PGR-free ½ MS or MS mediums at the *in vitro* rooting stage. The rooting status of lisianthus shoots in MS and ½ MS medium is shown in Figure 5. As can be clearly seen from the image, there is no difference in rooting between the two mediums. Especially as the waiting time increased, the roots surrounded the bottom of the jar. Rooted shoots that had completed 4 weeks were ready for hardening and transferring to external conditions.

It was observed that lisianthus has no problems with rooting and even rooting occurs in environments that are kept for a long time during the shoot propagation stage. However, rooting experiments were established, and results were obtained. As a result of this research, rooting occurred in both used mediums. Rooting medium using full-strength MS salts gives positive results in most plant species (Németh, 1986). However, some conditions where macro and micro elements are diluted in ½, $^1/_3$, or ¼ ratio can sometimes give better results for rooting (Skirvin et al., 1980; Lineberger, 1983). Uddin et al. (2017) also noted that PGR-free ½ MS medium is a suitable choice for rooting in lisianthus.

Lisianthus has been observed as a plant species that responds quickly to *in vitro* applications and does not require intensive doses of PGRs. This is also evident in the rooting phase and plain medium with low strength resulted in healthier plantlets. Both MS and semi-strength PGR-free MS mediums were observed to be successful since 100% rooting occurred in both mediums without any difference in rooting rates.

3.2.5. Aclimatization stage

Rooted lisianthus plantlets were planted in the peat: perlite (2:1) mixture and covered for 10 days. The plantlets, which were gradually acclimated to atmospheric conditions, continued to live in a healthy way at the rate of 100% and were transferred to the greenhouse. Just like the seedlings transferred to the outdoor conditions by transferring to the pots, the seedlings, which were adapted to the external conditions for 5 days by transferring directly to the viols in the greenhouse, continued their vitality without any loss. In addition to being a species that responds positively to tissue culture and can be easily reproduced, lisianthus has proven to be a plant with a strong adaptability with its high survival rate during the acclimatization phase to external conditions.

Peat: perlite (2:1) mixture was determined suitable for the transfer stage to external conditions. No infection was encountered and all plants developed healthily. Kabakci (1996), reported that the lisianthus seedlings obtained under *in vitro* conditions were first acclimatized to external conditions in the pumice medium and then transferred to the soil medium. At this stage, an impression was formed that different applications can be made on *ex vitro* rooting experiments.

The *in vitro* lisianthus shoots can be rooted at the same time during the transfer of external conditions in subsequent studies. Rooting was also

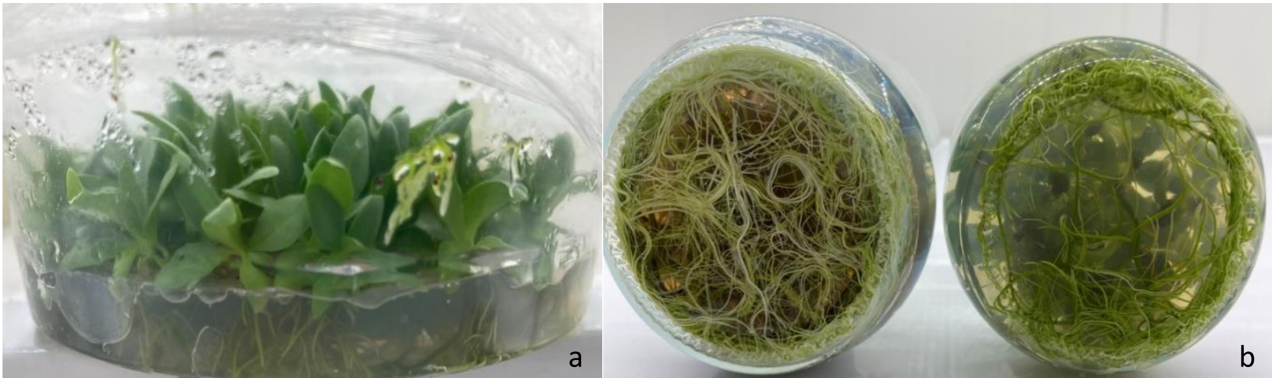

Figure 5. Formed roots of lisianthus shoots, 6 weeks after transferring to rooting mediums (a) and after 2 months to MS (left jar) and ½ MS (right jar) medium (b).

successfully achieved in ex vitro rooting experiments. Moreover, 87% of rooting occurred in the float hydroponic system. Among the shoots transferred directly to the soil mixture, 91% were rooted. It may be useful to address this issue in future studies. Thus, it seems possible to provide a more economical production cycle by reducing the time spent in *in vitro*. For the first time, Clapa et al. (2011), induced roots successfully and acclimatized the rooted shoots to external conditions. Ibrahim (2022), reported that *ex vitro* rooting was not obtained in *Pyrus* sp., while, using the float hydroculture technique, *in vitro* plantlets were successfully acclimatized in 4 weeks. Bejaoui (2022), achieved 100% rooting and acclimatization when transferring *in vitro Kalanchoe* shoots to the floating system. According to the study, *ex vitro* rooting enables tissue culture methods to be more affordable and allows for faster transportation of plantlets to greenhouses. Figure 6 shows lisianthus plantlets rooted in the float hydroponic system and Figure 7 directly transferred to the soil and also acclimatized. The seedlings that developed in our study were transferred to larger pots and grown in the greenhouse in Antalya (Figure 7).

4. Conclusions

Eustoma grandiflorum is a species prone to proliferation by tissue culture. Leaf explants are available for tissue culture propagation. When the appropriate medium is selected, direct shoot regeneration occurs after callus development on the cutting surfaces. Leaf explants achieved shoot differentiation in all mediums. It has been demonstrated that the leaf explants can be used successfully in the tissue culture of lisianthus when it comes to applications for organogenesis.

When the cytokinin and auxin structure is well adjusted, both shoot differentiation can be obtained as a sufficient number of starting materials, they can also benefit from each other, and shoots longer than 1.0 cm can be obtained. For the superficial sterilization of seeds, 20% commercial sodium hypochlorite can be used and after waiting for 15 minutes, it should be rinsed 3 times with sterile distilled water. There was no contamination problem because sterile seedlings were used as the explant source. The PGR-free MS medium used as the control medium, and no organ development took place. There was only swelling in the tissues and very slight swelling on the cut surfaces. There are PGR needs for *in vitro* regeneration. It can be concluded that MS medium containing 3 and 4.0 mg L^{-1} BAP could be a good starting medium for regenerating new lisianthus shoots from leaf explants. These treatments had the highest values both in shoot formation and the number of shoots longer than 1 cm. Although the number of shoots increased in high induction of NAA together with BAP, it was difficult to separate the regenerated shoots from each other during the sub-culturing phase, and they tend to develop in the form of bushes. In leaf explants, shoots begin to appear in the 6th week, and 10-15 shoots per explant can be obtained at the end of the 8th week in the above-mentioned mediums. Counts were made on 10 shoots in the subculture stage and it was aimed to reduce the standard error statistically.

Figure 6. Lisianthus shoots in *ex vitro* rooting and acclimatization periods (a), shoots placed for rooting in float hydroponic system in styrofoam viols (b), roots developed in water (c), healthy rooted lisianthus plantlets in the hydroponic culture, (d) lisianthus plantlets after 10 days of transfer to the soil mixture (in the greenhouse).

Figure 7. Lisianthus shoots in *ex vitro* rooting and acclimatization periods (a), transferring shoots to the soil mixture directly (b), *ex vitro* rooted lisianthus shoots (c) lisianthus plants after hardening in larger pots and grown in the greenhouse.

The highest number of shoots (152 shoots/10 explants) and shoots longer than 1 cm (95 shoots/10 explants) were obtained from 3.0 mg L^{-1} BAP medium. On the other hand, shoot number was also high in 1.0 BAP+0.5 NAA (151 shoots/10 explants) medium, and the number of shoots shorter than 1 cm got the maximum number (80 shoots/10 explants). It was determined that the 3.0 mg L^{-1} BAP alone can be used for both organogenesis from leaf explants and shoot proliferation in single node culture. *In vitro* shoots of the lisianthus species are easily rooted and both PGR-free MS and ½ MS mediums can be used for rooting at a 100% rate.

Peat and perlite mixture was determined suitable for acclimatization to external conditions. In the climate room, adaptation to external conditions was achieved easily under controlled conditions, and acclimatization was achieved quickly and successfully in plantlets planted in viols in less controllable conditions in the greenhouse. The survival rate was 100% in all treatments. The plants were grown healthy in the greenhouse.

The float hydroponic system and transfer to the soil mixture directly were both successful methods to obtain the rooted and the acclimatized shoots at the same time. Due to the continuation and improvement of studies on them, both techniques have the potential to be effective *ex vitro* rooting and acclimatization strategies for lisianthus or other readily rooted ornamental plants. It was revealed that the results obtained from the research can be used in breeding studies such as *in vitro* mutation and genetic transformation. This application can serve to accelerate the long cycles of ornamental plants.

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RESEARCH PAPER

Nematode Diversity Associated with Grapevines in İzmir, Manisa, Çanakkale, Balıkesir, and Bilecik Provinces in Türkiye

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Abstract

The study was conducted in İzmir, Manisa, Çanakkale, Balıkesir, and Bilecik provinces to determine nematode diversity and community structure. Thirty-six genera (İzmir 34 genera; Manisa 32 genera; Bilecik 21 genera; Çanakkale 28 genera) with different trophic groups were recorded: fungivores (3 genera; e.g., *Aphelenchoides* spp.), bacterivores (9 genera, e.g., *Achromadora* Cobb, 1913 and *Plectus* Bastian 1865 spp.), omnivore (4 genera; e.g., *Aporcelaimus* Thorne, 1936 and *Dorylaimus* Thorne & Swanger, 1936 and *Eudorylaimus* Andrassy, 1959 spp.), predators (3 genera; e.g., *Clarkus* spp.), herbivores (17 genera; e.g., *Boleodorus tylactus* Thorne, 1941). Twenty-six species of herbivores were identified, and the most common species were from the genera *Filenchus* Andrassy, 1954, *Geocenamus* Thorne & Malek, 1968; *Pratylenchus* Filipjev, 1934, and *Helicotylenchus* Steiner, 1945. *Dorylaimus* Dujardin, 1845; *Mesorhabditis* Osche, 1952; *Cephalobus* Bastian, 1865; *Acrobeloides* Cobb, 1924; *Mesodorylaimus* Andreassy, 1959; *Aphelenchus* Bastian, 1865, and *Ditylenchus* Filipjev were other commonly found nematode genera. *Meloidogyne incognita, M. javanica, Mesocriconema xenoplax, Longidorus elongatus, Xiphinema index*, *X. italiae, Praylenchus thornei*, and *P. neglectus* identified constitute a severe threat to grape production by causing crop damage or transmitting virus diseases.

1. Introduction

Grapevine (*Vitis vinifera* L.) is a perennial plant from the Vitaceae family, originating from Western Asia and Europe. The genus *Vitis* has more than 80 described species and consists of two subspecies, Muscadinia (2n = 40 chromosomes) and Euvit (2n = 38 chromosomes). More than 6,000 *V. vinifera* species are grown in different countries (Agulheiro-Santos et al., 2022. Grapevine adapted to semi-tropical or even tropical conditions from temperate climatic conditions, is among the world's most widely grown cultivated plants due to not being selected for soil requirements and being easily reproduced and consumed in different ways. In addition to consuming fresh fruits, their seeds are processed to produce jam, juice, jelly, vinegar, wine, plant extracts, and oil. Annual grape production worldwide is 7.8 million tons, of which 57% is processed, 36% is consumed fresh, and 7% is dried (Küpe et al., 2021). In Türkiye, where 1450 differentgrape varieties are grown, grape production contributes to 28% of fruit production, and vineyards constitute 15% of horticultural areas. While China, France, Spain, Italy, and Türkiye are the leading countries regarding production, the grapevine is grown in more than 90 countries (OIV, 2022).

Vineyard soil fauna maintains many beneficial or harmful organisms such as nematodes, fungi, viruses, algae, and protozoa. Nematodes with different feeding habitats, like fungal or plant feeding, takes an important place in the soil fauna. For instance, 300 plant feeder (herbivore) species and dozens from other trophic groups have been

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described in vineyards, and the total number of nematodes distributed in agricultural areas in the world is estimated at more than 500.000 (Hoschitz, 2004; Kennedy and Luna, 2005; Singh et al., 2013).

Herbivore plant-parasitic nematodes, one of the major pests that damage the underground parts of plants, play a significant role in transmitting some viral diseases to plants (Taylor and Brown, 1997). Herbivores damage vine roots by feeding, thereby affecting the plant's uptake of water and nutrients from the soil. *Xiphinema* spp., *Longidorus* spp., *Criconemoides* spp., *Pratylenchus* spp., and *Meloidogyne* spp. are known as the most important herbivore genera in vineyards (Abd Elgawad and Askary, 2015). In contrast to herbivores, free-living species are involved in the mineral cycle of soil and are foremost contributors to the soil food webs (Bongers and Ferris, 1999). As a result of consuming organic residues, they promote the release of nitrogen and mineralization of phosphorus and sulphur. Additionally, some fungal and bacterial feeder nematodes are able to feed on plant pathogens, reduce their populations, and serve as prey and food source for nematode-trapping fungi (Taher et al., 2017). Predator nematodes, on the other hand, feed on other nematode species and affect the nematode population in vineyard areas (Ingham et al., 1985).

Nematode distiribution in vineyards is increasing day by day as a result of the uncontrolled planting of infected rootstocks, the inadequacy of quarantine procedures, and the increase in precipitation and flooding due to global warming. Many plant-parasitic nematode species have been identified in vineyards in some locations in the country, and dozens of unidentified ones still remain in other provinces. Dozens of nematodes, including free-living species, can also be found in other provinces, and nematode-infested areas continue to increase. Therefore this research focused on determining the nematode fauna of vineyards in İzmir, Manisa, Çanakkale, and Bilecik provinces in the western part of Türkiye. The diversity and community structure (trophic groups of nematodes, abundance, classification) in vineyards were discussed and compared by calculating some diversity indices.

2. Material and Methods

2.1. Survey area information

İzmir and Manisa are located in the Aegean Region of Türkiye. Çanakkale and Bilecik are located in the Marmara Region in Türkiye. Many fruit trees, such as olives and cherries, and cultivated plants, such as corn, tomatoes, and sunflower, are grown in these provinces. On the other hand, vineyard areas have an important position in agricultural production in many districts of Manisa, especially in terms of the area they cover. While the grape is produced on a small scale in İzmir, Çanakkale, and Bilecik, production is carried out in more extensive areas in Manisa. During the field studies, the temperature in İzmir, Manisa, Bilecik, and Çanakkale ranged between 26-36°C, and the annual precipitation in the provinces was around 600-713 mm.

2.2. Survey and soil sample collection

A survey was conducted in September 2021 and June 2022 in vineyards in Manisa, Çanakkale, Bilecik, and İzmir provinces (Figure 1). During the study, randomly selected commercial vineyards were sampled, and 105 soil samples were collected at 0-60 cm soil depth from the rhizosphere of the grapevines (Table 1). Sampling was done at randomly selected vineyards at a distance of at least 1 km between them. The mean size of vineyards was 1.0-2.0 ha in İzmir, 1.0-4.5 ha in Manisa, 0.5-1.2 ha in Çanakkale, and 0.5-0.9 ha in Bilecik. The sampled location was recorded. Sampling in each vineyard was conducted by moving in a zigzag pattern between the rows and collecting soil from the rhizosphere of randomly selected vines at different points. Soil cores were collected from at least 7 points in each vineyard,

Figure 1. The study area map with the names of provinces marked.

Table 1. Sampling details of Manisa, İzmir, Çanakkale, and Bilecik provinces.

Provinces	Total vineyard area (ha)	Locations	Collected samples
İzmir	10 385	Kemalpaşa, Bayındır, Torbalı	25
Manisa	86 849	Saruhanlı, Akhisar, Turgutlu, Şehzadeler, Yunusemre	52
Çanakkale	4 565	Bayramiç, Bozcaada, Merkez	16
Bilecik	1 048	Söğüt	12

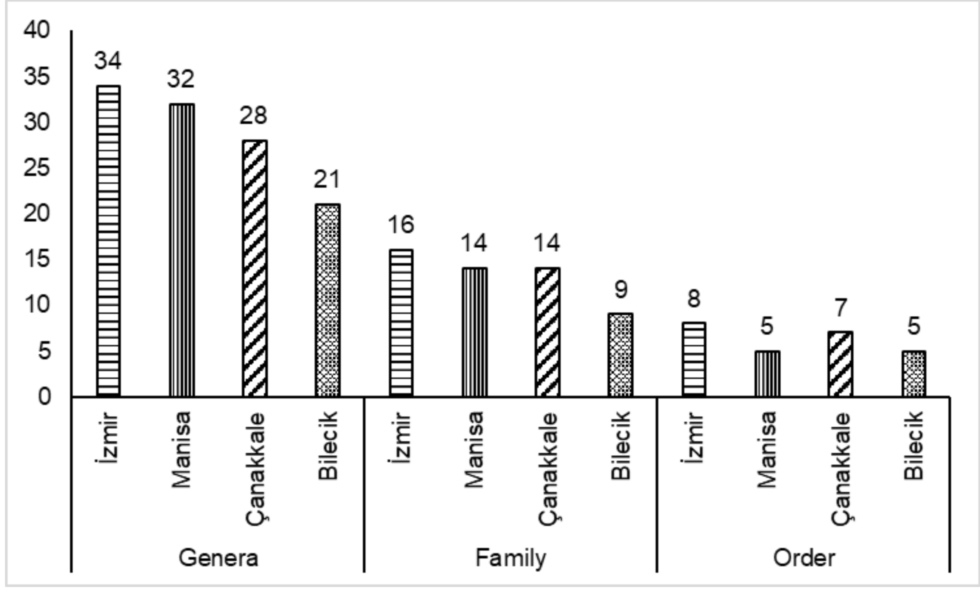


Figure 2. Genera, family, and order richness of İzmir, Manisa, Çanakkale and Bilecik.

and all were mixed to obtain 1 kg of soil. Collected soil samples were labelled, and transferred to the laboratory.

2.3. Extraction of nematodes from soil and identification

Soil nematodes were extracted by combining Cobb's (1913) decanting-sieving and centrifuge flotation methods of Jenkins (1964). Extracted nematodes were classified at the genus level by examining the morphology of females in nematode slides. Herbivores and Aphelenchids were identified at the species level. To prepare slides, extracted females were heat-killed, fixed in double-strengthened TAF solution, and mounted by the wax-ring method (Seinhorst, 1959). Root-knot nematodes were identified from the perineal pattern of females. The classifications of specimens were conducted based on Siddiqi (2000). Extracted nematodes were grouped based on feeding habitat and colonizer-persister values (c-p value 1-5) calculated based on life strategy (Bongers, 1990; Yeates et al., 1993; Neher et al., 2004).

3. Results

3.1. Nematode diversity of vineyards in İzmir, Manisa, Çanakkale, and Bilecik

In the nematode fauna of vineyards in İzmir, Manisa, Çanakkale, and Bilecik districts, species from eight orders, 17 families, and 36 genera were identified, and these were divided into two groups of plant-parasitic herbivore and free-living (bacterivore, fungivore, predator, and omnivore) nematodes (Figure 2).

Depending on the number of genera and identified families, the provinces ranked from higher to lower as İzmir, Manisa, Çanakkale, and Bilecik. Herbivores took first place with 17 genera, followed by bacterivores (9 genera), omnivores (4 genera), fungivores (3 genera), and predators (3 genera) (Figure 3).

Extracted nematodes belonged to five colonizer-persister (c-p1-5) functional groups. Nematodes from c-p2 were dominant, occurring in all sampled vineyards. (Figure 4).

Acrobeloides (90%), *Aphelenchus* (76.1%), *Aphelenchoides* (72.7%), and *Ditylenchus* (81.8%) were highly distributed in Manisa. The same genera were also frequent and abundant in İzmir and Çanakkale. The abundance of nematodes from these genera ranged between 6-45 individuals/100 cm^3 soils. In Bilecik, bacterivore *Cephalobus* and fungivore *Aphelenchus* were extracted from all sampled vineyards, and other common free-living genera were *Acrobeles* and *Aphelenchoides*. The least frequent nematodes in all survey areas belonged to predator *Clarkus* (2.5 ± 0.5 and 2 individuals 100 cm^3 soil) and *Plectus* (2 individuals 100 cm^3 soil) genera, which were only found in 4 vineyards in İzmir.

The classification of identified nematodes based on Siddiqi (2000) is given in Table 2.

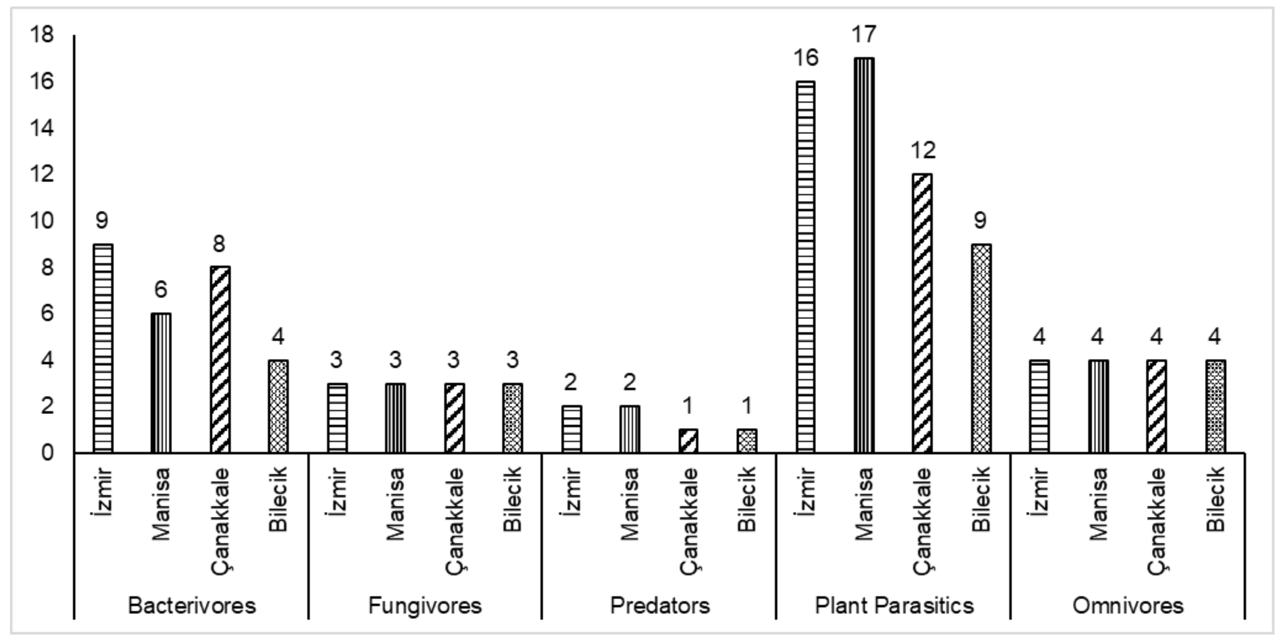


Figure 3. The composition of nematode genera in the point of feeding habitat in vineyards in four provinces.

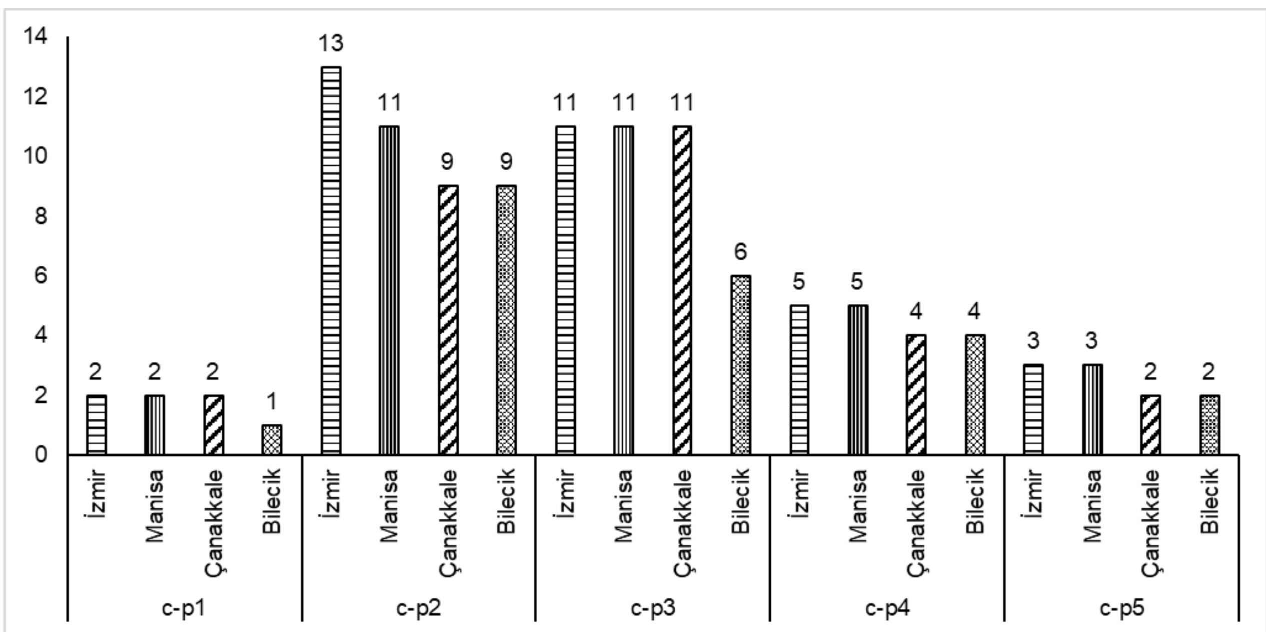


Figure 4. The number of nematode genera according to c-p classes.

3.2. Plant-parasitic nematodes in vineyards in İzmir, Manisa, Çanakkale and Bilecik

In this study, 17 herbivores nematode species belonging to the genera of *Boleodorus, Filenchus, Geocenamus, Helicotylenchus, Longidorus, Macroposthonia, Meloidogyne, Mesocriconema, Paratylenchus, Pratylenchus, Pratylenchoides, Psilenchus, Rotylenchus, Rotylenchulus, Tylenchus, Tylenchorhynchus,* and *Xiphinema* were identified. Sixteen were found in İzmir, 17 in Manisa, 14 in Çanakkale, and nine in Bilecik. At least one specimen of ectoparasite was found in all sampled vineyards, while endoparasites and semi-endoparasites were extracted from 51.8% of vineyards. Additionally, herbivores were classified into three colonizer-persister groups (c-p2, 3 and 5), and in all provinces, c-p3 species were leading (Figure 5). Considering the number of plant-parasitic species in provinces, 20 species were counted in Izmir, 23 species in Manisa, 14 species in Çanakkale, and 10 species in Bilecik. Table 3 represents the classification and distribution of plant parasitic nematode species.

Filenchus (occurrence: İzmir 95%; Manisa 97.7%; Çanakkale %100, Bilecik 57.1%) was widespread in survey areas. *Meloidogyne* spp., one of the most damaging plant parasites, was found in 9 vineyards in Manisa and two vineyards in İzmir. In addition, *Meloidogyne* was also found in two vineyards in Bozcaada, Çanakkale. The abundance in 100 cm^3 soil was 33 ± 6 individuals 100 cm^{-3} soil.

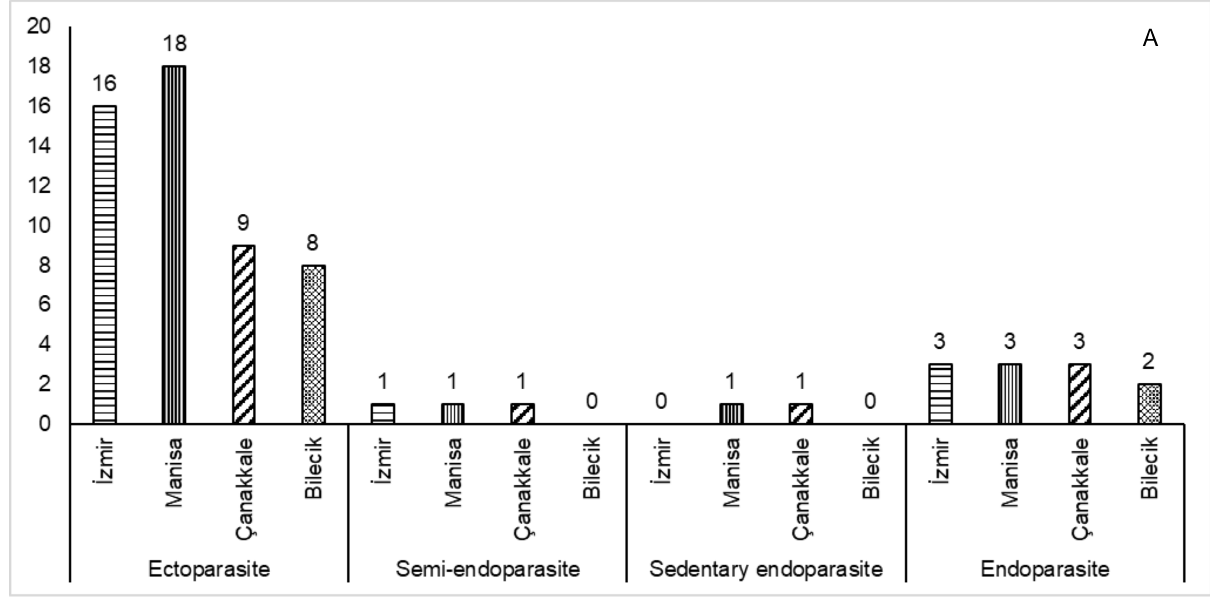
Table 2. Taxonomic classification of nematode genera and species and occurrence in İzmir, Manisa, Çanakkale, and Bilecik vineyards.

Genus	Order	Family	Functional guild/ c-p class	İzmir	Manisa	Çanakkale	Bilecik
Achromadora	Chromadorida	Achromadoridae	ba/c-p3	+	-	+	-
Acrobeloides	Rhabditida	Cephalobidae	ba/c-p2	+	+	+	+
Acrobeles	Rhabditida	Cephalobidae	ba/c-p2	+	+	+	+
Alaimus	Dorylaimida	Alaimidae	ba/c-p4	+	+	+	-
Aphelenchus avenae	Aphelenchida	Aphelenchoididae	fu/c-p2	+	+	+	+
Aphelenchoides sacchari	Aphelenchida	Aphelenchoididae		-	+	-	-
Aphelenchoides obtusus	Aphelenchida	Aphelenchoididae	fu/c-p2	+	+	+	+
Aporcelaimus	Dorylaimida	Aporcelaimidae	om/c-p5	+	+	+	+
Basiria	Tylenchida	Tylenchidae	pp/c-p2	+	+	-	-
Boleodorus	Tylenchida	Boleodorinae	pp/c-p2	+	+	+	+
Cephalobus	Rhabditida	Cephalobidae	ba/c-p2	+	+	+	+
Clarkus	Mononchida	Mononchidae	pr/c-p4	+	-	-	-
Ditylenchus myceliophagus	Tylenchida	Anguinidae	fu/c-p2	+	+	+	+
Dorylaimus	Dorylaimida	Dorylaimidae	om/c-p4	+	+	+	+
Eudorylaimus	Dorylaimida	Dorylaimidae	om/c-p4	+	+	+	+
Filenchus	Tylenchida	Tylenchidae	pp/c-p2	+	+	+	+
Geocenamus	Tylenchida	Merliniidae	p-p/c-p3	+	+	+	+
Helicotylenchus	Tylenchida	Hoplolaimidae	pp3	+	+	+	+
Longidorus	Dorylaimida	Longidoridae	pp/c-p5	+	+	-	-
Macroposthonia	Tylenchida	Criconematidae	pp/c-p3	+	+	+	-
Meloidogyne	Tylenchida	Heteroderidae	pp/c-p3	+	+	+	-
Mesodorylaimus	Dorylaimida	Dorylaimidae	om/c-p4	+	+	+	+
Mesorhabditis	Rhabditida	Rhabditidae	ba/c-p1	+	+	+	-
Paratylenchus	Tylenchida	Paratylenchidae	pp/c-p3	+	+	-	-
Plectus	Plectida	Plectidae	ba/c-p2	+	-	-	-
Pratylenchoides	Tylenchida	Pratylenchidae	pp/c-p3	+	+	+	-
Pratylenchus	Tylenchida	Pratylenchidae	pp/c-p3	+	+	+	+
Psilenchus	Tylenchida	Tylenchidae	pp/c-p2	+	+	-	-
Rhabditis	Rhabditida	Rhabditidae	ba/c-p1	+	+	+	+
Rotylenchus	Tylenchida	Hoplolaimidae	pp/c-p3	+	+	+	+
Rotylenchulus	Tylenchida	Rotylenchulidae	pp/c-p3	-	+	+	-
Seinura	Aphelenchida	Aphelenchoididae	pr/c-p4	-	+	-	-
Tripyla	Triplonchida	Tripylidae	pr/c-p3	+	+	+	+
Tylenchus	Tylenchida	Tylenchidae	pp/c-p2	+	+	-	+
Tylenchorhynchus	Tylenchida	Telotylenchidae	pp/c-p3	+	+	+	+
Xiphinema	Dorylaimida	Longidoridae	pp/c-p5	+	+	+	+
Wilsonema	Plectida	Plectidae	ba/c-p2	+	-	+	-

ba: bacterivore, fu: fungivore, om: omnivore, pr: predator, pp: plant parasitic.

In this study, many nematode species with different trophic groups were detected in İzmir, Manisa, Çanakkale, and Bilecik provinces. Of these plant-parasitic and bacterivore species were the most dominant. Some of the species that we identified have been found in vineyards in many studies. Species belonging to free-living nematode genera, such as *Acrobeles, Acrobeloides, Aphelenchus, Clarkus*, and *Plectus*, were found in California, the USA, and Austria. Likewise, in our study, *Clarkus, Plectus*, and *Wilsonema* were the least frequent in survey areas (All-Banna and Gardner, 1996; Hoschitz, 2004).

Several bacterivore genera were present in soil samples in İzmir, Manisa, Çanakkale, and Bilecik. They are highly tolerant to extreme soil conditions, are widely reported in agricultural areas in Türkiye. In the research conducted by Yıldız et al. (2017) in Bolu, *Cephalobus* and *Acrobeloides* species were found in all sampled fields. Again, the prevalence of these two genera was 100% in another study conducted by Akyazı et al. (2012) in the potato fields in the Ordu province. Similarly to these studies, in our study in İzmir, Manisa, Çanakkale, and Bilecik, species from *Acrobeloides* and *Cephalobus* came to the fore in vineyards regarding prevalence. For instance, in Bilecik Söğüt, İzmir Kemalpaşa, Çanakkale Bozcaada, and Bayramiç, most of the surveyed vineyards were found to be infested with *Acrobeloides*. Bacterivores consume and decompose the bacteria in the soil and release valuable nitrogen into the soil. Bacterivore nematodes can consume 10^6 bacterial cells daily (Blanc et al., 2006). Although they have shorter life spans than other group nematodes. These species have a higher reproduction rate. For example, *Acrobeloides nanus* from *Acrobeloides* genera can produce 2-3×10^5 new nematodes in one month (Wasilewska et al., 2011). Morever, *Cephalobus litoralis* of the genus *Cephalobus* can reproduce



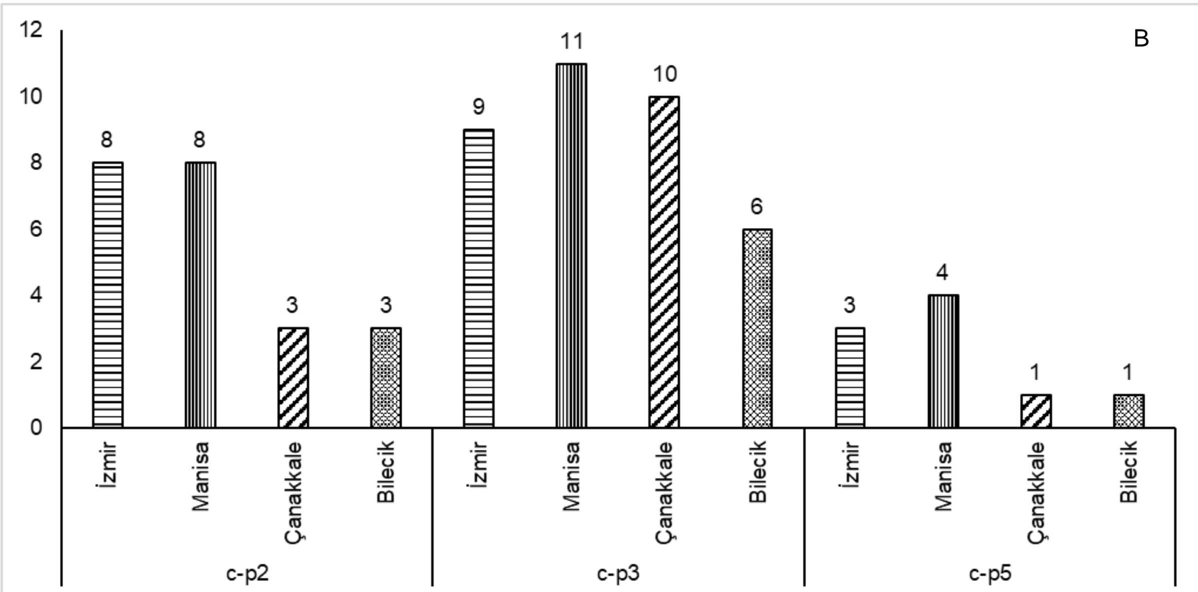


Figure 5. The number of identified genera of herbivore nematodes with different feeding strategies in İzmir, Manisa, Çanakkale, and Bilecik (A) and the number of herbivore nematode genera in three c-p values in İzmir, Manisa, Çanakkale and Bilecik (B).

very quickly parthenogenetically, can complete its life cycle in 72-90 days, and a female can lay 200-300 eggs (Saeed et al., 1988). Another species found widespread in vineyards were fungal feeders, especially *Aphelenchus avenae,* which was found in 80 of 105 soil samples. Considered as mostly fungi and mushroom feeder, there are few records of *A. avenae* being a plant parasite (Kumari, 2012). The nematode can feed on more than 90 fungal species belonging 50 genera (Mankau and Mankau, 1963). *Ditylenchus myceliophagus* and *Aphelenchoides* spp., commonly found in our study, can also feed on many pathogenic and non-pathogenic fungi, including *Agaricus bisporus* (Arrold and Blake, 1966).

Seventeen genera of plant-parasitic nematode with different feeding strategy were identified in survey area. Migratory ectoparasite species were found to be more predominant in four provinces. Among the herbivore nematodes in İzmir, Manisa, Çanakkale, and Bilecik, economically important species were *Meloidogyne* spp., *Pratylenchus thornei, P. neglectus, Longidorus elongatus, Xiphinema index, X. italiae,* and *Mesocriconema xenoplax*. *Xiphinema pachtaicum* was found in all provinces, but other species were not in detected in Çanakkale and Bilecik. *Longidorus elongatus* was extracted only from one vineyard in Akhisar, Manisa and one vineyard in Kemalpaşa, İzmir. All these species have been found in vineyards in many countries, including Spain, Iran, the USA, and Türkiye (Pinkerton et al., 1999; Téliz et al., 2007; Deimi and Mitkowski, 2010). *Meloidogyne* and *Xiphinema* species were reported in vineyards in

Table 3. Plant-parasitic nematode species extracted from soils collected from vineyards in İzmir, Manisa, Çanakkale, and Bilecik provinces.

Species	c-p	Feeding strategy	İzmir	Manisa	Çanakkale	Bilecik
Basiria graminophila	2	Migratory ectoparasite/ root fungal feeder	+	+	-	-
Boleodorus thylactus	2	Migratory ectoparasite	+	+	+	+
Filenchus sheri	2	Migratory ectoparasite	+	+	+	-
Filenchus thornei	2	Migratory ectoparasite	+	+	+	+
Filenchus cylindricus	2	Migratory ectoparasite	+	+	-	-
Geocenamus brevidens	3	Migratory ectoparasite	+	+	+	+
Helicotylenchus digonicus	3	Migratory ectoparasite	+	+	+	+
Helicotylenchus varicaudatus	3	Migratory ectoparasite	-	+	-	-
Longidorus elongatus	5	Migratory ectoparasite	+	+	-	-
Meloidogyne	3	Sedentary endoparasite	+	+	+	-
Mesocriconema xenoplax	3	Migratory ectoparasite	+	+	+	-
Paratylenchus nainianus	2	Migratory ectoparasite	+	+	-	-
Pratylenchoides alkani	3	Migratory endoparasite	+	+	+	-
Pratylenchus neglectus	3	Migratory endoparasite	+	+	+	+
Pratylenchus thornei	3	Migratory endoparasite	+	+	+	+
Psilenchus hilarulus	2	Migratory ectoparasite/ root fungal feeder	+	+	-	-
Rotylenchus cypriensis	3	Migratory ectoparasite	+	+	+	+
Rotylenchulus macrosoma	3	Semi-endoparasite	-	+	+	-
Tylenchorrhynchus cylindricus	3	Migratory ectoparasite	+	+	+	+
Tylenchus davainei	2	Migratory ectoparasite/ root fungal feeder	+	+	-	+
Xiphinema pachtaicum	5	Migratory ectoparasite	+	+	+	+
Xiphinema index	5	Migratory ectoparasite	+	+	-	-
Xiphinema italiae	5	Migratory ectoparasite	-	+	-	-

previous studies in Manisa and İzmir (Mistanoğlu et al., 2015).

Among all plant-parasitic nematodes identified in this study, *Meloidogyne incognita* and *M. javanica* were reported to be the most damaging on grapevines. Due to the galls formed on the vine root by these pests' damage, the vascular bundles are blocked, and the plant cannot uptake water and nutrients from the soil. Although many rootstocks such as Richter 110R, Salt Creek, and SO_4 have been reported as resistant to *Meloidogyne* species, resistance can vary depending on climatic conditions, the abundance of nematode populations, and soil conditions (Vega-Callo et al., 2021).

Xiphinema index, X. italiae, and *L. elongatus* are considered as other harmful nematodes because they can transmit virus diseases like grapevine fanleaf virus (GFLV) to healthy vines (Taylor and Brown, 1997). In this study these species were found in different locations of Türkiye. Elekçioğlu et al., (1994) stated *X. italieae* infection of the roots of grapevine in the southern part of Türkiye, while Mistanoğlu et al. (2015) revealed a 9.5% prevalence in western provinces. However, including this recent study, in none of these studies, the nematode population was found to be at a level that would cause economic damage.

Mesocriconema xenoplax was one of the other damaging ectoparasitic species found in four provinces. In this study's research area, the nematode was found in İzmir (4 vineyards), Manisa (5 vineyards), and Bilecik (one vineyard). Controversially, the prevalence rate of this species in vineyard areas in Thrace was previously reported as 52% in Türkiye (Öztürk et al., 2018). The species is known to cause root damage and 58% pruning weight reduction in grapevines (Forge et al., 2020). *Pratylenchus* species are also very damaging to the vine at high population level. In the sampling areas, the number of individuals in 100 cm^3 soil was 22±12, but the abundance in the roots was not examined.

Geocenamus brevidens was another nematode distributed in all provinces with a 68% occurrence rate. Furthermore, *Filenchus* species were highly distributed. These species and other tylenchid genera are species that do not cause severe grapevine damage even under heavy populations. In addition to feeding on plant roots, it has been stated in the literature that they also feed on fungi (Okada et al., 2005; Munawar et al., 2022).

In this study, the colonizer persister (c-p) values of nematodes ranged between 1-5. Of these Rhabditis, a c-p1 group nematode was found in four provinces, and was abundant in some locations. The c-p1 group include species with a short life cycle, tolerant to adverse environmental conditions, and abundant in soil due to its ability to multiply several times (Bongers and Ferris, 1999). The species population was counted as >70 individuals in 100 cm^3 in İzmir. The c-p5, on the other hand, is another group rarely found in survey areas and, includes species with a long life cycle, which are sensitive to adverse soil conditions, and reproduce very slowly and in small numbers (Bongers and Bongers, 1998). The number of individuals of *Longidorus* and *Xiphinema* species with c-p5 values in İzmir and Manisa was 1-10 in 100 cm^3 soil.

4. Conclusion

This study indicated the presence of free-living and plant parasitic nematode species in the

vineyards located in the western part of Türkiye. Bacterial and fungal feeders and plant feeders were highly distributed in İzmir, Manisa, Çanakkale, and Bilecik, and in contrast, a rare occurrence of predator species was observed. The nematode fauna of vineyards covered 37 genera of nematode species, including three plant parasitic nematodes vectoring viruses; *Xiphinema index, X. italiae,* and *Longidorus elongatus.* There are several economically damaging plant parasitic species such as *Meloidogyne* and *Mesocriconema xenoplax*. Several species like *Helicotylenchus varicaudatus, Rotylenchus cypriensis, and Tylenchorhynchus cylindricus* were also extracted, and these are considered not of economic importance due to low abundance.

In this study, several nematode species were identified from the western part of Türkiye. In more vineyards, extensive studies must be carried out periodically to determine nematode reproduction status to prevent infestation, especially of *Meloidogyne* spp., to new areas through infected rootstocks or soil, and apply sanitation in case of infestation.

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RESEARCH PAPER

Determination of the Efficiency of Moringa Leaf Extract Treatments on Seedling Vigor in Marigold

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Abstract

The effect of different planting depths on emergence characteristics was investigated in this study carried out using seeds of Marigold (*Tagetes patula*) collected from nature. In addition, the most effective dose that can be used in priming treatments was tried to be determined by examining the effects of moringa leaf extract treatments applied at different doses in terms of emergence rate, emergence time, survival seedling rate, true leaf emergence time, emergence speed index, coefficient of velocity of emergence and vigor index values. After the T test, it was determined that different planting depth treatments created statistically significant differences especially in the survival seedling rate. It has been observed that the fact that the planting is exposed causes an increase in the survival seedlings rate. In addition, following hydropriming and moringa treatments, the highest seedling emergence rate at both planting depths was achieved with hydropriming and moringa leaf extract (4 g L^{-1}) treatment. Moreover, moringa leaf extract (8 g L^{-1}) treatments exhibited the earliest emergence compared to other treatments. All treatment groups were statistically different compared to the control group in terms of emergence speed index, coefficient of velocity of emergence and vigor index values in both planting depths, and HP and M3 treatments were the treatment groups that gave the best results. As a result of the study, it has been determined that surface sowing can be recommended in marigold cultivation and moringa treatments improve the emergence characteristics.

1. Introduction

There are approximately 50 species in the *Tagetes* genus. These species are especially widespread in the United States, Argentina and Chile. In addition to being terrestrial, subaquatic and perennial species within the genus, they also have a lot of diversity in terms of flower and leaf characteristics. It is known that the leaves of the species in the Tagetes genus, like other members of the family, have secretory cavities containing fragrant and essential oils (Hinojosa Espinosa and Schiavinato, 2022).

In ornamental plant cultivation, marigold is grown in many different aspects all over the world. It has features such as being suitable for pot, field and garden cultivation, having a long flowering period (summer and autumn), being used as a cut flower and containing a wide variety of species within the family (Priyanka et al., 2013; Singh et al., 2016). Tagetes is a species that has recently become widely used in sustainable agriculture and organic farming practices, with its many useful components such as thiophenes, alkaloids, polyacetylenes, fatty acids, flavonoids and terpenes. It is used as a low-cost product because

it has antimicrobial and nematicidal effects and uses plant material of natural (biological) origin (Santos et al., 2015). In addition to these advantageous aspects, it is grown in many different species such as melon (Mavi and Atak, 2016), bell pepper (Mavi, 2016), tomato (Mavi and Uzunoğlu, 2020a), gourd (Mavi, 2020) and pepper (Mavi and Uzunoğlu, 2020b). With its allelopathic effect, it is used as an organic priming material in pre-sowing seed treatments.

Pre-sowing seed treatments aim to increase germination and emergence rates, provide uniform emergence, as well as improve seedling quality and increase the plant's resistance to stress factors. While marigold cultivation is generally carried out at 25°C, it is known that temperatures above 35°C prevent germination (Drennan and Van Staden, 1989). While the optimum temperature value is 25°C, low temperatures may have a negative effect on the emergence rate in early plantings. Like other species in the family, marigold is known as a species that requires light during emergence. The aim of the study is to break the dormancy of the species regarding its need for light during emergence by applying different planting depths. In addition, it was aimed to determine the effective dose for use in organic priming treatments with moringa leaf extracts used in different doses.

2. Material and Methods

The study was conducted in Hatay Mustafa Kemal University Seed Physiology Laboratory and unheated glass greenhouses between January 2023 and March 2023. Marigold (*Tagetes patula*) seeds, which are used as ornamental plants in the campus area, were used in the study. Plants whose seeds are taken have orange flower color and a layered structure. The thousand-seed weight obtained from the plant was determined as 1.76 g. It was observed that the seed colors were black and the seed width was 0.27 mm and the seed length was 18.36 mm.

Five different treatments doses obtained from hydropriming, which is a pre-sowing seed treatments, extract obtained from Tagetes erecta petals, which is an agent used in organic priming treatments, and Moringa leaf extracts, were used on marigold seeds.

Hydropriming treatments: The seeds were planted after keeping them at 25°C for 24 hours between filter papers moistened with 10 ml distilled water in 9 cm petri dishes (Gündüz et al., 2019).

Tagetes erecta treatments: The extracted Tagetes erecta petals were brewed in pure water at the rate of 4 g per 1 Liter of distilled water for 3-4 minutes and this brewing tea was used in seed treatments. After the prepared extract cooled, the seeds were moistened with 10 ml of extract between filter papers in 9 cm petri dishes and kept at 25°C for 24 hours (Mavi, 2016).

Moringa treatments: Moringa leaf extracts prepared as brewing tea as in tagates at 5 different treatments doses (1-2-4-8-16 g L^{-1}) are added to the seeds in each petri dish, 10 ml, and inoculated 24 hours between filter papers in 9 cm petri dishes. The treatments were carried out by keeping it at 25°C. Treatments and abbreviations for marigold seeds before planting are given in Table 1.

After priming treatments, all treatments and control group seeds were planted in 3×25 repetitions×seeds in peat: perlite (3:1) growing medium, each repetition in separate vials (195×103×63 mm). To examine the effect of planting depth on emergence for each treatment and control group, the vials were covered with a peat:perlite (3:1) mixture at two different planting depths, 0.2 cm and 1.0 cm.

During the seedling emergence test, seedling emergence and actual leaf emergence were counted for 30 days and the vials were kept in the greenhouse environment. During this period, the minimum temperature was measured as 7°C and the minimum temperature average was 10°C, the maximum temperature was 37°C and the maximum temperature average was 24°C. The total average temperature value was determined as 17°C. The rate of surviving seedlings was obtained by counting the seedlings that remained alive for 30 days and increasing the percentage. At the end of the census, mean emergence rate (%) and mean emergence time were calculated based on 3×25 repetitions × plants. Mean emergence time and true leaf appearance were calculated according to the values obtained from daily counts made during the

Table 1. Treatments and abbreviations for marigold seeds before planting.

Treatments	Abbreviations
Control	K
Hydropriming	HP
Tagetes erecta priming	TAG
Moringa leaf extract (1 g L^{-1})	M1
Moringa leaf extract (2 g L^{-1})	M2
Moringa leaf extract (4 g L^{-1})	M3
Moringa leaf extract (8 g L^{-1})	M4
Moringa leaf extract (16 g L^{-1})	M5

seedling emergence trial (Orchard, 1977). At the emergence stage, emergence speed index and coefficient of velocity of emergence were also determined (Kader, 2005). Vigor index value was calculated as the emergence speed index × coefficient of velocity of emergence. In the experiment, percentage values were subjected to angle transformation before statistical analysis, and real values were used in figures and tables. Statistical analysis of all data was analysed using the Duncan multiple comparison test in the SPSS 17.0 package program in order to reveal the differences between the treatments. Differences were determined at the $p<0.05$ significance level. In addition, in order to observe the difference created by different planting depths on emergence characteristics, T test was performed on the averages of all treatments in the same package program and data with a significance value less than 0.05 were taken into account.

3. Results and Discussion

After the emergence test performed at both planting depths, the highest emergence rate was obtained from HP treatments at both planting depths. Among Moringa and TAG treatments, it was determined that M3 treatments gave the highest results for both planting depths. While the emergence rate of control group seeds was determined to be 11% at 1 cm planting depth, this rate was 21% at 0.2 cm planting depth, and it is seen that the emergence rate increased by 10% by changing the planting depth without any treatments. At 0.2 cm planting depth, emergence rates were determined as 39% after HP treatments and 31% after M3 treatments. When the results are compared, the treatments increased the emergence rates varying between 2-18% at 0.2 cm planting depth. At 1 cm planting depth, the emergence rate increased by 6-22% (Figure 1).

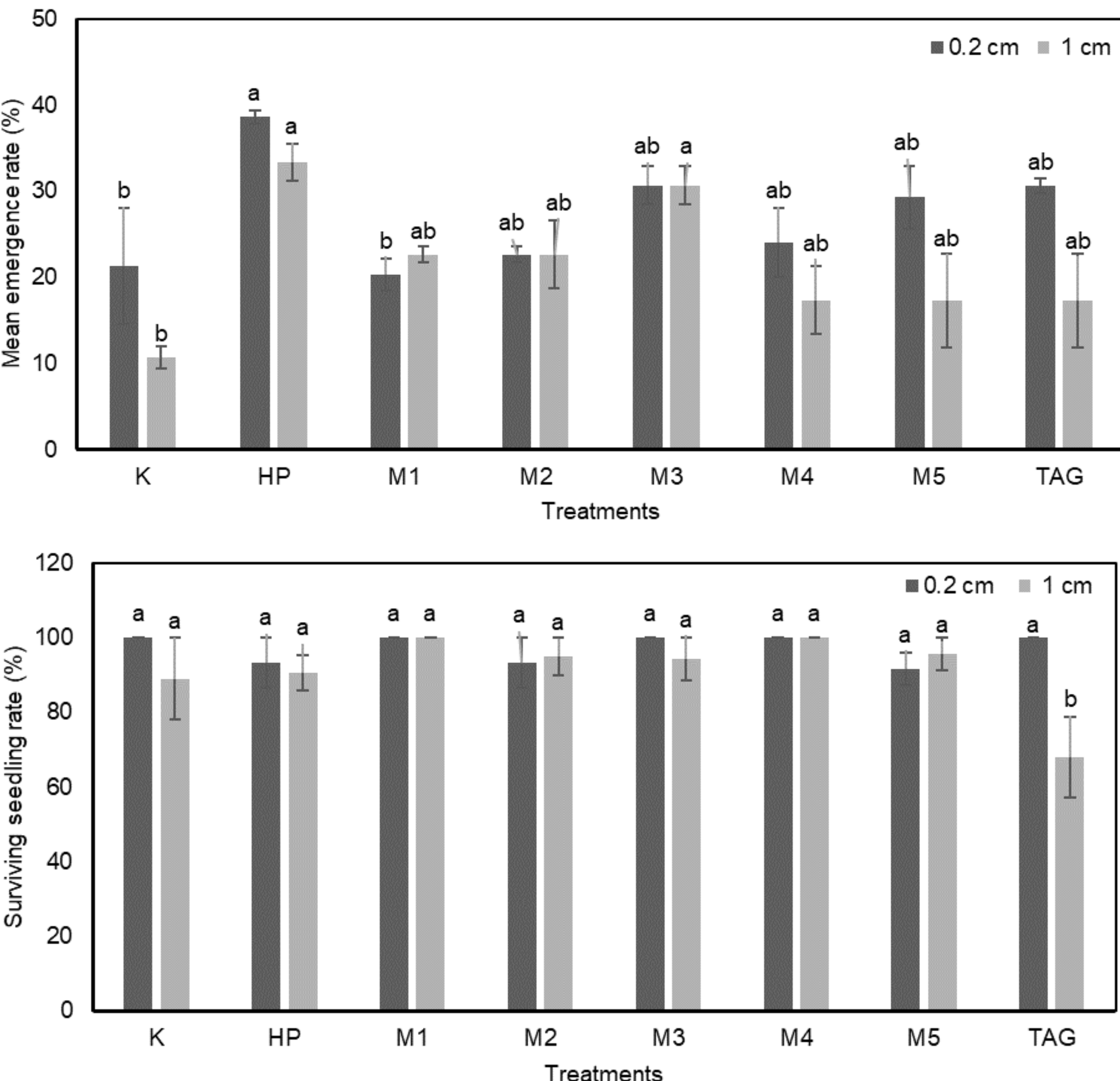

Figure 1. Effects of treatments and different planting depths on mean emergence rate (%) and surviving seedling rate (%) in marigold (K: Control, HP: Hydropriming, TAG: *Tagetes erecta* priming, M1: Moringa leaf extract (1 g L^{-1}), M2: Moringa leaf extract (2 g L^{-1}), M3: Moringa leaf extract (4 g L^{-1}), M4: Moringa leaf extract (8 g L^{-1}), M5: Moringa leaf extract (16 g L^{-1}).

Conway et al. (2003) stated that while the initial viability of the genotype coded E-1236 in African marigold was 89%, the viability increased to 96% after osmotic priming and solid matrix priming treatments. Ilbi et al. (2020) in their study on *T. erecta* and *T. patula* species, after the standard germination test of 20°C dark / 30°C light (16 hours / 8 hours), the average germination rate varied between 78-90%, while the highest emergence rate (90%) was obtained by keeping T. patula seeds at 5°C for 2 weeks. In their study on 3 different species, Kumar and Sharma (2012) examined the effects of different pre-planting practices and different planting environments and stated that the germination rate values for Tagetes species varied between 14.4-42.3%. It was observed that the highest germination rate value was obtained from the treatments of keeping it in the dark at 20°C for 2 days. While some of the literature results had higher outputs, they were similar to the results of Kumar and Sharma (2012). In this study, a low stress temperature of approximately 17°C was used. It is aimed to guarantee emergence with seed treatments, especially in early plantings in the spring period. In addition, it is seen that the effectiveness rate of the treatments is better understood when working with seed groups with low vitality values. As a result of the calculations of the survival seedling rate after the emergence test, it was determined that the effectiveness of the planting depth on the living seedling rate created statistically significant differences, while the treatments were not statistically significant in the living seedling rate (Figure 1).

After the emergence test on marigold, it was observed that while the average planting depths caused differences in the mean emergence time, they did not create significant differences in the true leaf appearance. At a planting depth of 0.2 cm, the earliest emergence time value was in the M5 treatments with 4.47 days in the earliest group, while the longest emergence time was in the control group with 8.37 days. At 1 cm planting depth, the earliest emergence was obtained from the M4 treatments with 4.4 days, while the groups with the longest emergence time were the control group with 6.34 days and the M5 treatments with 6.48 days (Figure 2). Afzal et al., (2009) stated that

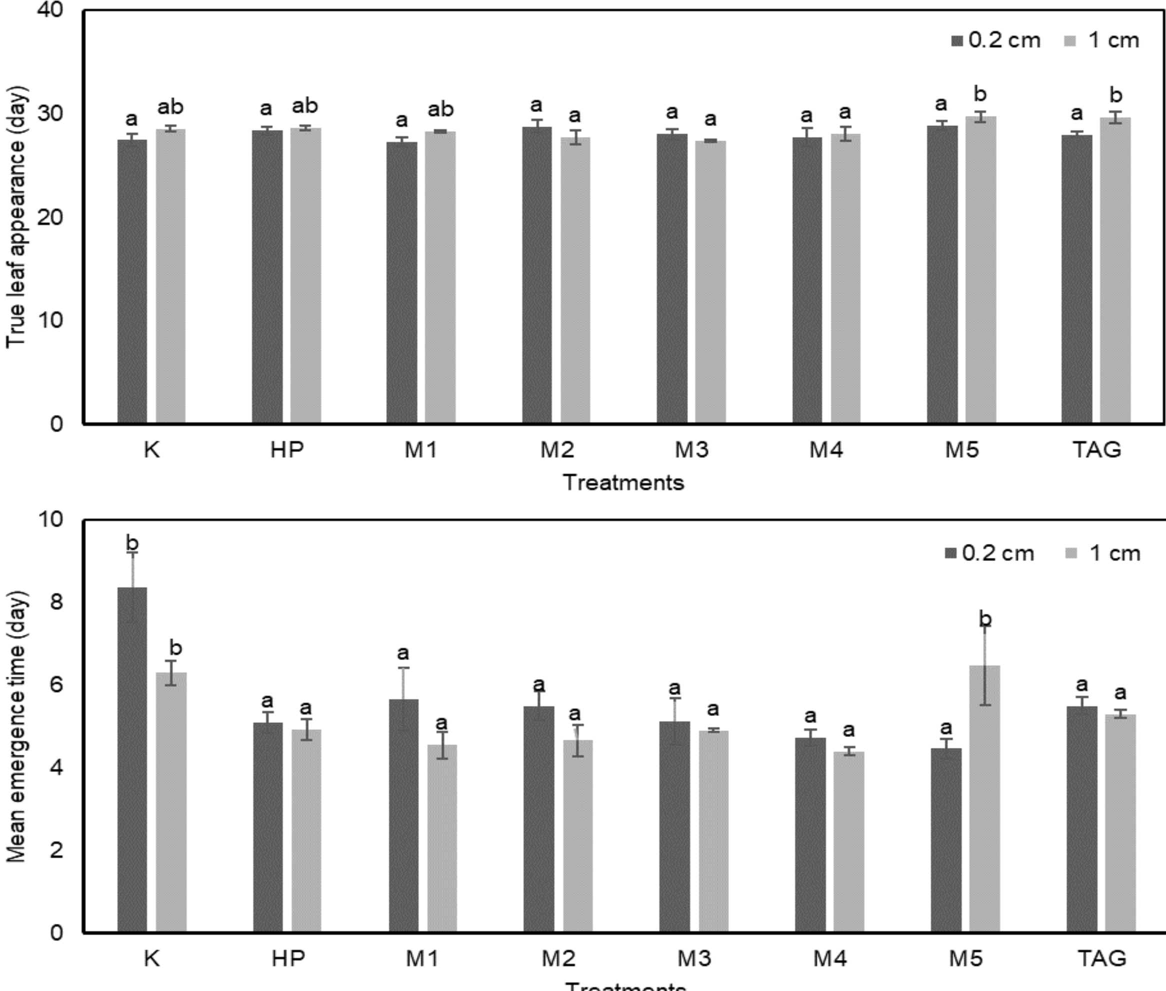

Figure 2. Effect of different pre-sowing seed treatments and planting depths on the mean emergence time and true leaf appearance in marigold. (K: Control, HP: Hydropriming, TAG: *Tagetes erecta* priming, M1: Moringa leaf extract (1 g L^{-1}), M2: Moringa leaf extract (2 g L^{-1}), M3: Moringa leaf extract (4 g L^{-1}), M4: Moringa leaf extract (8 g L^{-1}), M5: Moringa leaf extract (16 g L^{-1}).

Table 2. Effect of treatments and different planting depths on emergence speed index, coefficient of velocity of emergence and vigor index in marigold.

Treatments	0.2 cm			1 cm		
	ESI	CVE	Vigor index	ESI	CVE	Vigor index
K	0.74±0.4 d	12.78±0.7 b	285.76±142.0 c	0.44±0.1 c	15.78±1.0 b	168.83±25.6 c
HP	1.97±0.1 a	19.69±1.1 a	762.07±54.9 a	1.77±0.3 a	19.37±2.1 ab	656.40±124.0 a
M1	0.91±0.1 cd	18.24±2.2 a	372.92±74.9 bc	1.30±0.1 ac	22.10±1.5 a	504.76±60.4 ac
M2	1.04±0.1 bd	18.35±1.2 a	418.13±47.1 bc	1.29±0.4 ac	21.65±1.8 a	506.29±143.9 ac
M3	1.67±0.3 ac	19.97±2.2 a	627.49±134.1 ab	1.63±0.2 ab	20.40±0.2 ab	624.37±66.4 ab
M4	1.31±0.3 ad	21.17±0.8 a	498.17±105.7 ac	1.01±0.3 ac	22.75±0.5 a	395.75±110.2 ac
M5	1.73±0.3 ab	22.58±1.3 a	648.80±97.8 ab	0.74±0.3 bc	16.11±2.3 b	275.52±123.6 bc
TAG	1.48±0.0 ad	18.21±0.7 a	556.91±12.4 ac	0.86±0.4 ac	18.81±0.4 ab	331.59±148.2 ac

(ESI: Emergence speed index, CVE: Coefficient of velocity of emergence, K: Control, HP: Hydropriming, TAG: Tagetes erecta priming, M1: Moringa leaf extract (1 g L^{-1}), M2: Moringa leaf extract (2 g L^{-1}), M3: Moringa leaf extract (4 g L^{-1}), M4: Moringa leaf extract (8 g L^{-1}), M5: Moringa leaf extract (16 g L^{-1}).

Table 3. Determination of the effectiveness of different planting depths on emergence characteristics in marigold by T test.

Depth	Emergence rate (%)	Mean emergence time (day)	True leaf appearance (day)	Surviving seedling rate (%)	ESI	CVE	Vigor index
1	31	5.55	28.06	97	1.36	18.87	521.3
2	27	5.19	28.51	91	1.13	19.62	432.9
Sig	0.157	0.234	0.982	0.03	0.359	0.771	0.244

*In the depth column, number 1 indicates 0.2 cm planting depth, number 2 indicates 1 cm planting depth. Data with a significance value (sig) less than 0.05 are statistically significant in terms of depth. ESI: Emergence speed index, CVE: Coefficient of velocity of emergence.

halopriming treatments increased the seedling emergence rate, reduced emergence time and increased seedling quality. In another study, it was determined that germination time varied between 1.75-2.84 days after pre-sowing seed treatments (Ilbi et al., 2020). After the seedling emergence test, the emergence speed index, coefficient of velocity of emergence and vigor index values were calculated to determine the emergence power of the seed groups. When the changes in treatments and planting depths were examined, HP and M3 treatments were the treatments groups that gave the best results in terms of all values, while the results of 0.2 cm planting depth were seen to give better results compared to 1 cm planting depth (Table 2). HP treatments gave the highest results, with the emergence speed index value being 1.97 at 0.2 cm planting depth and 1.77 at 1 cm planting depth. The highest coefficient of velocity of emergence value was measured from the M5 treatments with a value of 22.58 at 0.2 cm planting depth, while this value was obtained from the M4 treatments with 22.75 for 1 cm planting depth. When the Vigor index values are examined, HP and M3 treatments are seen as the prominent treatments groups (Table 2.) It is known that different planting depth treatments cause changes in emergence rate in the Asteraceae family. A T Test was also conducted to observe the difference of different planting depths on the emergence characteristics of marigold, a member of the Asteraceae family (Table 3).

In the T Test performed by taking the mean of 24 replicates of 8 treatments, it was observed that while the mean emergence rate of 0.2 cm surface planting was 31%, this rate decreased to 27% in 1 cm deep planting. In addition, it has been found important to have a high rate of survival as well as a high survival rate in ornamental plant cultivation. While the rate of surviving seedlings at 0.2 cm planting depth was 97%, the rate of surviving seedlings at 1 cm planting depth decreased to 91%. As a result of the T test, planting depth was found to be statistically significant ($p<0.05$). Among all the features, the prominent feature was the survival rate of seedlings with a significance level of 0.03.

4. Conclusion

When all the results were evaluated, it was seen that there may be changes in the effectiveness levels of the treatments as the variation between repetitions increased with the increase in planting depth. Decreasing the planting depth is important to increase the effectiveness of the treatments and the emergence rate. In addition, starting to work with a seed batch with low viability proves the effectiveness of the planting depth and treatments. It is thought that the fluctuations seen in the test results may be due to the low test temperature, maturity differences between seeds collected from nature and high seed variation. When all the results are evaluated, it is seen that M3 treatments stands

out in terms of moringa leaf extract and can be used as an effective dose in future studies. Although the emergence rate of control group seeds is low in early stage cultivation at low temperatures, it is seen that this rate can be increased by 22% with treatments. Thus, it is thought that early seedlings obtained at low temperatures make a significant difference for the ornamental plant breeding sector.

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