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RESEARCH PAPER

HORTICULTURAL STUDIES

Asymbiotic Germination and Seedling Development of Terrestrial Orchid *Bletilla striata* Using in vitro and ex vitro Cultures

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Abstract

Procedures for asymbiotic germination and seedling development under in vitro and ex vitro conditions were investigated for *Bletilla striata*. Five different asymbiotic germination media (½ P6668 ¬Phytamax Orchid Maintenance Media, ½ P6668 ¬Phytamax Orchid Maintenance Media with coconut water, P723 *Phyto*Technology Orchid Seed Sowing Media, P723 *Phyto*Technology Orchid Seed Sowing Media with coconut water, and ½ MS media) for *in vitro* culture and sphagnum moss for *ex vitro* culture were examined for their effectiveness for 8 weeks on seed germination and seedling development of *B. striata*. Germination occurred in all media, however, the best germination rate was obtained in P723 medium with coconut water while the lowest frequency was obtained in sphagnum moss. Moreover, the highest leaf parameters of the seedlings of *B. striata* developed in ½ MS medium when the seedlings in sphagnum moss showed the lowest results. Furthermore, all germinated protocorms showed rhizoid formation in all media.

1. Introduction

The Orchidaceae is among the largest flowering plant families with about 28 500 species and 700 to 800 genera (Govaerts et al., 2018). Members of this family are distributed worldwide and are largely cultivated as ornamentals (Harrap, 2009). Orchid species are found in almost every place in the world (Arditti, 1990).

The East Asian endemic terrestrial orchid genus *Bletilla* has nine species distributed widely from north Myanmar and Indochina through Korea, China and Japan. *Bletilla cotoensis*, *B. foliosa*, *B. japonica*, *B. morrisonicola*, *B. ochracea*, *B. sinensis*, *B. striata*, *B. szetschuanica*, and *B. yunnanensis* species belong to the *Bletilla* genus (Dressler, 1993). This orchid is commonly known as Chinese ground orchid. The plant is an herbaceous perennial about 50 cm in height, with four to eight grass-like leaves, and a peduncle that forms in May to July. The plant has rose mauve coloured flowers in a racemous inflorescence (Tan, 1969).

Besides the ornamental characteristics, *Bletilla striata* has many compounds (bibenzyl, phenanthrene, dihydrophenanthrene and diphenanthrene) which have some biological effects, like antioxidant, antimicrobial etc. In China, people consume the tubers of this plant and were consuming with honey (Dong et al., 2014). Especially in China, *B. striata* is used in modern medicine also, not only traditional medicine. After tubers are collected from the plant, they are peeled and dried. People use that plant for bleeding, muscular damage, burns, skin wounds, ulcers and, liver tumours (Xiang et al., 2013; Wang et al., 2013; Peng et al., 2014; Zhang et al., 2019).

Orchid plants produce capsules, which are generally dry and mature, and each capsule has thousands of seeds and sometimes millions. The seeds of most flowering plants, have an endosperm,

which is the food reserve, while most of the orchid seeds do not have endosperm, or have an undeveloped endosperm. Thus, orchid seeds are different from other Angiosperms. (Arditti and Ghani 2000; Seaton et al., 2011). However, some orchid species have endosperm inside of the seed, like *Bletilla* or *Sobralia* orchids (Arditti, 1992; Tullock, 2005; Zhi-Hui et al., 2006). Some people call orchid seeds "naked seeds" or "dust seeds" because of the lack of endosperm, hence, the seeds need a mycorrhizal fungus association to develop (Rasmussen, 1995; McKendrick, 2000; Seaton et al., 2011). With germination, the embryo of the orchid seed becomes bigger to form a protocorm. Afterwards, rhizoids develop, first leaves and roots appear respectively (Arditti, 1992). Furthermore, the development of *B. striata* has four stages: embryo, protocorm, rhizome, and pseudobulb (Zhang et al., 2019).

Orchid seeds can be germinated under *in vitro* conditions with the help of orchid mycorrhizal fungi called symbiotic germination. Seeds also can be germinated in media, which are not inoculated with fungi called asymbiotic germination. In the symbiotic media, the fungus provides water, minerals and energy source to the orchid seeds. However, in the asymbiotic media, all nutrient requirements are provided in complex formulations. Furthermore, asymbiotic medium is efficient for seed growing, not used only at the germination stage, but also for seedling growth (Seaton et al., 2011). The asymbiotic orchid seed germination method is really important for breeding and conservation of rare and native species. Moreover, it is important for species, which are difficult to germinate. With the asymbiotic germination method, large numbers of plants can be produced at the same time quickly and efficiently (Stenberg and Kane, 1998).

In the 1800's, the asymbiotic germination method was found difficult by the people, however, Lewis Knudson improved the solution of Wilhelm Pfeffer and created the Knudson B solution in 1921. After a while, Lewis Knudson created the Knudson C germination medium in 1946. He made it possible to germinate orchids without using fungi. Moreover, people started to think that orchid seeds could be germinated with simple nutrient media, which contain sugar (Arditti and Ernst, 1984; Arditti, 1990; Arditti, 2008). After Lewis Knudson, many researchers created and improved a lot of asymbiotic orchid media such as MS (Murashige and Skoog), Fast, VW (Vacin & Went), MM (Malmgren Modified), RM (Reinert and Mohr), Curtis and Norstog. Moreover, there are some commercial media types like P6668 (Sigma Aldrich), P668, P723, B141, F522, T839, O156 (Phytotechnology Laboratory). The asymbiotic orchid medium contain macro and microelements, amino acids, polyol, vitamins, hydrolysates and autolysates, sugars, and gelling agents. Moreover, some formulations add some additional compounds to the asymbiotic orchid germination medium, like auxin, cytokinin, banana, pineapple juice, coconut water, anticontaminants, activated charcoal, etc. (Arditti and Ernst, 1993; Seaton and Ramsay, 2005; Arditti, 2008; Butcher and Marlow, 2008; Thomas, 2008; Seaton et al., 2011).

Terrestrial orchids are quite different than epiphytic orchids in terms of asymbiotic seed germination protocol (De Pauw et al., 1995). There are numerous previous studies about asymbiotic germination using different media formulations on several terrestrial orchids genera such as *Cypripedium* (Chu and Mudge, 1996; De Pauw et al., 1996; Szendrak, 1997; Yan et al., 2006; Bae and Choi, 2008; Klavina et al., 2009; Zhang et al., 2013; Huh et al., 2016; Huh et al., 2019), *Dactylorhiza* (Laurent et al., 2014; Gümüş et al., 2017), *Serapias* (Gümüş and Ellialtioglu, 2012; Bektas and Sokmen, 2016; Calevo et al., 2017; Acemi and Ozen, 2019), *Cephalanthera* (Szendrak, 1997; Hemrova et al., 2019), *Paphiopedilum* (Lee, 2007; Zeng et al., 2012), *Chloraea* (Pereira et al., 2017; Quiroz et al., 2017), *Bletia* (Dutra et al., 2008), *Geodorum* (Bhadra and Hossain, 2003), *Habenaria* (Stewart and Kane, 2006), *Peristylus* (Thakur and Dongarwar, 2017), *Anacamptis* (Magrini et al., 2019), *Bipinnula* (Pereira et al., 2015), *Pecteilis* (Kim et al., 2019), *Epipactis* (Hemrova et al., 2019), *Himantoglossum* (Szendrak, 1997; Dulic et al., 2019), *Spathoglottis* (Barrientos and Fang, 2019), *Anoectochilus* and *Haemaria* (Chou and Chang, 2004), *Calopogon* and *Socoila* (Kauth, 2005), *Gastrodia* (Godo et al., 2020), *Calanthe* (Bae and Kim, 2015), *Spiranthes* (Dulic et al., 2019), *Ophrys*, *Barlia*, and *Platanthera* (Szendrak, 1997; Calevo et al., 2017). Furthermore, optimization for asymbiotic seed germination protocol of *Bletilla striata* has been described by Szendrak (1997), Fu et al. (2006), Ye et al. (2010), Su-qin, (2010), Godo et al. (2011), Kulpa and Katron (2012), , Yili et al. (2012), Billard et al. (2013), Zhang et al. (2013), Song et al. (2014), Nie et al. (2016), Min et al. (2017), and Wei et al. (2018).

The objective of this experiment was to select the best germination and seedling development media for *Bletilla striata*.

2. Material and Method

2.1. Acquisition of orchid seeds

Bletilla striata seeds were donated by Thompson & Morgan Company. They were collected when they were ripe. The seeds were checked under the microscope (Wild Heerburg, Switzerland) and only seeds, which had an embryo were used. Besides, the *B. striata* seeds were extremely small (Figure 1).

2.2. Asymbiotic media screen

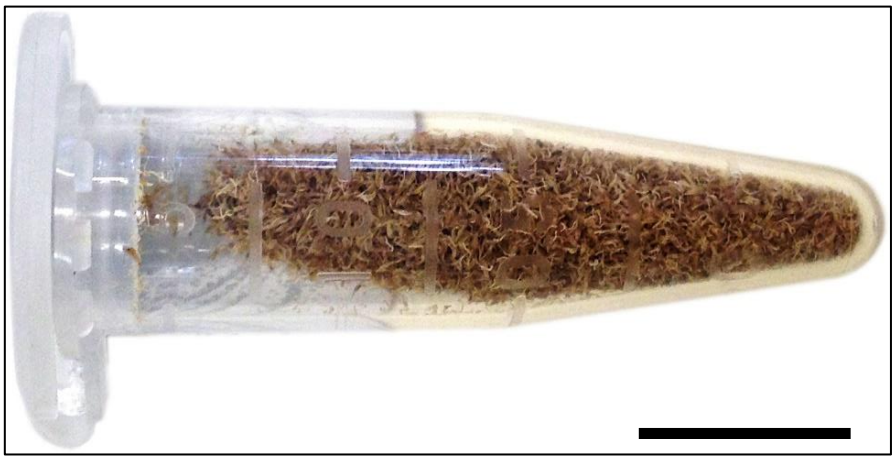

Figure 1. The seeds of *Bletilla striata* (Scale bar = 10 mm)

Table 1. Nutrient composition of germination media used for the asymbiotic seed germination of *Bletilla striata*

Nutrient elements	Formulations (mg L^{-1})	½ MS	½ P6668	P723
Macro elements	Ammonium Nitrate	825	412.5	412.5
	Calcium Chloride Anhydrous	166	83	83
	Magnesium Sulphate Anhydrous	90.35	45.175	75.18
	Potassium Nitrate	950	475	475
	Potassium Phosphate, Monobasic	85	42.5	42.5
Micro elements	Cobalt Chloride Hexahydrate	0.0125	0.0063	0.0063
	Cupric Sulphate Pentahydrate	0.0125	0.0063	0.0063
	Disodium EDTA Dihydrate	18.65	18.65	18.65
	Ferrous Sulphate Heptahydrate	13.9	13.9	13.9
	Boric Acid	3.10	1.65	1.65
	Manganese Sulphate	8.45	4.23	4.23
	Sodium Molybdate Dihydrate	0.125	0.0625	0.0625
	Potassium Iodide	0.415	0.2075	0.2075
	Zinc Sulphate Heptahydrate	4.30	2.65	2.65
Vitamins	Myo-inositol	50	50	100
	Nicotinic Acid (Free Acid)	0.25	0.5	1
	Pyridoxine Hydrochloride	0.25	0.5	1
	Thiamine Hydrochloride	0.5	5	10
Organics	Glycine	1		
	Peptone from Meat		1000	2000
	Activated Charcoal		1000	1000
	Sucrose	20000	10000	20000
	MES (Free Acid)		500	500

½ MS — Half-Strength Murashige and Skoog, P723 — *Phyto*Technology Orchid Seed Sowing Media
½ P6668 — Half-Strength Phytamax Orchid Maintenance Media (Sigma Aldrich)

For this research the following media were chosen:
1. ½ strength Phytamax P6668 orchid maintenance media (Sigma-Aldrich Co., UK).
2. ½ strength Phytamax P6668 orchid maintenance media + coconut water.
3. P723 Orchid seed sowing media (PhytoTechnology Laboratories, USA).
4. P723 Orchid sowing media + coconut water.
5. ½ strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) (Sigma-Aldrich Co., UK).
6. Sphagnum Moss (Gardman Company, UK) (Table 1).

½ MS were modified with 2.0% sucrose (Sigma-Aldrich Co., UK), and 0.8% agar (Oxoid–Termo Fisher Scientific, USA) were added to all media as a gelling agent. Moreover, 5.0% coconut water were added to ½ P6668 and P723 media. The aim of decreasing strength to half is reducing the salt concentration to stimulate germination. Besides, coconut water has some nutrients and natural phytohormones, however, generally it is used as a supplement, not as a single medium. All media were adjusted to pH 5.8 (Seaton and Ramsey, 2005) and were taken to autoclave at 117.7 kPa for 15 min at 121°C.

2.3. Surface sterilisation of the seeds

For sterilizing seeds, the pocket method was used (Seaton and Ramsay, 2005; Gümüş, 2009), as the seeds were so tiny. Filter papers (Whatman grade no: 1, 90 mm) were folded and stapled to prevent seed loss (Figure 2). 100 seeds were added per pocket.

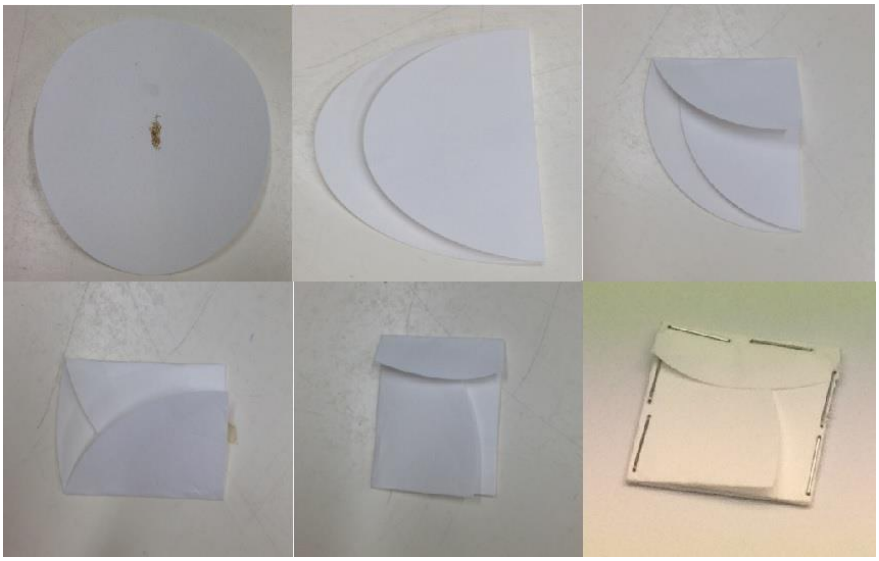

Figure 2. Folding and stapling of filter paper for the pocket method

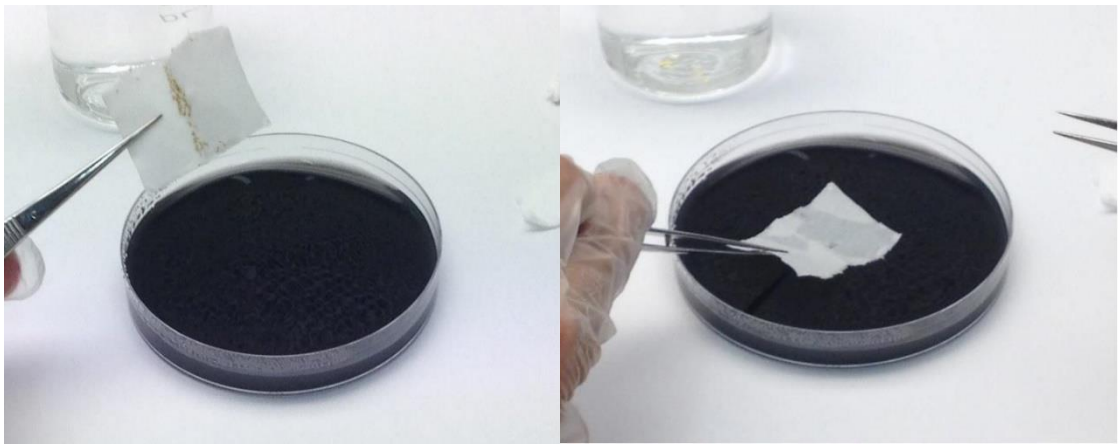

Figure 3. Sowing of the seeds

All the instruments, beakers, distilled water, filter papers, scissors, forceps, scalpels were covered with aluminium foil and sterilised in the autoclave for 20 min at 121°C. The pockets were placed with the pair of forceps in the beaker, which had sterile distilled water, and were left there for 5 min. After the water penetrated the filter papers, they were transferred to a beaker, which had bleach solution (10% sodium hypochlorite) and was left there for 10 min and agitated gently. After sterilising process, filter papers were rinsed in the sterile distilled water and transferred to an empty beaker to allow excess liquid to drain.

2.4. Sowing of the seeds

While the pocket was held with forceps, staples were cut with scissors and opened gently. The filter paper was dabbed on the petri dish (Termo Fisher Scientific, USA) and then the seeds placed in the media (Figure 3).

2.5. Incubation conditions

Seeds were kept for germination at the temperature between 22-26°C (Zhi-Hui et al., 2006; Kulpa and Katron, 2012) (recorded by TinyTag data logger). Dark treatment is not recommended by Zhi-Hui et al. (2006) and Kulpa and Katron (2012), therefore, petri dishes were kept between 2100 – 2600 lux (recorded by Lutron LX - 101 digital lux meter) for 14 hours in a day.

2.6. Checking for germination and seedling development

In the present study, 5 different parameters were observed: surface area of the leaves, length of the leaves, width of the leaves, number of leaves, and germination rate. 100 seeds were sown in each petri dish and 10 petri dishes were used for each media and parameter. For germination rate, all germinated seeds were counted. For the other parameters, 10 seeds were chosen randomly and were measured for each petri dish. The germination was determined when embryo ruptured the testa and became green. Germination rate was measured at 2nd, 3rd, 5th and 8th weeks, while the other parameters were measured at 3rd, 5th and 8th weeks. Leaf surface areas were measured according to Kindlmann and Balounova (1999) by formula of q x (length x width). In this case q was set as 0.5. The microscopic

photos were taken at 0.7 m (Invenio 3M Pixel CMOS Camera).

2.7. Statistical analysis

Obtained results were subjected to variance analysis employing a completely randomized design. Mean values for the examined plant traits were compared by Duncan multiple range test a = 0.05 by SAS software version 9.00.

3. Results and Discussion

Germination rates of the seeds of *B. striata* were observed at 2nd, 3rd, 5th, and 8th weeks. Over 8 weeks, the highest germination percentage was obtained in P723 media with coconut water with 71.31%, while the lowest rate was investigated in sphagnum moss with 25.86%. Moreover, ½ P6668 media had the second-highest germination rate with 64.05% (Figure 4).

P723 Orchid Seed Sowing Media with coconut water showed the best germination results after 8 weeks. This situation can be explained by that coconut water can increase the germination success of orchids. Coconut water (or coconut milk) is preferred sometimes because it contains some nutrients and natural phytohormones. (Arditti and Ernst, 1993; Seaton and Ramsay, 2005; Arditti, 2008; Butcher and Marlow, 2008; Seaton et al., 2011). Using coconut water on the germination of *B. striata* has not been used in previous researches. The present study shows that using coconut water with P723 media can increase the germination percentage of *B. striata*. In previous researches, half-strength MS media has been preferred for germination of Bletilla seeds (Wei et al., 2018). Many researchers have studied the effect of the combination of plant growth regulators with ½ MS media. According to the previous studies, the seeds can be germinated in higher rates with plant growth regulators. In the present study, ½ MS without plant growth regulators showed the third-highest germination rate was obtained. The reason of this can be explained that other media have activated charcoal and lesser salt concentrations with a comparison of ½ MS media. Activated charcoal is added to the orchid germination medium because this supplement adsorbs some compounds like phenols, vitamins and inorganic compounds and improves cell growth sometimes (Pan and van Staden, 1998; Thomas, 2008). Activated charcoal affects adsorbing compounds, however, it is still not clear that activated charcoal helps to adsorb plant hormones or not. Some researchers think that it has an effect on plant hormones like the other compounds (macro, micro minerals, etc.). Orchid seeds are not dependent on activated charcoal; however, it has a positive effect on seed germination and development (Pierik et al., 1988). Furthermore, activated charcoal has a positive effect on root development (Yan et al., 2006) and rhizome growth (Paek and Yeung, 1991). In the present study, germination rates reached to 71.31%. Moreover, Ye et al. (2010) got 90% germination rate with ½ MS + 6-benzyladenine (6-BA) 1.0 mg L^{-1} + 1% activated charcoal and Song et al. (2014) got almost 98% with the same medium components. Furthermore, Zhang et al. (2009) and Ding and Zheng (2016) got around 90% germination rate with ½ MS + 1.0 mg L^{-1} naphthalene acetic acid (NAA). Besides, Min et al. (2017) obtained 90% germination rate with ½ MS medium + 1.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} NAA. Moreover, Kulpa and Katron (2012) reached 89% germination frequency by Knudson C medium without any plant growth regulators. Unlike the other orchids, *B. striata* seeds store nutrients. Thus, there is a chance for direct sowing. In the present study, 25.86% germination frequency has been obtained by direct sowing of seeds on the sphagnum moss. Under the direct seed sowing conditions, spraying seeds with different nutrient solutions can increase the germination rate between 5% and 69.7% (Zhang et al., 2019). It is really important to demonstrate that *B. striata* seeds can germinate without auxin or cytokinin. There are a lot of factors that can affect seed germination success. For instance, the duration of the storage has a negative effect on the seeds of *B. striata* germination frequency. Hence, short-time storage is recommended (Zhang et al., 2019).

The percentage of rhizoid formation of the seedlings of *B. striata* were observed at 2nd, 3rd, 5th, and 8th weeks. After week 5, all the media showed the same rhizoid formation rate. Moreover, the seedlings in MS media and sphagnum moss showed slow rhizoid development in comparison with the other media at week 2 and 3 (Figure 5).

At the end of 8 weeks, all germinated protocorms formed rhizoid in all plant media. In the study by Godo et al. (2011), the effect of different wavelength of LED-lights on *B. ochracea* has been investigated. The highest rate of rhizoid formation was obtained by Orange LED-light with 71.7%.

The lengths of the leaves of the seedlings of *B. striata* were investigated at 3rd, 5th, and 8th weeks. After 8 weeks, the highest length of the leaves was obtained in ½ MS, ½ P6668, ½ P6668 media with coconut water, and P723 media with coconut water respectively in the same group. Moreover, it was observed that the length of the leaves in ½ MS media climbed sharply after 8 weeks. Besides, the lowest length of the leaves was seen in sphagnum moss (Table 2).

The widths of the leaves of the seedlings of B. striata was investigated at 3rd, 5th, and 8th weeks. Over an 8-week period, the highest width of the leaves of the seedlings of *B. striata* was obtained in ½ MS media. It was followed by ½ P6668, ½ P6668 media with coconut water, P723 media with coconut

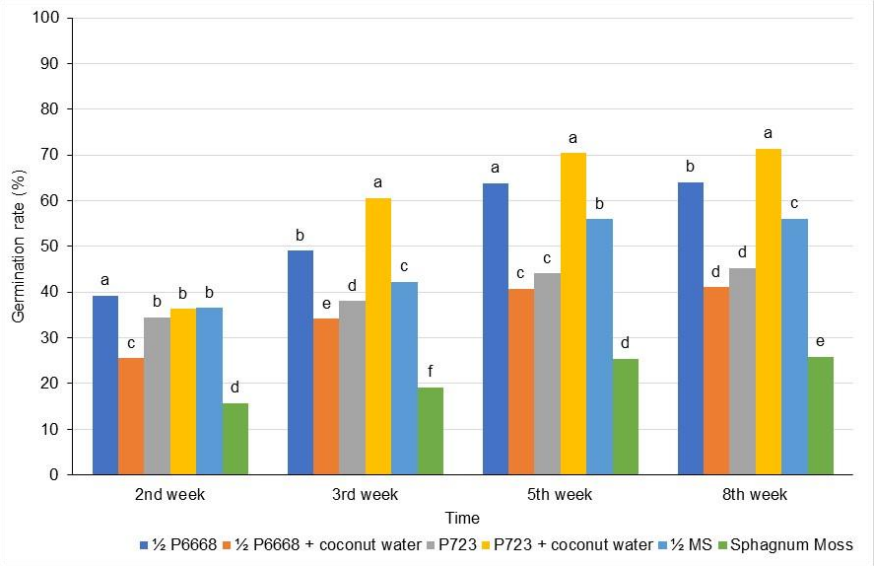

Figure 4. Comparative effects of culture media on germination rates of the seeds of *Bletilla striata* after 2, 3, 5, and 8 weeks (Bars with the same letters are not significantly different by Duncan's multiple range test at α = 0.05).

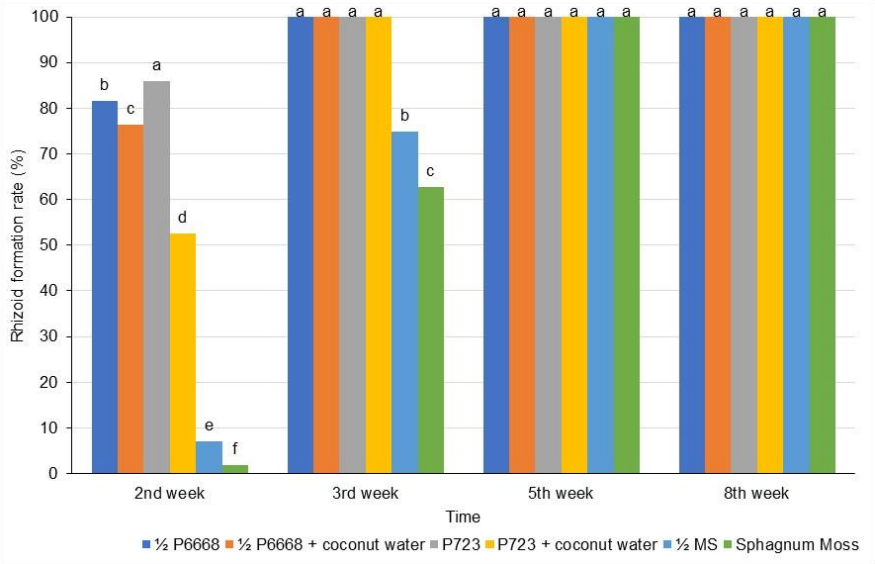

Figure 5. Comparative effects of culture media on the rhizoid formation rates of *Bletilla striata* after 8 weeks (Bars with the same letters are not significantly different by Duncan's multiple range test at α = 0.05.)

water respectively in the different group. Additionally, sphagnum moss had the lowest width of the leaves (Table 3).

The surface areas of the leaves of the seedlings of *B. striata* were investigated at 3rd, 5th, and 8th weeks. The highest width of the leaves of the seedlings of *B. striata* was obtained in ½ MS media, ½ P6668, P723 media with coconut water, and ½ P6668 media with coconut water respectively in the same group. Like for the other seedling development parameters, the surface area of the leaves belongs to *B. striata* in MS media increased rapidly after 5th week. Furthermore, the lowest surface area of the leaves was investigated in sphagnum moss (Table 4).

Results at the present study show that the highest leaf parameters of the seedlings of *B. striata* developed in ½ MS medium at the end of the 8 weeks. Seedlings in ½ P6668 media, P723 media with coconut water, and ½ P6668 media with coconut water followed that respectively. Leaf parameters of seedlings in ½ MS media climbed sharply between week 5 and week 8. A possible reason is that ½ MS media has a two times higher salts concentration than the other media. These results showed that the seedlings at ½ P6668 medium, ½ P6668 medium with coconut water, P723 medium, and P723 medium with coconut water should have been transferred to the proliferation medium with higher salts for better seedling development. After germination, when the protocorms are formed, explants can be transferred to the proliferation media. Ye et al. (2010) indicated that the best proliferation media for *B. striata* is MS + 1.0 mg L^{-1} BA + 0.15 mg L^{-1} NAA. Moreover, Yili et al. (2012) stated that MS + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA performed the best shoot induction. Furthermore, Min et al. (2017) showed MS +

Table 2. Comparative effects of culture media on length of the leaves of *Bletilla striata* after 3, 5, and 8 weeks

Media	Length of the leaves (cm)		
	3rd week	5th week	8th week
½ P6668	0.83 a	2.13 a	2.84 a
½ P6668 + coconut water	0.67 b	1.56 b	2.75 a
P723	0.59 b	1.25 b	2.05 b
P723 + coconut water	0.58 b	1.54 b	2.57 ab
½ MS	0.41 c	0.93 c	2.95 a
Sphagnum Moss	0.35 c	0.65 c	0.93 c

Measurements represent the mean of 100 seedlings per treatment. Measurements with the same letters are not significantly different by Duncan's multiple range test at α = 0.05.

Table 3. Comparative effects of culture media on the width of the leaves of *Bletilla striata* after 3, 5, and 8 weeks

Media	Width of the leaves (cm)		
	3rd week	5th week	8th week
½ P6668	0.26 a	0.60 a	1.21 b
½ P6668 + coconut water	0.27 a	0.57 a	1.17 b
P723	0.26 a	0.46 ab	0.91 b
P723 + coconut water	0.22 b	0.54 a	1.12 b
½ MS	0.20 ab	0.30 bc	1.60 a
Sphagnum Moss	0.15 b	0.20 c	0.46 c

Measurements represent the mean of 100 seedlings per treatment. Measurements with the same letters are not significantly different by Duncan's multiple range test at α = 0.05.

Table 4. Comparative effects of culture media on the surface area of the leaves of *Bletilla striata* after 3, 5, and 8 weeks

Media	The surface area of the leaves (cm^2)		
	3rd week	5th week	8th week
½ P6668	0.11 a	0.66 a	2.15 ab
½ P6668 + coconut water	0.10 a	0.48 ab	2.07 ab
P723	0.09 ab	0.33 bc	2.03 b
P723 + coconut water	0.06 ac	0.43 ab	2.10 ab
½ MS	0.04 bc	0.16 cd	2.35 a
Sphagnum Moss	0.03 c	0.06 d	1.15 c

Measurements represent the mean of 100 seedlings per treatment. Measurements with the same letters are not significantly different by Duncan's multiple range test at α = 0.05.

Table 5. Comparative effects of culture media on the number of the leaves of *Bletilla striata* after 3, 5, and 8 weeks

Media	The number of the leaves		
	3rd week	5th week	8th week
½ P6668	1.00 a	1.35 a	1.77 ab
½ P6668 + coconut water	1.00 a	1.22 a	1.75 ab
P723	1.00 a	1.17 a	1.06 bc
P723 + coconut water	1.00 a	1.25 a	1.49 b
½ MS	0.85 a	1.12 a	2.54 a
Sphagnum Moss	0.60 b	1.00 a	0.21 c

Measurements represent the mean of 100 seedlings per treatment. Measurements with the same letters are not significantly different by Duncan's multiple range test at α = 0.05.

0.5 mg L^{-1} 6-BA + 0.2 mg L^{-1} NAA + 50.0 g L^{-1} mashed potato as the best proliferation media. Besides, some studies demonstrated that directly inducing cluster buds without proliferation process is possible (Ding and Zheng, 2016). Moreover, Fu et al. (2006) indicated that coconut water can induce proliferation as well. At the present study, it was observed that cluster buds and leaves can be formed without proliferation process and using media without plant growth regulators.

The numbers of the leaves of the seedlings of *B. striata* were investigated at 3rd, 5th, and 8th weeks. The highest number of the leaves of the seedlings of *B. striata* was obtained in ½ MS, ½ P6668, and ½ P6668 media with coconut water after 8 weeks. Moreover, seedling grown in the sphagnum moss showed the lowest number of the leaves (Table 5). The number of leaves at the end of 8 weeks reached to 2.54 with ½ MS media. Billard et al. (2013) found similar results as 2 to 3 leaves with ½ MS after 7 weeks. In another study by Kulpa and Katron (2012), plant growth regulators in different amounts have been combined with Knudson C media. After 13 weeks, researchers obtained 3.0 leaves per seedling with Knudson C, Knudson C + 0.20 mg L^{-1} IBA, and Knudson C + 0.50 mg L^{-1} IBA, 4.33 leaves with Knudson C + 0.50 mg L^{-1} BAP, and 5.66 leaves with Knudson C + 0.20 mg L^{-1} NAA. As it is seen in the previous research, the number of leaves can be increased with auxin and cytokinin hormones.

The seeds of *B. striata* germinated, and seedlings developed in all media. Besides the measurements of the parameters, microscope images were observed at the 2nd, 3rd, 5th, and 8th weeks (Figure 5, 6, 7, and 8).

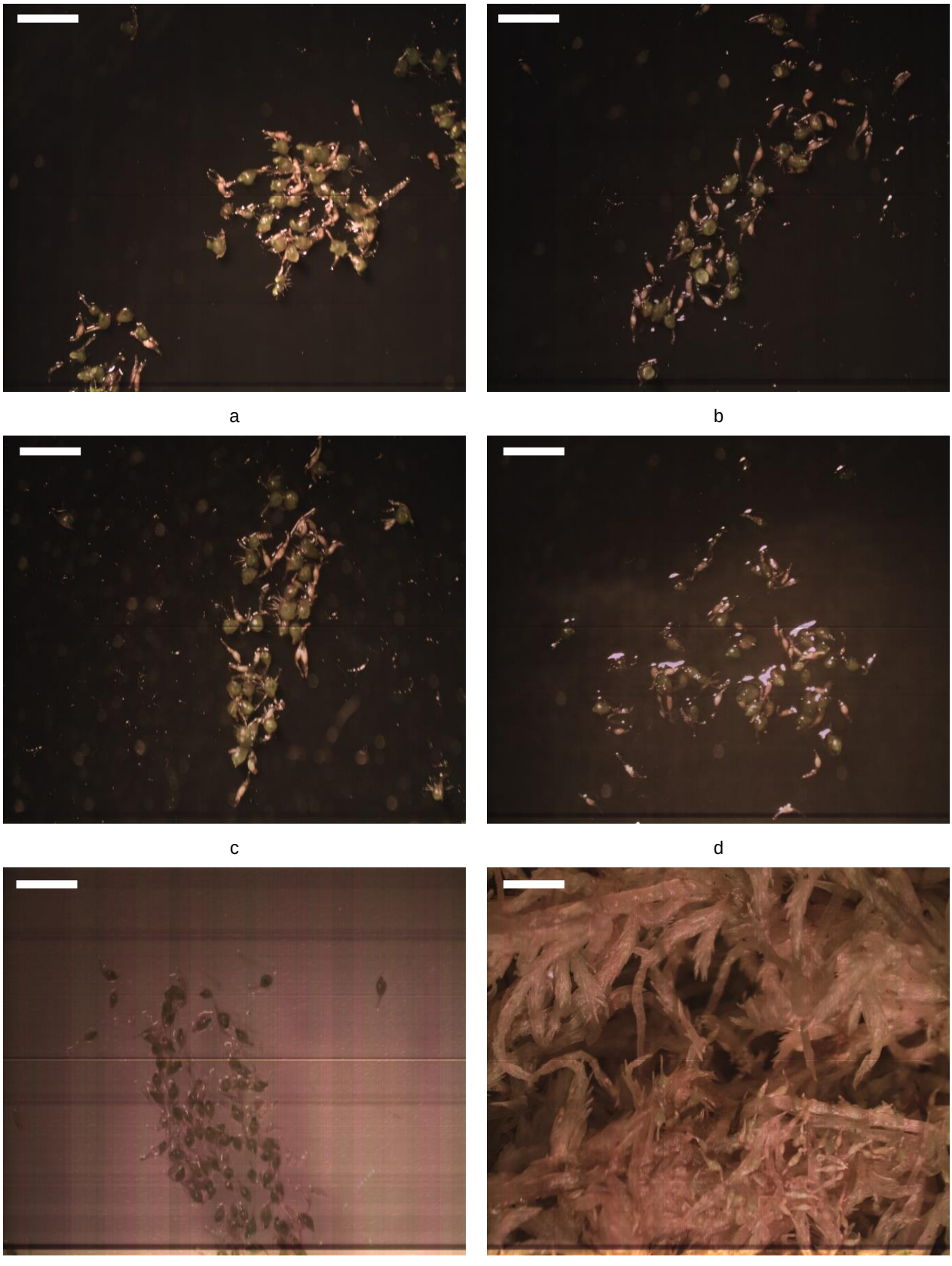

Figure 5. Asymbiotic seed germination, protocorm and seedling development of *Bletilla striata* in different media after 2 weeks period. (a) ½ strength Phytamax P6668, (b) ½ strength P6668 + coconut water, (c) P723, (d) P723 + coconut water, (e) ½ strength MS, (f) Sphagnum Moss. Scale bars = 10 mm.

Figure 6. Asymbiotic seed germination, protocorm and seedling development *of Bletilla striata* in different media after 3 weeks period. (a) ½ strength Phytamax P6668, (b) ½ strength P6668 + coconut water, (c) P723, (d) P723 + coconut water, (e) ½ strength MS, (f) Sphagnum Moss. Scale bars = 10 mm.

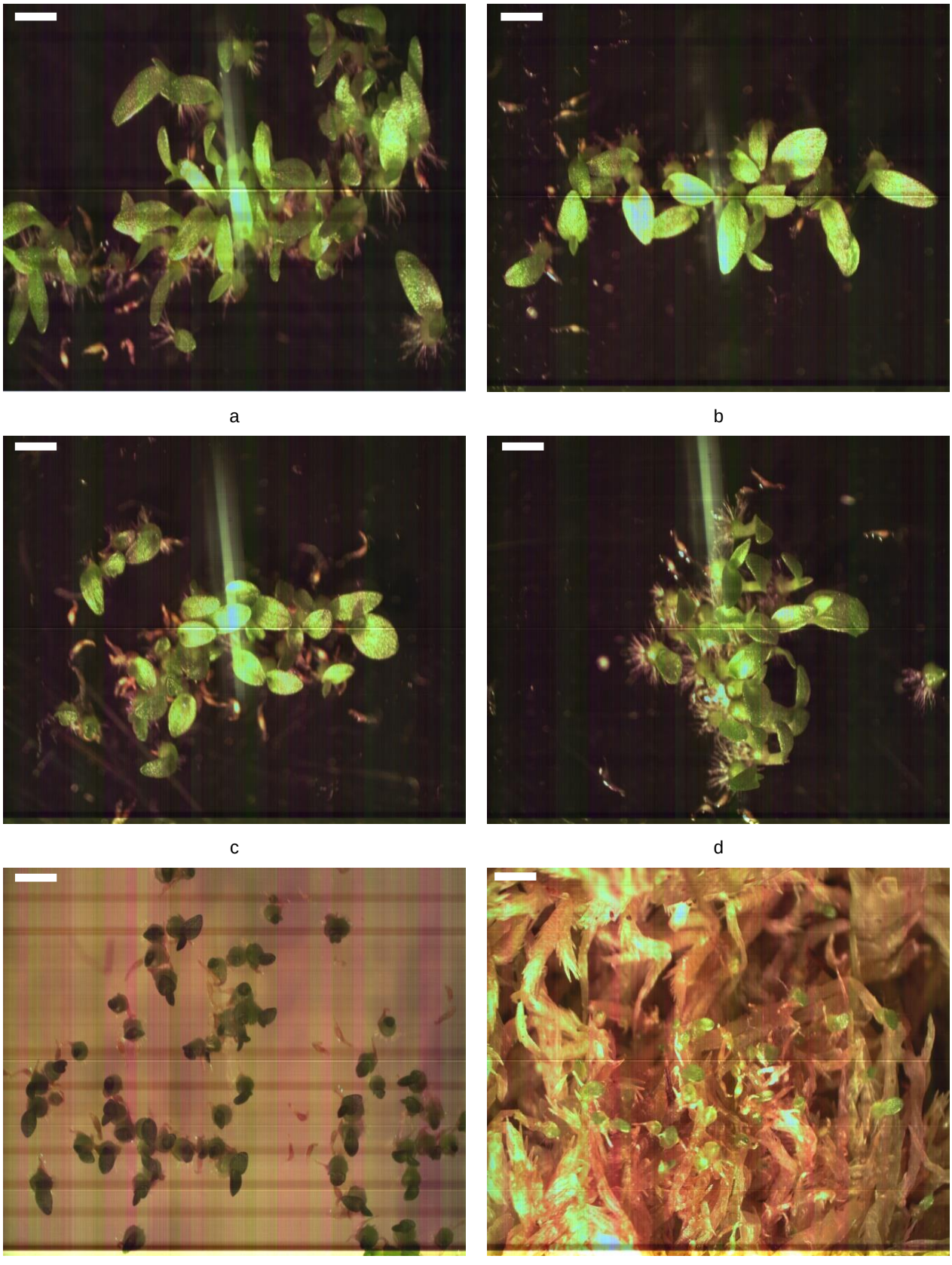

Figure 7. Asymbiotic seed germination, protocorm and seedling development of *Bletilla striata* in different media after 5 weeks period. (a) ½ strength Phytamax P6668, (b) ½ strength P6668 + coconut water, (c) P723, (d) P723 + coconut water, (e) ½ strength MS, (f) Sphagnum Moss. Scale bars = 10 mm.

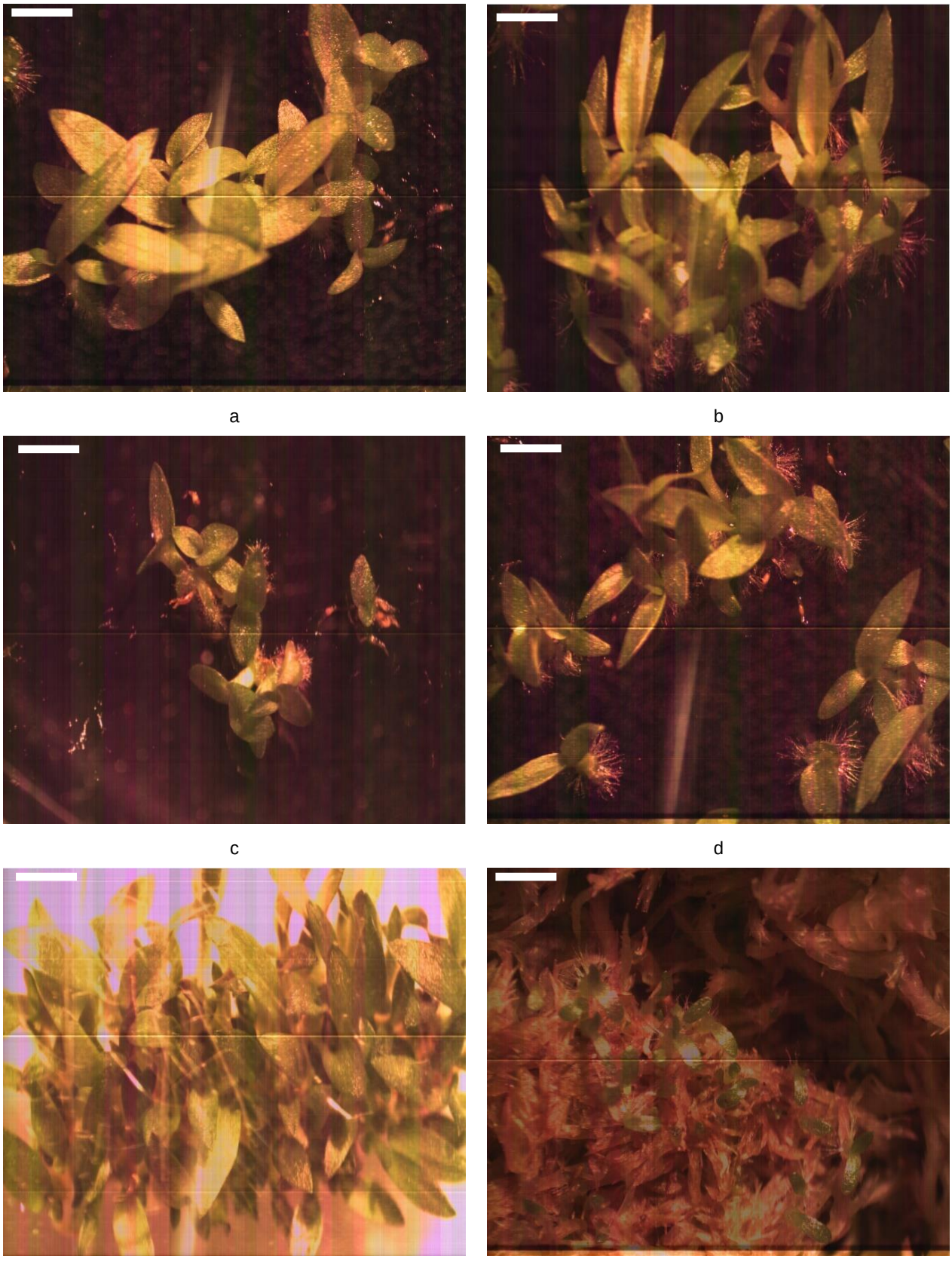

Figure 8. Asymbiotic seed germination, protocorm and seedling development of *Bletilla striata* in different media after 8 weeks period. (a) ½ strength Phytamax P6668, (b) ½ strength P6668 + coconut water, (c) P723, (d) P723 + coconut water, (e) ½ strength MS, (f) Sphagnum Moss. Scale bars = 10 mm.

4. Conclusion

The results show that the seeds of *Bletilla striata* can be germinated and seedlings can be developed successfully under in vitro and ex vitro conditions. According to the present study, there were statistically significant differences between nutrition media in terms of germination and seedling development parameters. Different components like macro elements, micro elements, vitamins, and organics showed significant differences for asymbiotic in vitro germination. Different combinations of plant growth regulators with commercial orchid media can be investigated for further researches. Furthermore, different nutrition sprays can be applied to the sphagnum moss after the germination process in the future studies.

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RESEARCH PAPER

Effects of Calcium Treatment on Physical and Biochemical Changes of Cold-Stored Sweet Cherry Fruit

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Abstract

In the present study, sweet cherry fruit (*Prunus avium* cv. 0900 Ziraat) were dipped into calcium (Ca) gluconate (calcium concentration of 1.5%) and distilled water (as control). The treated fruit were stored at 1 ± 0.5°C and 90% relative humidity for 4 weeks in two different packages. At weekly intervals, weight loss, fruit firmness, colour changes, soluble solids content, titratable acidity, sensory analysis, total phenolic and anthocyanin content of fruit and total chlorophyll content of stem were evaluated. As a result, Ca treatment positively affected sweet cherry fruits by maintaining firmness and decreasing respiration rate of sweet cherry fruit. Moreover, Ca treatment generally delayed the skin browning, and maintained fruit quality. Stem chlorophyll decomposition was retarded with Ca throughout cold the storage. Considering the sensory analysis, storage life of fruit could be prolonged at least up to 1 week by Ca1 (treated Ca-Glu and stored in plastic box with lid) treatment compared to other treatments.

1. Introduction

Sweet cherry is one of the most attractive fruit for consumer in the world because of its own taste and aroma. Cherries are rich in nutrients such as vitamins (C, A and K), minerals (potassium, magnesium etc.). Additionally, sweet cherry fruit contains bioactive compounds (polyphenols, anthocyanins etc.) which have positive effects on health. Increasing the consumption of sweet cherry is associated with these compounds (Le Nguyen et al., 2020). Sweet cherry fruit is generally consumed as fresh therefore, the quality parameters that affect consumers are mostly important. The main quality criterias widely used sweet cherry fruit are firmness, fruit skin and stem colour, acidity and sugar content (Göksel and Aksoy, 2014). Especially sweetness, firmness and colour of sweet cherry fruit influence consumers' acceptance (Göksel et al., 2013). Because of the high respiration rate, harvested sweet cherry fruit are susceptible to handling, storage, transportation and marketing. The main causes of quality losses during cold storage are water loss, softening, colour deterioration, stem browning, pitting and fungal decays (Martínez-Romero et al., 2006). Therefore, postharvest treatments and appropriate storage conditions to maintain postharvest quality are very important for sweet cherry fruit (Miranda et al., 2020).

Pre/postharvest treatments of calcium (Ca) play an important role to increase storability and promote postharvest quality of cherries. After harvest, Ca dipping and vacuum infiltrations treatments are one of the most used techniques to increase the calcium content of cell wall. Ca affects the cell wall structure, membrane integrity and cell wall strength in fruit (Dong et al., 2019). In addition, it is reported that Ca applications can reduce decay rate and disorders during storage (Wang et al., 2014; Michailidis et al., 2017). Exogenous Ca treatments and the positive

effects of it on the fruit quality during cold storage have been stated in many studies (Wójcik and Wawrzyńczak, 2014; Michailidis et al., 2017; Hosein-Beigi et al., 2019; Öztürk et al., 2019). But these reported effects on the quality may vary depending on the source of Ca, treatment timing, concentration, environmental conditions and cultivars. Although Ca treatments have positive effects on fruit quality, it has been stated that sometimes inorganic salts like calcium chloride ($CaCl_2$) change the fruit taste-aroma (Monsalve-Gonález et al., 1993). But using the organic salts such as calcium gluconate (Ca-Glu), lactate and citrate can suppresses the undesirable taste-aroma (Hernández-Muñoz et al., 2006).

Previous studies have shown that the storage temperature and techniques such as modified atmosphere, controlled atmosphere and dynamic controlled atmosphere are effective in prolonging the postharvest life of stone fruit by delaying quality losses. With these informations in this study, the effects of Calcium glucanate on physical and biochemical changes of 0900 Ziraat sweet cherry fruit during during storage were investigated.

2. Material and Methods

2.1. Material

Sweet cherry (*Prunus avium* cv. 0900 Ziraat), which have standard size and colour, were hand-harvested (16 June 2020) at commercial maturity stage in Isparta-Turkey. Cherry trees were 8 years old and grafted on *Prunus mahaleb* L. rootstocks. Immediately after harvest, fruit were transfered to the laboratory within one hour, and undesireable parts and injured fruit were removed. After visual examination, fruit were divided into two lots. Fruit were dipped into a 4°C Ca-Glu solution (calcium concentration of 1.5%) for 30 sec or control (distilled water). Tween 20 (0.1%) was added to all solutions (including control) as the spreading adhesive. The doses and dipping time of Ca-Glu (Califast, Crops MCS) was chosen according to Akhtar and Rab (2014). Following each treatment, sweet cherry fruit were air-dried for 30 min at room conditions then fruit (750 g) were placed into plastic boxes (18 cm × 11 cm × 7.5 cm). After treatments control and Ca-Glu treated fruits were placed into plastic boxes with lid or without lid. Plastic boxes with lid (1 kg) have 8 perforation holes with 5 mm diameter. As a result, the study was arranged as 4 different treatments; Control group in plastic box with lid (C1), control group in plastic box without lid (C2), Ca-Glu in plastic box with lid (Ca1) and Ca-Glu in plastic box without lid (Ca 2). All boxes were stored at 1 ± 0.5°C and 90% relative humidity (RH) for 4 weeks, and fruit samples were analyzed at weekly intervals. Each box represented a replication.

2.2. Physical and chemical analysis

Weight loss: The boxes of cherry fruit were weighed initially and recorded. At each analysis day, the same boxes were weighed, and weight loss was determined by the difference between the initial and final weights. Weight losses of cherry fruit were expressed as percentage (%).

Fruit firmness: Fifteen cherries from each replicate were used for firmness evaluation. Firmness was measured using a texture analyzer machine (Lloyd Instruments LF Plus) with a 50 N load cell (5 mm cylindrical probe). The results were expressed as Newton (N).

Colour determinations: Fruit stem and skin colour (20 stem and fruit for each replicate) were determined using colorimeter (Minolta CR-300). Values of L*, a* and b*, which used to define a three-dimensional colour space, were measured. The a* and b* values were used to calculate Chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] and Hue angle ($h° = \tan^{-1} b^*/a^*$) values.

Soluble solids content (SSC) and titratable acidity (TA): Fruit juice was extracted, and the SSC (%) was determined by using a digital refractometer (Atago-PAL1). For TA, fruit juice (10 mL) was titrated with 0.1 N sodium hydroxide up to pH 8.1, and results were expressed as percentage.

Respiration rate: Respiration rate of fruit was measured by using gas chromatography. Fruit (140-150 g) were placed in a 1 L glass jar, hermetically sealed, for 1 h in room temperature. Gas sample was taken by a gas-tight syringe, and injected into a gas chromatography (Agilent 6890N) equipped with a thermal conductivity detector. Results were expressed as $mLCO_2\ kg^{-1}\ h^{-1}$.

Total phenolic and anthocyanin content: Cherry fruit were extracted for these analyses according to procedure used by Ağlar et al. (2017). Extracted supernatants were stored at −20°C until the day of analysis. Total phenolic content and total anthocyanin content were determined by using spectrophotometric method according to Singleton and Rossi (1965) and Giusti et al. (1999), respectively. The results were expressed as mg gallic acid equivalent (GAE) on FW basis (mg GAE $100\ g^{-1}$) for total phenolic content and mg in kg cyanidin 3-glucoside (cy-3-glu) on FW basis ($mg\ kg^{-1}$ cy-3-glu) for total anthocyanin content.

Stem chlorophyll content: Chlorophyll extraction and stem chlorophyll analysis were performed to procedure described by Göksel (2011). The results were calculated as mg in 100 g ($mg\ 100g^{-1}$).

External appearance and taste: External appearance and taste of fruit were determined with hedonic test. Two scales from 1 to 9 (≤1-4: poor, 9: excellent) and from 1 to 5 (1: very poor, 5: excellent) were used for external appearance and taste evaluation, respectively (Erbaş et al., 2015).

2.3. Statistical analysis

The study was conducted to completely factorial randomized design with 3 replicate (each box

Table 1. Anova for dependent variables for treatments, storage period and their interactions for sweet cherry

Variables	Storage periods (SP)	Treatments (T)	SP × T
Weight loss	**	*	ns
Respiration rate	NS	NS	NS
Fruit firmness	**	*	NS
External appearance	**	**	**
Taste	**	**	NS
Fruit skin colour L*	**	NS	NS
Fruit skin colour C*	**	NS	NS
Fruit skin colour h°	**	NS	NS
Stem colour L*	**	*	NS
Stem colour C*	**	*	NS
Stem colour h	**	NS	NS

* NS represents non-significance at p < 0.05; ** Represents significance at the 0.01 level; * Represents significance at the 0.05 level.

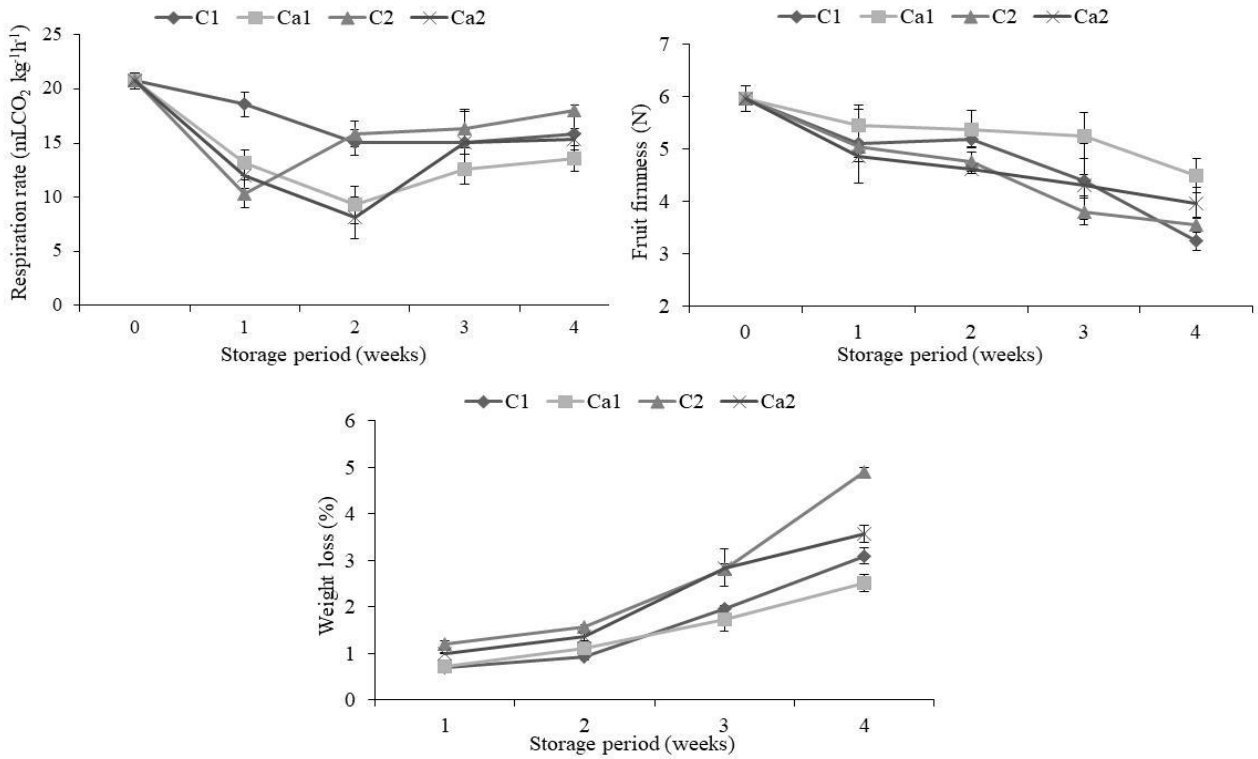

Figure 1. Respiration rate, fruit firmness and weight loss of 0900 Ziraat sweet cherry fruit during cold storage
(Data are mean ± S.E. C1: Control group in plastic boxes with lid; Ca1: Ca group in plastic boxes with lid, C2: Control group in plastic boxes without lid; Ca2: Ca group in plastic boxes without lid).

containing 750 g fruit was regarded as a replication). The data was analyzed using Minitab 18 statistics software. The differences among means were compared with Tukey's range test (5%).

3. Results and Discussion

3.1. Respiration rate, fruit firmness and weight loss

The respiration rate of fruit at harvest was 20.74 mLCO_2 kg^{-1} h^{-1}. When fruit were placed to cold storage, respiration rate decreased sharply in all treatments but this decrease was higher in Ca treated fruit compared to control. The lowest respiration rates were 13.53 mL CO_2 kg^{-1} h^{-1} (Ca1) and 15.37 mL CO_2 kg^{-1} h^{-1} (Ca2) at the end of the storage. Although there was no statistically difference between treatments and control group (Table 1), Ca dip treatments reduced, relatively, respiration rate of sweet cherry fruit (Figure 1). The decrease in the respiration rate of fruit might be due to the delaying of senescence processes. Ca treatments resulting in decreased respiration rate and delayed maturity have been reported in previous studies carried out on climacteric or non-climacteric fruit (Lara et al., 2004; Shafiee et al., 2010; Wang et al., 2014). Moreover, in the present study, the reduced oxygen and increased carbon dioxide by using the boxes with lid contributed to reduce respiration rate in sweet cherry fruit. Accordingly, as seen in Figure 1, the lowest respiration rate was obtained from combination of boxes with lid and Ca treatment at the end of the storage.

Fruit firmness of sweet cherries is one of the main quality attributes for consumer acceptance. Ca treatments significantly affected the fruit

firmness of cherry fruit (Table 1). Fruit firmness, which was 5.96 N at harvest, decreased with prolonging storage in all treatments (Figure 1). During cold storage, the delay in softening has been reported by Manganaris et al., 2007 and Naser et al., 2018 in Ca treated peach and persimmon, respectively. Ca treated fruit had greater fruit firmness compared to control groups. Especially, fruit in Ca1 treatment more firm than those of Ca2, and the softest fruit were observed in control groups at the end of storage (Figure 1). As known, increased weight loss can result in reduced fruit firmness (Mitcham et al., 1998). In the present study, a negative relationship between weight loss and fruit firmness was observed. The results indicated that Ca applications retarded fruit softening, especially in plastic boxes with lid depending on decreased weight loss (Figure 1). Likewise, Wang et al. (2014) indicated that pre or postharvest Ca applications generally contribute fruit firmness by increasing turgor pressure of cell and decreasing water loss of tissue. On the other hand, Serrano et al. (2005) reported that the high oxygen concentration in storage increased the softening of tissues in sweet cherry fruit.

Weight loss is one of the major problems which responsible for quality loss for stored cherries, and is related to loss of water vapor (Mitcham et al., 1998). In the present study, weight loss of sweet cherry fruit increased as storage time proceeds in all treatments. At the end of the storage, C2 treatment had the highest weight loss (4.89%) followed by Ca2 (3.57%), C1 (3.10%) and Ca1 (2.52%) treatments. Ca1 treatment showed the lowest weight loss, together with the lowest firmness loss (Figure 1). Garcia et al. (1996) reported the same effect on weight loss for strawberries, which were immersed in Ca solution. Weight or water loss is generally linked to moisture evaporation through the fruit skin and respiration rate of fruit. It is thought that lower weight losses in C1 and Ca1 treatments can be attributed to higher RH in these boxes. Similarly, Serrano et al. (2005) reported that the high RH inside the boxes might be responsible for the retarded softening and water loss.

3.2. Soluble solids content (SSC) and titratable acidity (TA)

Changes in the SSC and TA of cherry fruit are shown in Table 2. Regardless of treatments, the SSC of cherries showed fluctuation during storage but decreased compared to initial value (12.97%) at the end of the storage. Similar fluctuation in the SSC of sweet cherries throughout storage was declared by (Giacalone and Chiabrando, 2013; Wang et al., 2014). At the 3th weeks of storage, the SSC of cherries reached the maximum levels (except for Ca1 treatment) and then slightly decreased in all treatments. It is considered that these results are related to the weight loss and respiration rate of cherries. This thought was supported by Ca1 treatment, which had the lowest weight loss (Figure 1). Similarly, Güneyli et al. (2018) reported that the SSC was influenced by the weight loss of fruit causing, usually, an increase in the SSC of sweet cherries. On the other hand, Kluge et al. (1996) expressed that sugar decreases during storage might be caused by the use of sugars in respiration.

The TA of cherries decrease overtime compared to initial value (0.89%) varying between 0.71% (C1 and C2) and 0.79% (Ca1) at the end of the storage. The Ca1 (0.83%) was the best treatment for maintaining the acidity of cherries followed by Ca2 (0.80%), C1 (0.80%) and C2 (0.77%). Accordance with the present study, the TA losses were delayed by Ca treatments in previous research of Wang et al. (2004). Maintaining TA with Ca1 treatment can be explained by its suppressing effect on fruit metabolism, especially respiration rate (Figure 1). It has been reported that acids are a major component of respiration process (Kays and Paull, 2004), and the use of organic acids as substrates in respiration and enzymatic reactions reduce acid content in fruit (Certel et al., 2004).

3.3. Fruit skin and stem colour

Fruit (bright red) and stem (green) colour is the most important quality attributes for consumers. The change in skin and stem L* (lightness) values of fruit showed similar trend in all treatments. As seen in Figure 2, L* values of stem and fruit decreased, in general, during cold storage but Ca1 treatment was most effective on fruit and stem lightness. Moreover, Ca1 treatment had higher hue angle (h°) and chroma values (both skin and stem) than the other treatment (data not shown). Hue angle values decrease with increasing maturity, and the vividness of the colour become apparent with the increase of the C* values in sweet cherries (Mozetic et al., 2004). In the present study, L*, C* and h° values decreased gradually, and Ca1 treatment delayed their reductions slightly. Considering the stem and skin colour values, it could be said that Ca1 treatment delayed the colour deterioration of sweet cherries. Wang and Long (2015) indicated that delaying colour change or darkening by Ca treatments may be associated with its prevention of senescence in sweet cherries.

3.4. Sensory analysis

Sensory tests of cherries are presented in Figure 3. The taste and external appearance scores of sweet cherry fruit reduced with prolonging storage time. Boxes and Ca treatments affected sensory quality of sweet cherries. Cherries in Ca1 treatment had the best sensory quality scores compared to other treatments. The quality loss of cherries started to increase especially after 2th weeks of storage. At the 4 weeks of storage, the higher external appearance score (5.00) was obtained from Ca1

Table 2. Soluble solids content (SSC) and titratable acidity (TA) of 0900 Ziraat sweet cherry fruit during cold storage

Parameters	Treatments (T)	Storage periods (weeks)					Means
		Harvest	1	2	3	4	
SSC (%)	C1	12.97	13.00	12.97	13.17	12.90	13.00 a
	Ca1		11.07	11.83	12.37	12.83	12.21 b
	C2		12.93	12.93	13.10	12.80	12.95 a
	Ca2		11.70	11.60	13.20	12.77	12.45 ab
	Means	12.97 ab	12.17 c	12.33 bc	12.96 a	12.83 ab	
TA (%)	C1	0.89	0.81	0.83	0.77	0.71	0.80 ab
	Ca1		0.82	0.81	0.81	0.79	0.83 a
	C2		0.79	0.72	0.73	0.71	0.77 b
	Ca2		0.78	0.76	0.82	0.76	0.80 ab
	Means	0.89 a	0.80 ab	0.78 ab	0.78 ab	0.74 b	

P values	Storage periods (SP)	Treatments	SP × T
SSC	*	**	NS
TA	**	**	NS

Means followed by the same letter in the same column are not statistically significant (*P*<0.05). NS represents non-significance at p <0.05; ** Represents significance at the 0.01 level; * Represents significance at the 0.05 level. C1: Control group in plastic boxes with lid; Ca1: Ca group in plastic boxes with lid, C2: Control group in plastic boxes without lid; Ca2: Ca group in plastic boxes without lid.

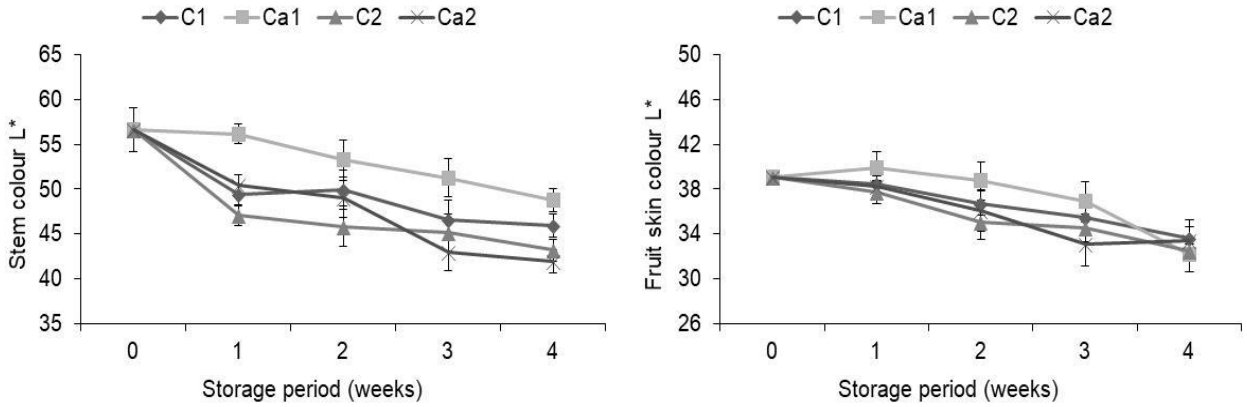

Figure 2. Fruit skin and stem colour L* values of 0900 Ziraat sweet cherry fruit during cold storage
(Data are mean ± S.E. C1: Control group in plastic boxes with lid; Ca1: Ca group in plastic boxes with lid, C2: Control group in plastic boxes without lid; Ca2: Ca group in plastic boxes without lid).

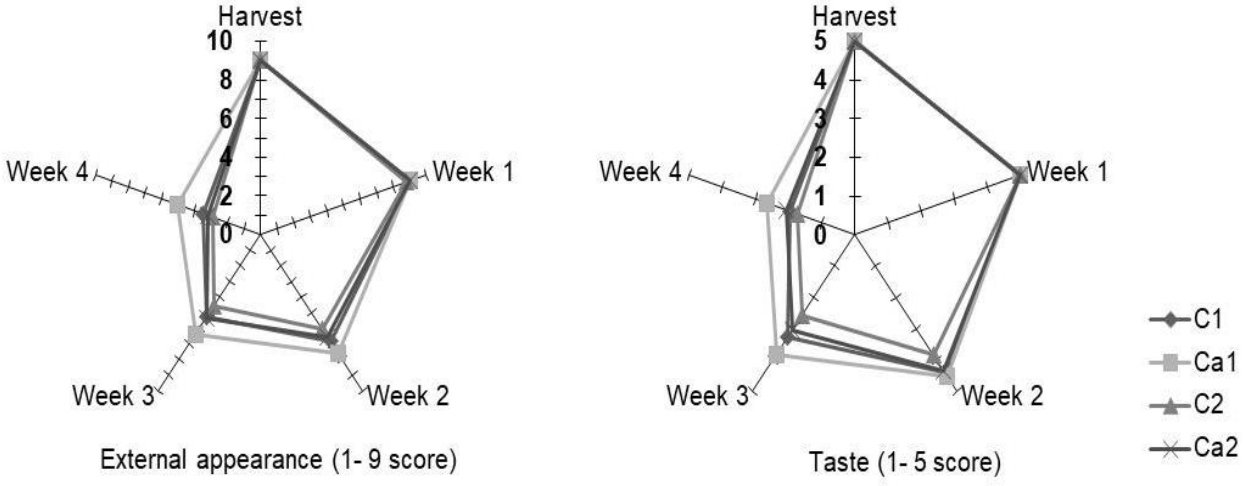

Figure 3. External appearance and taste of 0900 Ziraat sweet cherry fruit during cold storage
(C1: Control group in plastic boxes with lid; Ca1: Ca group in plastic boxes with lid, C2: Control group in plastic boxes without lid; Ca2: Ca group in plastic boxes without lid).

Table 2. Total phenolic content, anthocyanin content and stem total chlorophyll content of 0900 Ziraat sweet cherry fruit during cold storage

Parameters	Treatments	Storage period (weeks)			Means
		Harvest	2	4	
Total phenolic content (mg 100g^{-1})	C1	32.85	41.11	49.47	41.14 ^{ns}
	Ca1		40.66	44.97	39.49
	C2		46.29	60.52	46.55
	Ca2		43.45	52.54	42.95
	Means	32.85 c	42.88 b	50.21 a	
Total anthociyanin content (mg kg^{-1})	C1	13.02	17.22	18.17	16.14 ^{ns}
	Ca1		16.72	19.42	16.39
	C2		19.80	17.02	16.61
	Ca2		19.81	19.52	17.45
	Means	13.02 b	18.39 a	18.53 a	
Total chlorophyll content of stem (mg 100g^{-1})	C1	5.82	3.85	2.41	4.02 ab
	Ca1		4.70	2.71	4.41 a
	C2		3.45	1.72	3.66 b
	Ca2		3.72	2.64	4.06 ab
	Means	6.18 a	3.93 b	2.37 c	
	P values		Storage period (SP)	Treatments (T)	SP × T
	Total phenolic content		**	NS	NS
	Total anthociyanin content		**	NS	NS
	Total chlorophyll content of stem		**	**	NS

Means followed by the same letter in the same column are not statistically significant (*P*<0.05). NS represents non-significance at p < 0.05; ** Represents significance at the 0.01 level; * Represents significance at the 0.05 level. C1: Control group in plastic boxes with lid; Ca1: Ca group in plastic boxes with lid, C2: Control group in plastic boxes without lid; Ca2: Ca group in plastic boxes without lid.

treatment, while the lowest score (2.89) was determined in C2 treatment. Similar trend was also observed in taste evaluation. The best (2.67) and the worst score (1.72) was determined in Ca1 and C2 treatments, respectively at the end of the storage. Cherries with marketable scores (≥ 5) were only determined from Ca1 treatments (5.00) at the 4th weeks of storage. C2 treatments (4.61) lost their marketable quality at the 3th weeks of storage. C1 (5.28) and Ca2 (5.33) treatments preserved the marketable quality at the 3th weeks of storage. It was reported that Ca treatment was proved to delay tissue weakening, increase membrane integrity and reduce decay rate in fruit (Manganaris et al., 2005). At the same time, Wang et al. (2014) stated that Ca is effective in maintaining the fruit quality and reducing the disorders that may occur during cold storage. Similar results were also determined in present study.

3.5. Total phenolics, anthocyanin content and stem total chlorophyll content

Phenolic compounds have important effects on the fruit quality such as colour, taste and aroma (Göksel, 2011). During cold storage, total phenolic content of cherry fruit increased in all treatments. The highest total phenolic content (60.52 mg GAE 100g^{-1}) was determined from C2 treatment, whereas the lowest value (44.97 mg GAE 100g^{-1}) was measured in Ca1 treatment at the end of the storage. The Ca treatments affected the total phenolic content of cherry fruit depending on the packaging. The fruit in plastic boxes with lid had the lowest phenolic content, while fruit placed in open plastic boxes gave the highest values (Table 3). Higher phenolic content values can be attributed to higher water loss and increased stress conditions caused by open plastic boxes. As a stress factor, the cold air circulation in the atmosphere surrounding fruit can affect them in open boxes compared to closed ones with lid. It is known that the accumulation of phenolic compounds in fruit increases under stress conditions (Li et al., 2018), and fruit stored in plastic boxes with lid might have been exposed to less stress in the present study.

Anthocyanins (responsible to red colour of fruit and vegetables) are important phenolic compounds in cherry fruit (Aghdama et al., 2013). In the present study, according to general means, the anthocyanin content of fruit increased during storage (Table 3), accordance with several authors who reported similar increase (Bernalte et al., 2003; Ağlar et al., 2017). Total anthocyanin content of cherry fruit at harvest was 13.02 mg kg^{-1}. Total anthocyanin content of fruit in Ca1 was lower (16.72 mg kg^{-1}) than other treatments on the 2th week of storage. Anthocyanin contents of cherry fruit in C1 and Ca1 treatments increased slightly up to 2 weeks, on the contrary of other two treatments. Treatments did not affect total anthocyanin content of cherry fruit (P<0.05). However, fruit stored in closed boxes with lid had lower (C1: 16.14 mg kg^{-1}; Ca1: 16.39 mg kg^{-1}) anthocyanin contents compared to those without lid (C2: 16.61 mg kg^{-1}; Ca2: 17.45 mg kg^{-1}) (Table 3). This can be explained by the proportional increase of anthocyanin contents in open boxes

depending on higher water loss and quick ripening in fruit. Similarly, Giacalone and Chiabrando (2013) stated that modified atmosphere packaging (MAP) treatments delayed anthocyanin biosynthesis of sweet cherry fruit.

Stem colour, one of the most important indicators for determining the freshness of cherry fruit, changes during storage because of the chlorophyll breakdown. Göksel (2011) declared rapid declines in total chlorophyll content of stems throughout cold the storage, similar to the findings of the present study (Table 3). Compared to initial value (5.82 mg $100g^{-1}$) the lowest decrease in chlorophyll content was obtained from Ca1 and Ca2 treatments. These results showed that Ca treatments had a positive effect on total chlorophyll content of stems. Sweet cherry fruit stored in boxes with lid also had the low chlorophyll losses compared to boxes without lid. This result can be attributed to higher oxidation and senescence processes in fruit stems stored in open boxes.

4. Conclusions

In conclusion, it is found that postharvest Ca treatments could reduce respiration rate and maintain fruit firmness of sweet cherry fruit during cold storage. Moreover, Ca treatments had positive effects on maintaining sweet cherry skin colour, stem colour and sensory quality. But these positive effects were more noticeable when fruit were stored in boxes with lid compared to open boxes. According to sensory scores, the fruit in Ca1 treatment, which had the best results for storage life and quality, could be stored with good quality for 3-4 weeks. This treatment was followed by C1 (3 weeks), Ca2 and C2 (2 weeks) applications.

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RESEARCH PAPER

HORTICULTURAL STUDIES

Determination of Leaf Area of Some Vegetable Plants Grown under Greenhouse Condition by Non-Destructive Methods

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Regression

Abstract

The use of a mathematical model approach, which is a non-destructive method to determine the leaf area, has been used safely in recent years. The most important reason for this is that the application is easy and the application cost is very low. The most important disadvantage of this method is that the derived models need calibration according to different crop varieties. In the study, firstly, the width (W) and length (L) measurements of each individual leaf taken from the crops were correlated with the actual area of the leaf and crop-specific models were developed. The actual area (LA) of each leaf used in the sampling was determined by the ImageJ software. In the study, 7 different models were derived for each crop species (1, $LA=a+bL^2+cL^2/W^2+dW^2+eLW$; 2, $LA=a+bL^2+cW^2+dLW$; 3, $LA=a+bL^2+cW^2$; 4, $LA=aL^2+bW^2$; 5, $LA=aL^b+cW^d+e$; 6, $LA=a+bLW$; 7, $LA=aLW$). The coefficients in the models were determined separately for each crop species. In order to evaluate the performance of the models, some performance criteria such as determination coefficient (R^2) and root mean square error (RMSE), relative error (RE), mean bias error (MBE) and Willmott index (d) were used. In this study, leaf areas were estimated with high accuracy (R^2=0.96 for tomato; R^2=0.99 for pepper, eggplant and cucumber) with these models without using expensive measuring devices.

1. Introduction

The leaves of each crop varieties have a characteristic size. Moreover, there is a direct relationship between the width, length and area of the leaf (Al Mamun Hossain et al., 2017; Hinnah et al., 2014; Ray and Singh, 1989). For this reason, a large number of equations developed in order to estimate the leaf area by using leaf width and length values. The characteristic leaf dimension can show variability even in different varieties of the same species of plant. Therefore, as can be seen in Table 1, a large number of models were developed by researchers. Table 1 shows the different models used to estimate the leaf area of different crop species and varieties in different environments (Karaca, 2020).

There are two destructive and non-destructive categories which include counting squares on millimeter graph paper, hand-planimetry, the gravimetric method, dot counting, photoelectric planimetry, air-flow, linear measurements of leaves, leaf weighing, detached leaf counting, and the rating in determining leaf area (Pandey and Singh, 2011). In the destructive method, the leaves are separated from the plant body and the area is determined with the help of various scanning planimeters. However, destructive methods require more intensive labor and higher costs. In recent years, the portable types of these instruments were developed and the leaf area was measured without being destructive. However, in this method, leaf size, shape and texture are limiting and do not suitable for every plant leaf (Rouphael et al., 2010).

Table 1. Models used to predict leaf areas of different crop species in different growing environments

Environments	Plant	Equation	Source
Open Field	Eggplant	$LA = 0.66LW - 0.00008\mathrm{LW}^2$	(Rivera et al., 2007)
Open Field	Eggplant	$LA = 0.6537LW + 3.14$	(Ogoke et al., 2015)
Greenhouse	Eggplant	$LA = 0.4395LW^{1.0055}$	(Hinnah et al., 2014)
Greenhouse	Cucumber	$LA = 0.859LW + 2.7$	(Blanco and Folegatti, 2003)
Greenhouse	Cucumber	$LA = 10.3602 + 0.7001W^2$	(Bozkurt and Keskin, 2018)
Greenhouse	Cucumber	$LA = -28.5522 + 0.8301L^2$	(Bozkurt and Keskin, 2018)
Greenhouse	Cucumber	$LA - 3.6852 + 0.5202W^2 + 0.2252L^2$	(Bozkurt and Keskin, 2018)
Open Field	Cucumber	$LA = 210.61 + 13.358W + 0.5356LW$	(Cho et al., 2007)
Greenhouse	Tomato	$LA = 0.347LW - 10.7$	(Blanco and Folegatti, 2003)
Greenhouse	Tomato	$LA = 0.2695L^{0.4759}\mathrm{W}^{1.4184}$	(Schwarz and Kläring, 2001)
Greenhouse	Tomato	$LA = 0.2633L\mathrm{W}^{1.1175}$	(Dumas, 1990)
Open Field	Pepper	$LA = 0.604LW$	(Ray and Singh, 1989)
Open Field	Bell pepper	$LA = 0.57LW$	(Padrón et al., 2016)
Greenhouse	Green pepper	$LA = -8.28 + 1.89L + 2.5W + 0.0028LW$	(Cemek et al., 2011)
Greenhouse	Chili Pepper	$LA = 0.498LW + 0.054$	(Aminifard et al., 2017)

Non-destructive methods are widely used because they do not require the leaves to be plucked, therefore do not adversely affect plant growth and allow measurements to be repeated throughout the growing period. Simple and accurate mathematical models that reveal the relationship between leaf area and leaf dimensions (width and length) have been developed by many researchers for many plant species as they eliminate the dependence on expensive measuring devices and, save time (Carmassi et al., 2007).

In this study, empirical models were derived and tested to estimate the leaf area depending on the leaf width and length for four different crop species (tomato, eggplant, pepper and cucumber) commonly grown under greenhouse conditions in Antalya. For this purpose, seven different models were developed for each crop Different statistical performance criteria were used to determine the performance of these models.

2. Material and Methods

This study was carried out under the conditions of a lysimeter in a plastic greenhouse in the experimental area of the Faculty of Agriculture, Akdeniz University. The first and second seasons of the experiment were conducted from 14.09.2018 to 21.02.2019. Anıt F1 in tomato, Ayda F1 in cucumber, Corsica F1 in eggplant and Buket F1 in pepper varieties, which is suitable for both autumn and spring cultivation in Antalya Region, were used as crop material.

One hundred thirty-five leaves were used for each crop species to derive leaf area prediction models. Leaf length (L) was measured with a ruler, disregarding the petiole (except tomato). Leaf length in tomato was measured as the distance from the base of the petiole to the distal tip. Leaf width (W) was determined by measuring the longest width of the leaves perpendicularly to the midrib. The actual leaf area was obtained with ImageJ® software after scanning the measured leaves (Ferreira and Rasband, 2012) (Figure 1). The relationship between leaf dimensions and leaf area (LA) was determined by seven models for each crop.

Model 1: $LA=a+bL^2+cL^2/W^2+dW^2+eLW$
Model 2: $LA=a+bL^2+cW^2+dLW$
Model 3: $LA=a+bL^2+cW^2$
Model 4: $LA=aL^2+bW^2$
Model 5: $LA=aLb+cWd+e$
Model 6: $LA=a+bLW$
Model 7: $LA=aLW$

The coefficients (a, b, c, d and e) in the models were derived separately for each plant species.

In order to determine the most accurate model, some statistical performance criteria including coefficient of determination (R^2) (Equation 1), root mean square error (RMSE) (Equation 2), relative error (RE) (Equation 3), mean bias error (MBE) (Equation 4), the Willmott index of agreement (d) (Equation 5) were used.

$$R^2 = \frac{\left[\sum_{i=1}^{n}(X_i-\ddot{X})(Y_i-\hat{Y})\right]^2}{\sum_{i=1}^{n}(X_i-\ddot{X})^2\sum_{i=1}^{n}(Y_i-\hat{Y})^2} \quad (1)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(X_i-Y_i)^2}{n}} \quad (2)$$

$$RE = \frac{RMSE}{\bar{Y}} \quad (3)$$

$$MBE = \frac{\sum_{i=1}^{n}(X_i-Y_i)}{n} \quad (4)$$

$$d = 1 - \frac{\sum_{i=1}^{n}(X_i-Y_i)^2}{\sum_{i=n}^{n}\left((X_i-\hat{Y}_i)+(Y_i-\hat{Y}_i)\right)^2} \quad (5)$$

Where n is number of observations, Xi is the indirectly estimated LA, Yi is directly measured LA and $\ddot{X}$ is mean value of estimated LA and $\hat{Y}$ is mean value of measured LA. RMSE, RE and MBE are 0, while R^2 and d are 1 indicate that the prediction model is perfect. In the study, "data analysis" and "solver" add-ons of Microsoft Excel program were used to calculate the coefficients of the models.

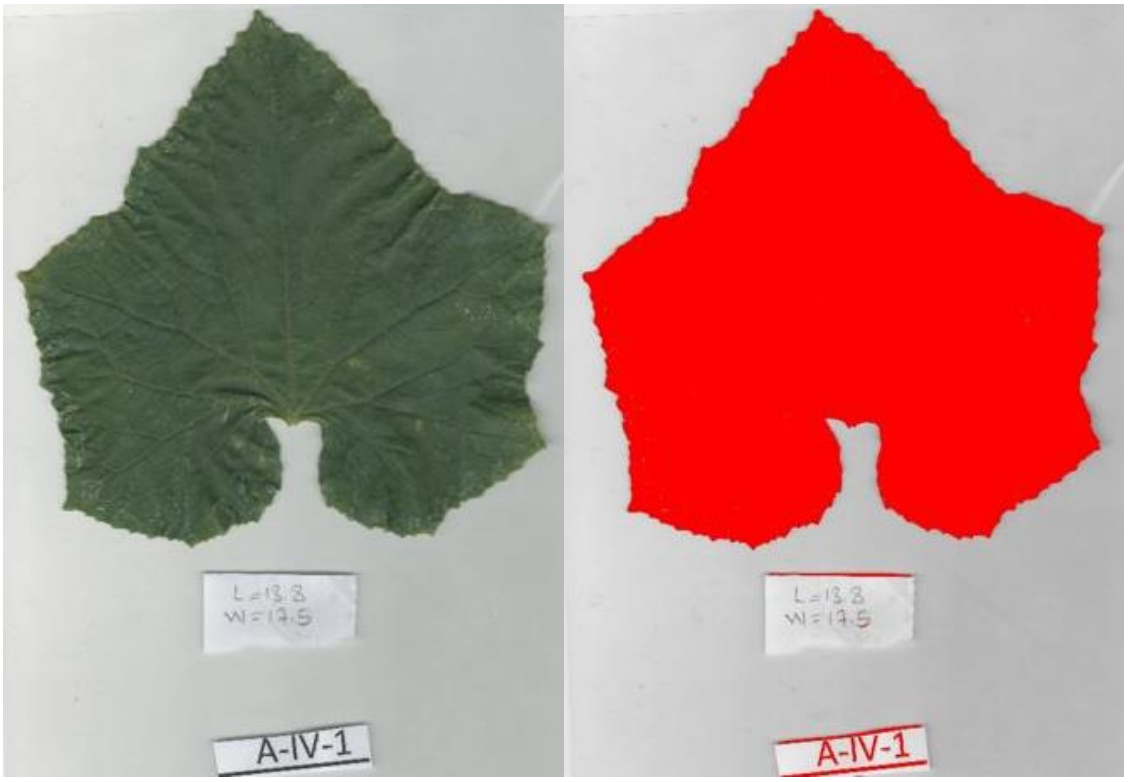

Figure 1. Determination of actual leaf area with ImageJ software

Table 2. Average data comparison of length, width and area of leaves in four different crop obtained by ImageJ® software

Plant	Dimension	Mean	SD	Min	Max
Tomato	Length (cm)	23.6	7.4	11.9	39.0
	Width (cm)	27.5	8.0	11.2	42.6
	Area (cm^2)	315.6	187.2	44.8	885.2
Eggplant	Length (cm)	17.7	5.7	8.9	28.5
	Width (cm)	11.0	4.1	5.2	19.4
	Area (cm^2)	138.5	83.2	35.7	305.2
Pepper	Length (cm)	7.8	2.5	4.1	11.8
	Width (cm)	3.9	1.2	1.9	6.3
	Area (cm^2)	20.4	11.4	5.8	46.3
Cucumber	Length (cm)	13.1	3.2	7.6	18.0
	Width (cm)	16.7	4.9	9.2	23.6
	Area (cm^2)	191.4	98.1	62.6	359.6

3. Results and Discussion

The mean, maximum (Max), minimum (Min) and standard deviation (SD) for area of leaves, length (L) and width (W), were given in Table 2.

In many studies (Aminifard et al., 2017; Hinnah et al., 2014; Ogoke et al., 2015; Padrón et al., 2016), leaf sizes were used to determine the leaf area. Therefore, in this study, LA was related to L, W and L×W in variations. The coefficients of the models used to determine the leaf areas of four different crops were given in Table 3 and the performance criteria of these models in Table 4.

Although the R^2 value is considered as a measure of accuracy in determining the performance of the estimation equation, it is not sufficient to be certain. The most important thing is to obtain a low error (Cemek et al., 2011). The most accurate result is obtained when RMSE, RE and MBE are equal to 0 and d and R^2 equal to 1 (Karaca et al., 2018). For this purpose RMSE, RE, MBE, and d values in Table 4 were also examined.

R^2 values of all models except for Model 5 in the tomato were 0.96. The lowest RMSE value (37.67) in tomato is determined in Model 1. RMSE values of Models 2, 3 and 6 are close to model 1. However, the RMSE performances of Models 4, 5 and 7 are lower than other models. When the MBE value was investigated, it was seen that Model 6 gave the best result. Models 4, 5 and 7 performed lower than other models. When d performance criterion was examined, although the results were very close to each other, Model 4 and 7 had lower performance compared to other models (Table 4). Blanco and Folegatti (2003) reported that the predictive performance (R2) of LA values of tomato plants with three different non-destructive models was between 0.95 and 0.98. On the other hand, Schwarz and

Table 3. The coefficients of the models used to determine the leaf area of four different crops

No	Model	Crop	Equation Coefficient				
			a	b	c	d	e
1	$LA = a + bL^2 + cL^2/W^2 + dW^2 + eLW^*$	Tomato	-42.46	-0.48	6.48	-0.41	1.31
		Eggplant	31.53	1.26	-9.51	2.75	-3.15
		Pepper	4.72	-0.37	-0.91	-1.69	2.19
		Cucumber	-38.56	-2.35	50.75	-1.18	4.20
2	$LA = a + bL^2 + cW^2 + dLW$	Tomato	-35.98	-0.38	-0.33	1.13	-
		Eggplant	3.00	0.86	2.04	-2.05	-
		Pepper	0.97	-0.47	-1.76	2.42	-
		Cucumber	-3.81	-0.52	-0.22	1.53	-
3	$LA = a + bL^2 + cW^2$	Tomato	-46.04	0.15	0.28	-	-
		Eggplant	4.22	0.19	0.50	-	-
		Pepper	0.98	0.15	0.57	-	-
		Cucumber	-3.96	0.43	0.39	-	-
4	$LA = aL^2 + bW^2$	Tomato	0.11	0.28	-	-	-
		Eggplant	0.20	0.50	-	-	-
		Pepper	0.16	0.59	-	-	-
		Cucumber	0.38	0.41	-	-	-
5	$LA = aL^b + cW^d + e$	Tomato	-19.17	-317.04	0.10	2.39	5.00
		Eggplant	-19.17	-317.04	6.06	1.36	-25.41
		Pepper	-19.17	-317.04	2.87	1.52	-3.19
		Cucumber	-19.17	-317.04	0.43	2.10	15.17
6	$LA = a + bLW$	Tomato	-45.08	0.42	-	-	-
		Eggplant	3.99	0.62	-	-	-
		Pepper	0.96	0.59	-	-	-
		Cucumber	-6.29	0.85	-	-	-
7	$LA = aLW$	Tomato	0.38	-	-	-	-
		Eggplant	0.64	-	-	-	-
		Pepper	0.61	-	-	-	-
		Cucumber	0.83	-	-	-	-

*a, b, c, d and e: Equation coefficient; LA: Mean leaf area (cm^2); L: Leaf lenght (cm^2); W: Leaf width (cm^2)

Kläring (2001) used two different methods to estimate the LA values of tomato plants: leaf length measurements from the stem and the rachis where the first pinnate starts. In that study where ten different non-destructive models were tested, the researchers stated that the R^2 prediction performance was between 0.73 and 0.91 and they recommended that the leaf length should be measured from the stem. Similarly, in our study, leaf length was measured from the stem. In addition, the performances obtained from the models had a high performance as in the literature.

R^2 values in the eggplant ranged from 0.99 to 0.94. Model 5 was the lowest performing model compared to the others with 0.94, while the other models showed very close results (Table 4). While the lowest RMSE value was in Model 1 (8.88), the highest RMSE value was in Model 5 (19.34). Similar to R^2, the performances of other models in RMSE value showed results close to Model 1. When the MBE criterion was examined, the highest performance belonged to Model 6 with -0.000002 while models 1, 4 and 7 had relatively worse performance. Rivera et al. (2007) analyzed six different models to estimate the LA value of the eggplant and determined that the R^2 values of these models were between 0.73 and 0.97 and the MSE values were between 725 and 88 cm^2. Hinnah et al. (2014) estimated the LA values of the eggplant with twelve different models and determined that the R^2 values of the models used were between 0.92 and 0.98, the RMSE values were between 33.2 and 77.8 cm^2, and the MAE values were between 23.36 and 68.59 cm^2. Therefore, in our study, it was determined that the performances of the models tried to estimate the LA value of the eggplant were similar to previous studies.

In the pepper plant, R^2 values of all models except Model 5 were determined as 0.99. Model 5, with a value of 0.94, was the model with the lowest performance compared to other models. When the RMSE performances of the models were evaluated, again Model 5 showed lower performance than other models. RMSE values of all models were close to each other. When the MBE values were examined, the highest performance was obtained in Model 6 (-0.000004), similar to other crops. d performance criteria were 1.00 for all models. Cemek et al. (2011) derived prediction models to determine LA of pepper plant under different salt and water stress. Researchers have stated that the models they developed to estimate the leaf area of green peppers grown under different stress conditions and levels could be used reliably. Aminifard et al. (2017) added leaf fresh and dry weight parameters to some of these models, unlike other studies in the literature, in order to estimate LA value. They reported that new parameters based on leaf weights did not give good results in predicting LA value. In our study, it was determined that the models used to predict LA values of pepper had high performance similar to the literature.

Since R^2 performance values of all models in the cucumber plant varied between 0.98 and 0.99, there

Table 4. The performance criteria of the models used to determine the leaf area of four different crops

Models	Crop	Performance criteria				
		R^2	RMSE	RE	MBE	d
1	Tomato	0.96	37.67	0.1194	0.0041	0.99
	Eggplant	0.99	8.88	0.0643	0.0048	1.00
	Pepper	0.99	1.07	0.0525	0.0000	1.00
	Cucumber	0.99	10.92	0.0570	-0.0027	1.00
2	Tomato	0.96	37.69	0.1194	0.0034	0.99
	Eggplant	0.99	9.53	0.0690	0.00002	1.00
	Pepper	0.99	1.13	0.0555	0.00001	1.00
	Cucumber	0.99	11.09	0.05791	0.00003	1.00
3	Tomato	0.96	38.61	0.1223	0.00006	0.99
	Eggplant	0.98	10.47	0.0758	0.00000	1.00
	Pepper	0.99	1.28	0.0626	0.00000	1.00
	Cucumber	0.99	11.11	0.0580	0.00001	1.00
4	Tomato	0.96	43.53	0.1379	-8.7819	0.98
	Eggplant	0.98	10.67	0.0773	1.0774	1.00
	Pepper	0.99	1.37	0.0669	0.2397	1.00
	Cucumber	0.99	11.21	0.0586	-0.5644	1.00
5	Tomato	0.95	41.83	0.1325	2.6158	0.99
	Eggplant	0.94	19.34	0.1401	0.0015	0.99
	Pepper	0.94	2.68	0.1309	0.0000	0.99
	Cucumber	0.98	13.53	0.0707	0.0149	0.99
6	Tomato	0.96	38.82	0.1230	0.0001	0.99
	Eggplant	0.98	11.17	0.0810	0.0000	1.00
	Pepper	0.99	1.22	0.0597	0.0000	1.00
	Cucumber	0.99	11.24	0.0587	0.0000	1.00
7	Tomato	0.96	43.71	0.1385	-8.9499	0.98
	Eggplant	0.98	11.36	0.0823	1.0953	1.00
	Pepper	0.99	1.31	0.0641	0.2364	1.00
	Cucumber	0.99	11.57	0.0604	-1.192	1.00

R^2: Determination coefficient; RMSE: Root mean square error; RE: Relative error; MBE: Mean bias error, d: Willmott index of agreement.

is no significant difference between the models. When the RMSE values were examined, the best performance was in Model 1 (10.92). The performances of all models except Model 5 (13.53) were found close to Model 1. Since the d values of the models vary between 0.99 and 1.00, there was no difference between the models according to this performance criterion. Blanco and Folegatti (2003) derived different LA prediction models in different salinity and grafting conditions of cucumber. Bozkurt and Keskin (2018) found a high and non-linear relationship between leaf area and leaf length and width of cucumber grown in greenhouse conditions. Researchers also derived special models for different deficit irrigation applications (I_{120}, I_{100}, I_{80}, I_{60}, I_{40}, and I_{20}) and reported that all estimation models provide highly accurate predictions. Cho et al. (2007), differently, models were developed using leaf length, leaf width, SPAD value and different combinations of these variables to predict cucumber leaf area. But, the researchers determined that when the SPAD value was used, the performance of the prediction models decreased.

When the general performances of the models developed for the crops grown in the study were examined, it was determined that all models were acceptable. On the other hand, it was determined that model number 5 had the lowest R^2 and d value and the highest RMSE, RE and MBE values in all varieties. For this reason, the Model 5 had the lowest performance among all models. Models 1 and 6 performed the highest performance compared to other models. In addition, the models and coefficients given in Tables 3 and 4 can be used safely for tomato, eggplant, pepper, and eggplant plants grown under greenhouse conditions.

4. Conclusion

In this experiment, firstly, seven different models were derived in order to determine the leaf area with a non-destructive method of four economically important horticulture greenhouse crops. Then, the performances of the derived models were examined. Performance analysis proved the accuracy of the models, and Model 1 ($LA=a+bL^2+cL^2/W^2+dW^2+eLW$) and Model 6 ($LA=a+bLW$) gave the best performance. However, since it is easier to use, model 6 was suggested to use for estimation of the LA value of tomatoes, eggplants, peppers, and cucumber grown under greenhouse conditions.

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RESEARCH PAPER

HORTICULTURAL STUDIES

Determination of Morphological and Physiological Changes of Ornamental Cabbage (*Brassica oleracea* var. *acephala*) Against Boron Toxicity in Phytoremediation

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Abstract

Boron toxicity in agricultural lands limits plant breeding as a plant nutrition problem. Some plants are able to tolerate high levels of heavy metals at potentially toxic doses, accumulate them in their bodies and remove them from the soil. In this study, it was aimed to determine the morphological and physiological responses of ornamental cabbage in phytoremediation against boron toxicity. This study was conducted under controlled greenhouse conditions, 4 different boron doses (0, 10, 25, and 50 mg kg^{-1} B) were applied to 2 different soil structures (acidic (S1) and alkaline (S2) soils). Toxicity symptoms were appeared at 50 mg kg^{-1} B. At alkaline soil, plant weights and visual properties of plants were found to be low in quality. Results show that as the boron dose increased, shoot-root fresh and dry weight, stomatal conductance, chlorophyll content, relative water content (RWC) were decreased. Boron accumulation in the shoot, root and whole plant was at 50 mg kg^{-1} B. As a result, it is understood that ornamental cabbage used for decontamination of boron element by phytoremediation method for the first time in this study. We suggest that it has a potential to as hyper-accumulator plant for the remediation of boron-contaminated soil.

1. Introduction

Environmental pollution by heavy metals is severely threatened human and animal health and agricultural production. Heavy metal pollution in the soil has risen as a result of the increase in industrial production, mining activities and pesticide and fertilization activities used in agriculture in recent years.

Boron, which may occur commonly in the soil and/or in groundwater, is the source of high boron concentration in the soil. The amount of boron in the soil may also increase due to fertilization, irrigation and mining site (Nable et al., 1997). Boron toxicity is common in agricultural lands where there are thermal power plants using lignite coal or compost fertilizers are used. In addition, B levels that may be toxic to plants can be found in salty and sodic soils (Gence, 2015).

Heavy metal contaminated soils can be remediated different remediation technologies such as soil replacement, soil isolation, vitrification, encapsulation and bioabsorption (Alaboudi et al., 2018). Phytoremediation techniques provide cleaning and stabilization of contaminated soils and groundwater through hyperaccumulator plants and trees (Paz-Alberto and Sigua, 2013). Hyperaccumulator plants are defined as plant species that can accumulate heavy metals (Ali et al., 2013; Khalid et al., 2017). The most well-known heavy metal collector hyperaccumulator species are belongs to Brassicaceae (Kumar et al., 1995; Gall and Rajakaruna, 2013). The important of *Brassica* species in phytoremediation is derived from extensive above ground biomass production and rapid growth (Mourato et al., 2015).

Boron is fundamental micronutrients for plants. Boron takes the form of boric acid by plants, mostly

through range between B levels that cause toxicity and deficiency in plants (Chapman et al., 1997; Goldberg, 1997; Yau and Ryan, 2008; Brdar-Jokanovic, 2020). Soil boron concentrations are proposed to scale 0.0–0.2 ppm as low, 0.21–0.60 ppm medium, 0.61–1.10 ppm high, 1.2–3.0 ppm very high, and >3.0 ppm as toxic (de Abreu et al., 2005; Brdar-Jokanovic, 2020). The most common boron toxicity visible symptom in plants is the existence of burns, which develop as chlorotic and/or necrotic spots, often found on the edges and tips of the mature leaves (Nable et al., 1997; Ramila et al., 2015; Garcia Sanchez et al., 2020). With boron toxicity, root growth is also often inhibited and roots are shortened (Schnurbusch et al., 2010).

Some edible plants under the *Brassica* genus are noted accumulate comparatively large amounts of toxic heavy metals (Mourato et al., 2015.) The aim of study is to evaluate the potential of ornamental cabbage to be hyperaccumulator plant to remediate boron contaminated soils and to be alternative for edible plants.

2. Material and Methods

2.1. Plant materials and treatments

This study was conducted in the greenhouse with automatic temperature and relative humidity control (at 13-17ºC temperature and 40-45% relative humidity) and laboratories of the Department of Horticulture in Ankara University and Soil, Fertilizer and Water Resources Central Research Institute in Ankara, Turkey. Two different soil structure (S1: acidic and S2: alkaline soils) and four different boron doses (0, 10, 25, and 50 mg kg^{-1} B) was used in this experiment. The pH degrees of studied soils were 7.49 and 5.87, respectively. Table 1 shows chemical and physical properties of studied soils. Ornamental cabbage seedlings with 3-4 true leaves were planted in pots 15×17×22 cm (a plant per pot) in October 18, 2017. The pots were irrigated at the level of field capacity with tap water. Boron was applied as boric acid (H_3BO_3, 17.5% B) with irrigation water (25 October). Plants were grown for eight weeks. In order to determine the changes in plants according to soil type and boron concentrations, every week three plants were harvested for observations and measurements. Experiments were carried out randomized plots with a factorial design with 3 replications for soil type and boron dose.

2.2. Determination of plant growth characteristics

2.2.1. Plant shoot and root fresh and dry weight measurements

The parts of the plants that are divided into two as root and shoot parts of each plant are weighed on a precision scale in grams on a weekly basis. After their fresh weights measured, the samples were dried in the oven set at 65ºC until they reach constant weight then their dry weight was measured.

2.3. Measurements of physiological properties

2.3.1. Chlorophyll content

Before harvest, the chlorophyll amounts of the plants were measured each week by using Minolta Chlorophyll Meter (SPAD-502). Chlorophyll measurements (SPAD values) were taken as three readings on the 5 leaves of each plant, based on the central part of the leaf.

2.3.2. Stomatal conductance (g_s)

Decagon SC-1 model porometer was used to determine stomatal conductance. It was determined by making measurements on the same leaf randomly determined each week before harvest between 13.00-14.00 p.m.

2.3.3. Relative water content (RWC)

Leaf samples taken before harvest were immediately weighed and their fresh weights (FW) were measured, the samples were kept in pure water for 4 hours and then their turgor weight (TW) was measured. Finally, the leaf samples were dried in an air circulation drying cabinet at 65°C for 24 hours and their dry weight (DW) was measures

Table 1. Chemical and physical properties of studied soils

Proporties	Acidic soil (S1)	Alkaline soil (S2)
Texture class	Clay (C)	Clay Loam (CL)
EC (dS m^{-1})	0.71	0.92
pH	5.87	7.49
$CaCO_3$ (%)	0.90	37.60
Available P (P_2O_5) (kg ha^{-1})	41.0	110.00
Available K (K_2O) (kg ha^{-1})	340.00	780.00
Organic Matter (%)	1.80	1.86
Available Fe (mg kg^{-1})	16.24	15.44
Available Cu (mg kg^{-1})	1.81	2.59
Available Zn (mg kg^{-1})	3.35	2.42
Available Mn (mg kg^{-1})	4.89	16.68
Available B (mg kg^{-1})	0.00	0.00

(Dhanda and Sethi, 1998). The relative water content of the leaves was calculated with the help of the equation below:

RWC (%) = [(FW-DW) / (TW-DW)] (×) 100

2.3.4. Boron analysis in plant

The shoots and roots of the plants to be sampled were washed first with tap water and then with pure water, then placed in a paper bag and dried until they reached a constant weight at 65°C. Plant shoots and roots were ground finely to pass through a 200 µm sieve for analysis. 0.25 g of shoots and root samples was first digested with nitric acid (HNO_3) in a microwave device, then these samples were transferred to a 50 mL Erlenmeyer flasks and completed with deionized water and filtered through the blue tape filter paper. Total Boron in the plant solution obtained by method of wet decomposition was determined in Shimadzu UV-160 Spectrophotometer according to the vanadomolybdophosphoric yellow color method (Kacar and İnal, 2008). The boron content of the sieves obtained according to the method of wet decomposition was determined in Varian 720-ES ICP-OES (Kacar and İnal, 2008).

2.3.5. Statistical analysis

All data was statistically analysed using the MSTAT-C. The significant differences were compared with LSD test at P< 0.05.

3. Results and Discussion

3.1. The effect of B on plant shoot-root fresh and dry weights

Plant species that form large biomass, grow rapidly, form a wide root system and can be cultivated and harvested easily are considered to be an ideal hyper-accumulator plant species (Paz-Alberto and Sigua, 2013). The biomass of the plant selected for phytoremediation is one of the factors to be considered during the removal of toxic heavy metals (Alaboudia et al., 2018).

Effects of soil type on the fresh and dry weights of shoots and roots under four different B doses are shown in Table 2. The effect of "soil type and B dose" treatments interaction was found statistically significant (P<0.05) in terms of shoot fresh and dry weights, while it was not significant for root fresh and dry weights (P>0.05). The highest shoot fresh weight in plant under boron stress was obtained 'S2×25 mg kg^{-1}B' (85.08±4.30 g $plant^{-1}$) in S2 soil, while the lowest shoot fresh weight was 'S1×50 mg kg^{-1} B' (47.00±5.00 g $plant^{-1}$) in S1 soil. The highest shoot dry weight was determined in the combination of 'S2×10 mg kg^{-1} B' (17.44±1.31 g $plant^{-1}$). The lowest shoot dry weight was found to be at a dose of 50 mg kg^{-1} B' in S1 soil, showing similarity with the lowest shoot fresh weight ('S1×50 mg kg^{-1} B', 8.30±1.13 g $plant^{-1}$) (Table 3).

The effect of B doses on both shoot fresh weight and shoot dry weight showed itself as of the 5th week and its effect increased as the stress period increased. Similar findings were obtained in both root fresh weight and root dry weight (Figure 1). It was also determined that the plants grown in S2 soil had lower root fresh and dry weight than the plants grown in S1 soil. In agreement with conducted study by Eraslan et al. (2007) toxicity symptoms were seen on the tips and edges of the old leaves of the plants in this study. It has been reported that the most severe boron toxicity symptoms occurred 50 mg kg^{-1} B in tomato and pepper plants (Eraslan

Table 2. ANOVA for shoot - root fresh and dry weights, stomatal conductance, chlorophyll content, relative water content

Source of variation	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Stomatal conductance	Chlorophyll (SPAD value)	Relative water content
Boron dose (BD)	**	**	**	**	**	**	**
Soil type (ST)	**	**	NS	NS	**	**	NS
BD x ST	*	*	NS	NS	**	NS	*
CV (%)	5.37	12.82	8.55	19.51	5.99	10.09	5.33

CV: Coefficient of variation; **: P<0.01 is significant at probability level; *: P<0.05 is significant at probability level; NS: Not significant

Table 3. The effect of 'Soil type × B dose' interaction on the fresh and dry weights of shoot, root fresh and dry weights, stomatal conductance, chlorophyll and relative water content

Soil type	Boron dose (mg kg^{-1})	Shoot fresh weight (g $plant^{-1}$)	Shoot dry weight (g $plant^{-1}$)	Root fresh weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Stomatal conductance (mmol $m^{-2}s^{-1}$)	Chlorophyll (SPAD value)	Relative water content (%)
S1	Control	77.17±5.01 c*	17.15±3.07 a	27.58±2.50	8.38±1.43	113.18±11.51 b	25.00±2.95	75.33± 3.30 a
	10	59.33±1.53 d	11.55±0.88 bc	25.62±1.01	4.81±1.16	107.22±5.33 bc	17.34±1.90	69.57±1.51 ac
	25	60.67±1.53 d	10.10±1.01 cd	19.17±2.02	4.76±1.00	94.02±2.00 de	15.10±3.00	72.66±5.51 ab
	50	47.00±5.00 e	8.30±1.13 d	13.00±1.00	2.99±0.92	84.00±8.00 e	15.07±0.90	60.08±4.00 e
S2	Control	91.00±6.00 a	17.61±1.86 a	26.30±2.46	7.66±0.86	203.23±7.01 a	30.55±2.06	75.48±1.39 a
	10	81.17±1.52 bc	17.44±1.31 a	24.33±1.53	5.21±1.00	119.08±4.00 b	24.37±1.52	67.01±4.00 bd
	25	85.08± 4.30 ab	14.20±2.03 b	20.33±2.52	4.25±0.67	107.24±8.60 bc	23.18±3.02	63.20±5.01 de
	50	59.00±2.00 d	9.01±1.00 cd	16.51±0.50	3.16±0.81	98.19±4.01 cd	24.04±1.00	65.18±2.12 ce
LSD (%5)		6.51	2.93	-	-	11.99	-	6.33

*: Means with the same letter within column are significantly different (P<0.05). S1: Acidic soil, S2: Alkaline soil

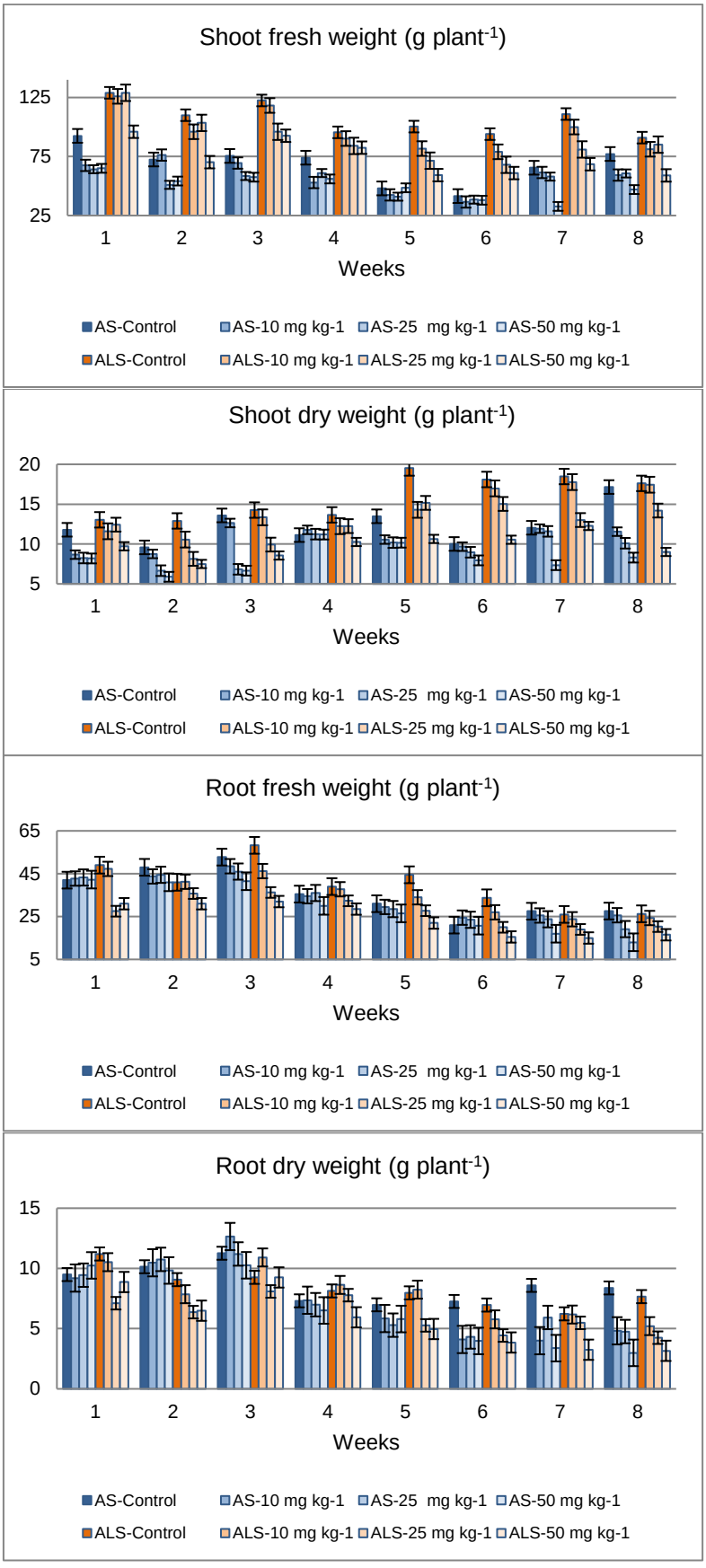

Figure 1. Weekly change of the effect of B applications on shoot-root fresh and dry weight, stomatal conductance, chlorophyll and RWC of ornamental cabbage in two different soil types. (AS: Acidic soil, ALS: Alkaline soil. $P<0.05$ is significant at probability level).

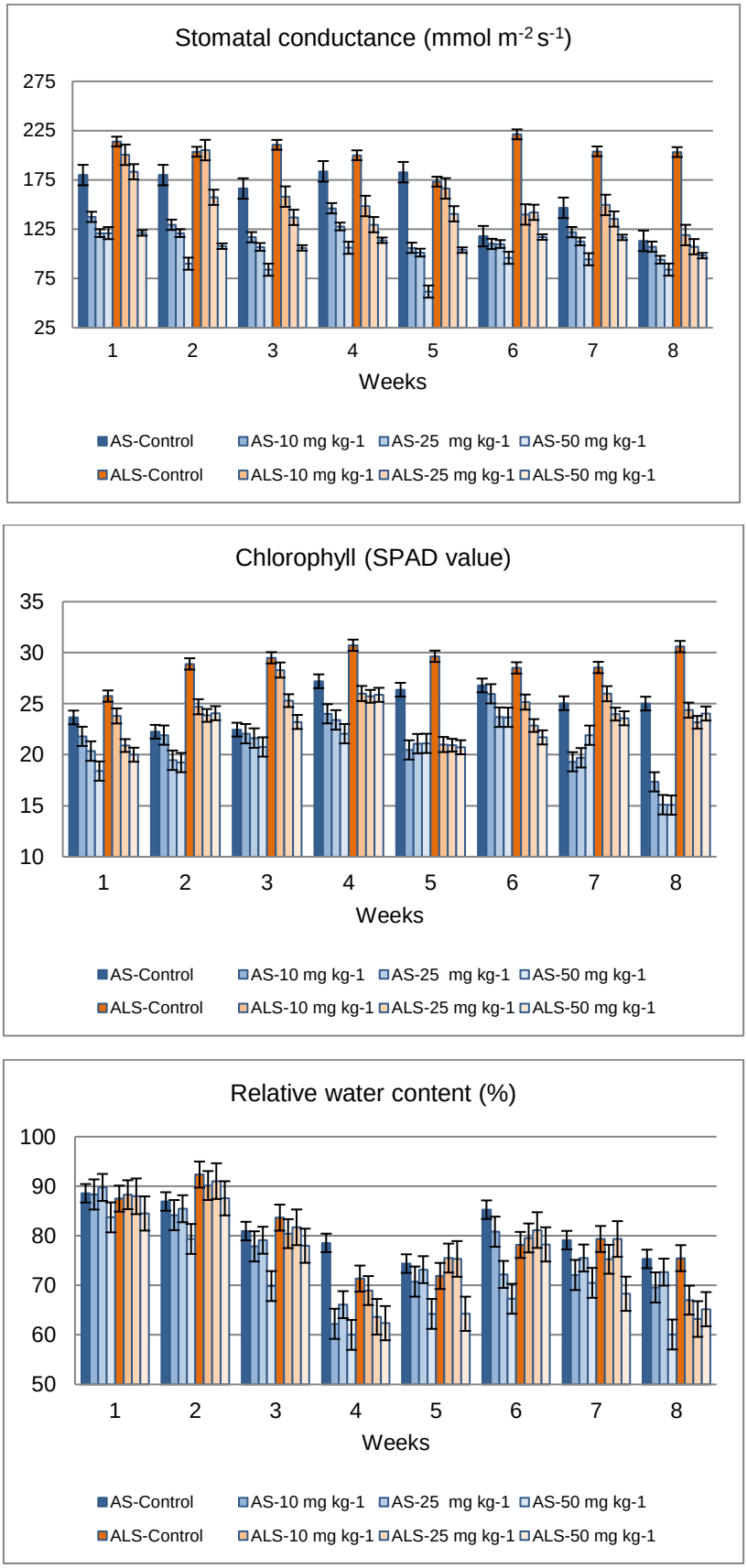

Figure 1 (continued). Weekly change of the effect of B applications on shoot-root fresh and dry weight, stomatal conductance, chlorophyll and RWC of ornamental cabbage in two different soil types. (AS: Acidic soil, ALS: Alkaline soil. P<0.05 is significant at probability level).

et al., 2007). The occurrence of boron toxicity symptoms at 25 mg kg^{-1} B doses in our study is consistent with the study conducted by Akıncı (2006).

In this study, shoot-root fresh and dry weight showed an alteration according to boron dose and soil type during boron stress and decreased compared to control plants. A similar result has been reported in corn (Güneş et al., 2000a), tomato (Güneş et al., 2000b), potato (Ayvaz, 2009), purslane plants (Samet and Çakılı, 2016; 2019) for shoot fresh weight, in pepper (Akıncı, 2006; Eraslan et al., 2007), corn (Palta and Gezgin, 2011), canola (Koohkan and Maftoun, 2016; purslane plants (Samet and Çakılı, 2016; 2019) and buckwheat plant (Yazıcı and Korkmaz, 2020) for shoot dry weight.

Since boron regulates lignin biosynthesis through the formation of stable phenolic acid borate compounds, it has been reported that it limits excess B lignin biosynthesis (Marschner, 1995). Boron toxicity also causes negative physiological effects such as decreased cell division, shoot and root growth (Nable et al., 1997; Liu and Yang, 2000; Reid et al., 2004).

It is reported in different studies in the literature that high levels of boron applications lead to a decrease in root weight. Ayvaz (2009) reported that there was a decrease in root fresh weight in parallel with increasing B doses on potato plant. Similar findings were also obtained in the root fresh and dry weights of barley varieties and barley grass (Keskin, 2010), *Vicia sativa* plant (Karaömerlioğlu, 2011), green bean genotypes (Akoğlu, 2013). Due to the application of B, toxic effects were observed on the leaves. This situation can be explained by the fact that the B element is carried upward due to transpiration and accumulates in the leaves and the boron accumulated in the leaves shows toxic symptoms at the leaf tips (Kacar and Katkat, 2007).

3.2. The effects of B on stomatal conductance, chlorophyll, and relative water content (RWC)

In our study, the interaction of 'soil type x B dose' was found to be important in terms of stomatal conductance and relative water content ($P<0.05$), while the interaction of 'soil type × B dose' was not statistically significant ($P>0.05$) in terms of chlorophyll content. The highest stomatal conductance was measured in leaves of plants grown in S2 soil. The combination of 'S2 × 10 mg kg^{-1} B' has been determined to have both the highest stomatal conductivity (119.08± 4.00 mmol m^{-2} s^{-1}). Especially, when the stomatal conductance of plants grown in S1 soil and S2 soil were compared, it was determined that plants growing in S1 soil had lower stomatal conductance than S2 soil. The RWC of the plants applied with B stress decreased compared to control in both soil types. The highest RWC was determined as 'S1×25 mg kg^{-1} B' (72.66% ± 5.51) (Table 3).

It was determined that B doses decreased stomatal conductivities in both soil types compared to the control starting from the first week. Especially as the dose B increased (25 and 50 mg kg^{-1} B) stomatal conductance decreased compared to the control. The decreases in chlorophyll values differed depending on the duration, severity and soil type of the stress. In the early weeks, losses in chlorophyll values were observed in both soil types with the increase in dose. These losses started to become evident in the following weeks and continued towards the last weeks. It was determined that the relative humidity contents of the plants were close to the control in the early weeks of the stress, and they showed a significant decrease in S1 soil compared to the control in parallel with the increase in the B dose at the 4^{th}, 5^{th} and 6^{th} weeks. In terms of relative moisture content, it was determined that ornamental cabbage plants under boron stress preserved their turgority better in S2 soil than S1 soil, and this was noticeable at 25 mg kg^{-1} B level at 5^{th}, 6^{th} and 7^{th} weeks. Towards the last weeks, the decrease in relative moisture content in plants, especially when 50 mg kg^{-1} dose B was applied, continued (Figure 1).

Stomatal conductance is determined by measuring the carbon dioxide entering or water vapour exiting from the stomata of the leaves per unit time (Erdal, 2016). Closure of the stomata occurs as a result of the direct interaction of toxic metals with guard cells and the initial effects of metal toxicity on the root and stem. Root-derived ABA or ABA-derived signals in metal-stressed plants may play a role in stomatal movement. As a result of the exposure of plants to toxic metal concentrations such as Cd, Co, Ni, Pb and Zn, it has been reported in many studies that stomatal conductivity decreases or causes stomatal resistance to increase (Rucinska-Sobkowiak, 2016). In a study on the response of Clementine mandarins inoculated on two different rootstocks to B toxicity, it was determined that B application reduces stomatal conductance (Papadakis et al., 2004a). In a study investigating the effect of boron toxicity on stomata behaviour in grapevine plants, it was found that stomata resistance was increased in the leaves of plants treated with excess B at 20 and 30 mg kg^{-1} B levels (Güneş et al., 2006). Simon et al. (2013) reported that stomatal conductivity decreases with increasing B concentrations in *Jatropha curcas* plant. When the studies conducted were examined, the results obtained that increasing B doses decrease stomatal conductance were found to be consistent with our findings that stomatal conductivity decreased with the prolongation of the stress period with increasing B doses. It was reported that B application reduced the chlorophyll content in Clementine mandarins (Papadakis et al., 2004a), mung bean (Hasnain et al., 2011), *Brassica juncea* (Varshney et al., 2015), canola (Koohkan and Maftoun, 2016), purslane plants (Samet and Çakılı, 2016).

Boron toxicity, which is one of the abiotic stress factors for plants, causes various morphological and physiological changes in plants. Under these conditions, it has been reported that the RWC of the leaves in the plants and decreases in the rate of photosynthesis occurs with the decrease of the leaf water potential (Lawlor, 2002; Akoğlu, 2013). Chlorosis formation and decreases in relative water content are among the known signs of boron toxicity (Ramila et al., 2016). In a study conducted by Keskin (2010), it was stated that the relative moisture content in the leaves of Tokak and Hamidiye variety wheat decreased with increasing B applications, and this decrease occurred in the highest dose of B, 500 mg kg^{-1} B application. Depending on the sampling time, differences in the relative water content values of the *Puccinellia distens* plant were revealed, while the relative water content values of the plant increased in the 30th day sampling, it was stated that the 60th day sampling decreased. In green bean genotypes, different concentrations of B were applied for 10 and 20 days and it was found that leaf proportional water content decreased in parallel with increasing B concentration. It has been determined by Akoğlu (2013) that in green bean genotypes, different concentrations of B were applied for 10 and 20 days, and that the leaf proportional water content values generally decreased in parallel with the increasing B concentration. Although there was no significant difference between the 2.0, 4.5, and 7.0 mg L^{-1} B applications, the relative water content was significantly reduced with B applications (Simon et al., 2013). In a study conducted on different boron doses in wild wheat (*Triticum boeticum* L.), it was reported that the relative water content of B application decreased with the increase in boron stress time and boron (500 mg kg^{-1} B) dose (Uygan, 2014).

3.3. Boron content

The interaction of 'Soil type × B dose' was statistically significant in differences between the amounts of B accumulated in the shoot, root, and plant (Table 4).

In both soil types, the highest amount of B accumulation in the shoot is seen at 50 mg kg^{-1} B. It was determined that the plants growing in S1 soil accumulated more B than S2 soil and it was also statistically significant. In both soil types, there is no statistical difference between the 25 mg kg^{-1} B dose in terms of boron accumulation in plants. There is a remarkable increase in the amount of boron in the shoot of boron applications compared to the control (Figure 2).

When B accumulation in the root was examined, the applied B doses showed a significant increase compared to the control. The highest B accumulation occurred at 50 mg kg^{-1} B as in the root. There is no statistical difference in the application of 50 mg kg^{-1} B dose in plants growing in S1 and S2 soil structure. Similarly, there is no statistical difference in the B accumulation in the

Table 4. ANOVA for boron content in shoot, root and whole plant

Source of variation	B in shoot	B in root	B in whole plant
B dose (BD)	**	NS	**
Soil type (ST)	**	**	**
BD x TT	**	**	**
CV (%)	3.51	5.24	3.65

CV: Coefficient of variation; **: P<0.01 is significant at probability level; *: P<0.05 is significant at probability level; NS: Not significant.

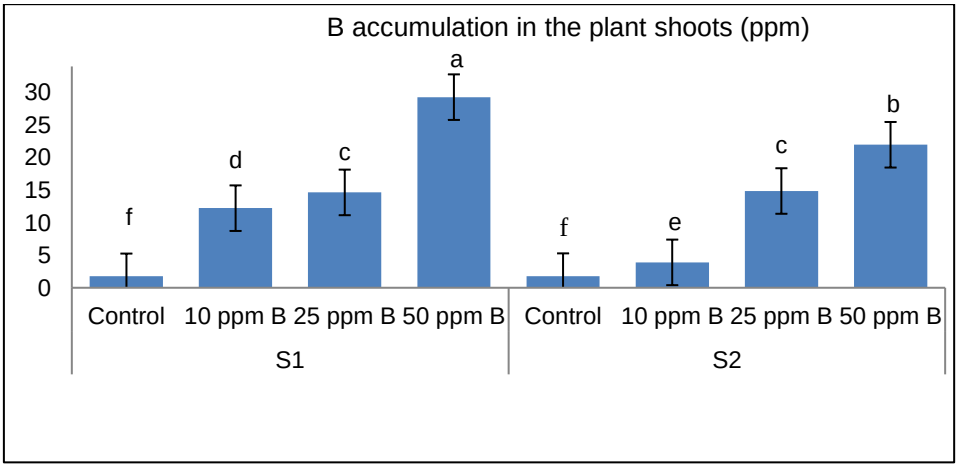

Figure 2. Boron accumulation in the plant shoot in two different soil types (S1: Acidic soil, S2: Alkaline soil. P<0.05 is significant at probability level).

root in the application of 10 mg kg^{-1} B dose in both soil structures. In the application of 25 mg kg^{-1} B dose, there was more B accumulation in S1 soil than in S2 soil and the difference between them was found statistically significant (Figure 3).

The soil type with the highest B accumulation in the whole plant in ornamental cabbage was S1 soil. The highest increase was 50 mg kg^{-1} B, consistent with B accumulation in the shoot and root. Boron accumulation in whole plant was followed by 25 mg kg^{-1} B and 10 mg kg^{-1} B, respectively. When the B doses of both soil types were examined separately, a statistical difference was found between them. It is seen that there is an increase in the boron doses applied to the plant compared to the control (Figure 4).

In terms of soil type, the highest boron accumulation occurred in S1 soil compared to S2 soil. B uptake in plants decreases in parallel with the increase in soil pH and excessive calcification. When the pH of the soil is 6.3-6.5, the B uptake is at the highest level. In addition, due to the ability of boron to be adsorbed by clay minerals, soils with high clay content accumulate more B than sandy soils (Kacar and Katkat, 2007). The pH of the S2 soil was 7.49 and the boron accumulation in the plant was lower than the S1 soil because it showed a calcareous soil feature. However, the fact that ornamental cabbage is a landscape plant and other morphological features were evaluated, the plants growing in S2 soil developed large corolla, making S2 soil stand out compared to S1 soil.

In the study on cotton varieties, it was reported by Harite (2008) that there are significant increases in shoot and root B contents with B applications compared to control. In another study by Keskin (2010), it was reported that the B accumulation in both the shoot and the root increased as the B dose increased. Examining the B accumulation capacities in *Medicago sativa* and *Vicia sativa* plants, it was notified that the roots, shoots and leaves of the plants showed resistance to the highest dose of 50 ppm and that B accumulation was the highest at this B dose (Karaömerlioğlu, 2011). In a study investigating the B tolerance of corn varieties, it was determined that the dose at

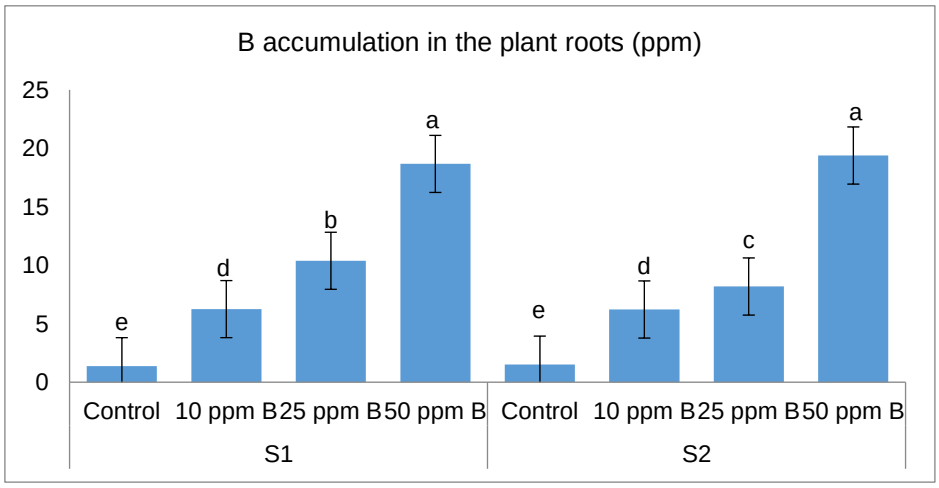

Figure 3. Boron accumulation in the plant root in two different soil types (S1: Acidic soil, S2: Alkaline soil. P<0.05 is significant at probability level).

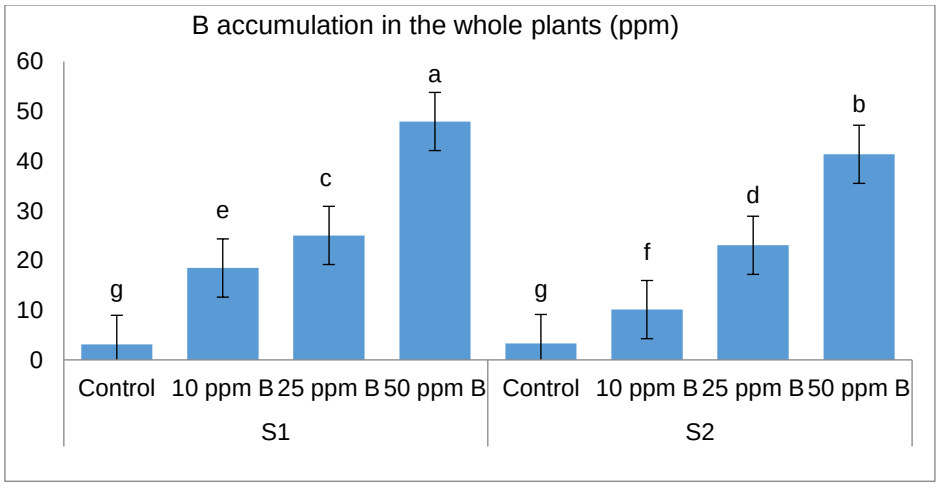

Figure 4. Boron accumulation in the whole plant in two different soil types (S1: Acidic soil, S2: Alkaline soil. P<0.05 is significant at probability level).

which boron toxicity symptoms occur was 40 mg kg^{-1} for all varieties and the highest B uptake occurred at this dose (Palta and Gezgin, 2011). Kookhan and Maftoun (2016) reported that 40 mg kg^{-1} B soil application in canola plant significantly increases the B concentration in shoots. In studies conducted in potatoes and green beans, B accumulation increased in parallel with the increase in B dose in both leaf and root (Akoğlu, 2013). Metwally et al. (2018) informed that canola plant shoots include a higher concentration of B than roots. The amount of boron removed by the shoot in the buckwheat plant increased with the increasing boron applications (Yazıcı and Korkmaz, 2020). Our results showed that boron was mostly accumulated in the shoot of plant, thus supporting previous studies.

4. Conclusion

This study was carried out to identify the ability of ornamental cabbage as hyper-accumulator plant potential and removal of boron from contaminated soil. The obtained results showed that ornamental cabbage has the ability to accumulate B in shoots and roots. Increasing B doses reduced stomatal conductance and relative water content and caused losses in chlorophyll content. As a result, it has been revealed that the ornamental cabbage plant is a potentially usable plant for the removal of boron from the soil by phytoremediation. The use of ornamental cabbage only for ornamental and landscaping purposes seems promising for this plant to come to the forefront in environmental remediation studies.

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RESEARCH PAPER

HORTICULTURAL STUDIES

Alteration of Antioxidant Activity and Total Phenolic Content during the Eight-Week Fermentation of Apple Cider Vinegar

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Abstract

Apple is one of the delicious fruit consumed by people. Apple cider vinegar was made through the traditional method and the changes occurred during the 8 week fermentation period were determined in this research. Total titratable acidity, pH, total soluble solids (°brix), total phenolic contents, Oxygen Radical Absorbance Capacity (ORAC) and Trolox Equivalent Antioxidant Capacity (TEAC) assays, phenolic contents were determined. Total phenolic substance, ORAC and TEAC values increased significantly weekly and reached the highest level in the 3rd week. Total phenolic substance, ORAC and TEAC values of 3rd week apple vinegar were determined as 1110.63 mg GAE L^{-1}, 10.92 mM and 21.11 µmol TE mL^{-1}, respectively. Apple vinegar samples had gallic acid, catechin, epicatechin, chlorogenic acid, and p-coumaric acid. The major phenolic substances in apple vinegar were gallic acid and chlorogenic acid. While gallic acid value of 3rd and 4th week apple vinegar were detected 11.91 and 23.69 mg L^{-1}, respectively; chlorogenic acid value of 4th and 5th week apple vinegar were found 46.36 and 49.71 mg L^{-1}. Antioxidant activity and phenolic substances values were not significant reduction during the acetic acid fermentation. In this study, the formation process of the functional and sensory properties of apple cider vinegar due to the change in the weekly antioxidant and bioactive component content of apple cider vinegar was emphasized.

1. Introduction

Apple is a fruit commonly consumed by humans. In addition to this consumption as fruit, apple can also be turned into different products (such as jam, puree, apple wine, vinegar). Polyphenolic ingredients in apple composition positively affect human health (Boyer, 2004; Francini and Sebastiani, 2013). There are over 8000 polyphenols which has known as antioxidants in nature. Polyphenols protect our body against damage caused by free radicals (Ganesan and Xu, 2017). Briefly, polyphenolics have been asserted to effective on human health (preventing chronic disease such as cancer, heart attack, hypertension, and diabetes) (Halliwell, 2007). Each of polyphenols may have private health impact (Manach et al., 2004). Apple vinegar comprises of polyphenols such as chlorogenic acid, gallic acid, catechin, epicatechin (Budak et al., 2011). Chlorogenic acid which is abundant in apples has been also indicated to inhibit DNA damage in vitro (Kasai et al., 2000) and displayed a preservative effect against cardiovascular diseases (Laranjinha et al., 1994). Budak et al. (2011) indicated that total phenolic content, chlorogenic acid, antioxidant (ORAC and TEAC) activities values of apple vinegar were the higher determined by surface (traditional) methods with maceration than submersion (industrial) methods with and without maceration. Besides,

different phenolic contents (gallic acid, epicatechin, chlorogenic acid etc.) were detected in apple cider vinegars while chlorogenic acid had been identified as the predominant phenolic content in apple vinegar samples (Budak et al., 2011).

Apple cider vinegar is one of the most commonly known in vinegar types. Although the first known usage of vinegar dates back to a century ago (Johnston and Gaas, 2006; Tan, 2005), vinegar has been widely used in food industry in recent 20 years. There are different kinds of vinegar which are balsamic, cane, champagne, cider, vinegar, distilled, malt, rice wine, sherry, wine (Tan, 2005). Vinegar has a double fermentation processes using different raw materials. These stages are ethanol and acetic acid fermentations. In addition, vinegar is produced by different production methods. While the fermentation in the traditional method (a surface-slow method) occurs on the surface of a barrel following wine or cider; the fermentation in the industrial method (a submersion (quick) method); consists a fermentator in the continuous oxygenation, optimum temperature (Tan, 2005). Acetic acid bacteria are responsible for vinegar production (Ley et al., 1984). Vinegar should contain at least 4% acetic acid (TSE, 2016). The final quality of vinegars depend on the selection of appropriate starter culture, starting material, the production method, maturation and aging (Mas et al., 2014).

The aromatic compounds, polyphenolic compounds and antioxidant activity of vinegar change during the vinegar formation process. Budak et al. (2014) reported that vinegar has high antioxidant and antibacterial activity. Vinegar has been found to be effective in cholesterol metabolism and reducing liver fat. Du et al. (2019) determined that apple pulp obtained by cold pressing technology has significant high antioxidant capacity and bioactive compounds. They reported that vinegar which has high bioactive content can be produced from this pulp. Chlorogenic acid, caffeic acid, phlorizin, gallic acid, coumaric acid, ferulic acid and vanilla acid detected 6.56, 3.03, 1.76, 0.35, 0.33, 0.24, 0.06 mg L^{-1} in apple cider vinegar, respectively (Du et al., 2019). In other study, antioxidant analyzes were performed on the filtered (FAV), clarified (CAV) and packaged (PAV) of apple vinegar samples in the industrial vinegar process (Bakir et al., 2016). They determined that total phenolic content of CAV, FAV, PAV had 383, 357, 459 mg GAE 100 mg^{-1}; TEAC value of CAV, FAV, PAV had 570, 587, 1256 mg TEAC 100 mL^{-1}, respectively. It was observed that apple vinegar contain gallic acid, syringic acid, caffeic acid, p-hydroxybenzoic acid, catechin, and p-coumaric acid (Bakir et al., 2016).

So far, we have not found any previously published studies on determining the weekly antioxidant activity and phenolic components of the apple cider vinegar process. In this study, weekly changes of antioxidant properties and bioactive substances were determined during the apple cider vinegar process.

2. Material and Methods

Apples were harvested in Isparta (in two different regions). Food Engineering laboratory in Suleyman Demirel University was used to convert apples into vinegar and analyzes. Figure 1 shows traditional vinegar production methods. During apple cider and vinegar formation, samples were taken weekly. Apple juice sample was coded as V0. Samples taken during ethanol fermentation and acetic acid fermentation at 1, 2, 3, 4, 5, 6, 7, and 8 weekly samples were coded as V1, V2, V3, V4, V5, V6, V7, and V8, respectively.

Total titratable acidity, pH, total soluble solid (°Brix) of samples were detected according to AOAC (1992) methods. Total titratable acidity of juice, cider, vinegar was expressed as malic acid, lactic acid, acetic acid, respectively. pH meter (WTW, Inolab, USA) and Abbe refractometer (Bellingham Stanley Limit 60/70 Refractometer, England) were used in pH and total soluble solids measurements. Ethanol content of apple cider samples were detected with alcoholometer (Dujardin-Salleron, France).

Folin-Ciocalteu method was used for determination of total phenolic content and "mg GAE L^{-1}" was used to express the values (Singleton et al., 1999).

The hydrophilic ORAC-Fluorescein method were used to detect the Oxygen Radical Absorbance Capacity (ORAC) (Davalos et al., 2005). ORAC values were kinetically calculated in BioTek Instruments (Winooski, Vermont, USA) and indicated as "µmol TE mL^{-1}".

Total antioxidant capacity was made according to the method determined by Seeram et al. (2005). "mM TE" was used in order to express the TEAC assay.

The identification and quantification of phenolic compounds in samples carried out a high-performance liquid chromatography (Shimadzu, Kyoto, Japan) according to Caponio et al. (1999). The system includes a pump (LC-10ADvp), autosampler (SIL-10AD vp), a DAD detector (λmax=278), system controller (an SCL-10Avp), degasser (DGU-14A), column oven (a CTO-10Avp), column (Inertsil ODS-3V C18) (GL Sciences Inc.). Standart chromatogram was shown Figure 2. Phenolic compounds were stated as "mg L^{-1}".

Yeasts were grown on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) at 25°C for 5 days. It was added lactic acid (0.14%) (Özdemir et al., 2015). Acetic acid bacteria were counted on Glucose Yeast Extract Agar (GYC, Merck, Darmstadt, Germany) with cycloheximide (100 ppm) at 30°C for 5-7 days (Yetiman, 2012).

Vinegar productions was done in duplicate and two in parallel and all experiments were repeated

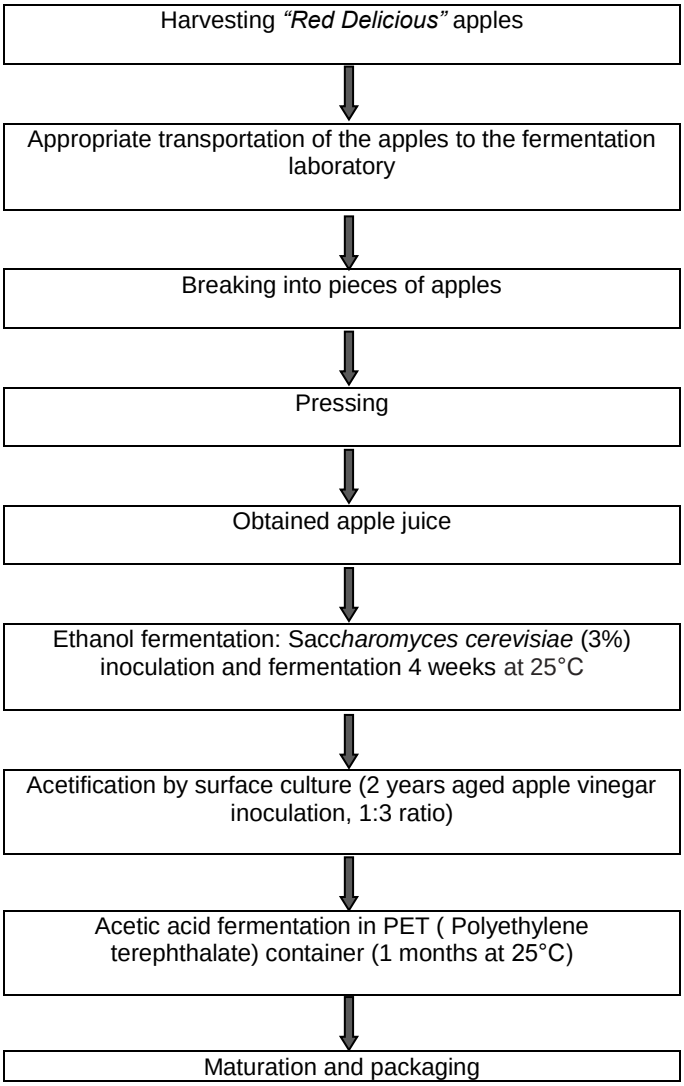


Figure 1. Flow chart of apple cider vinegar

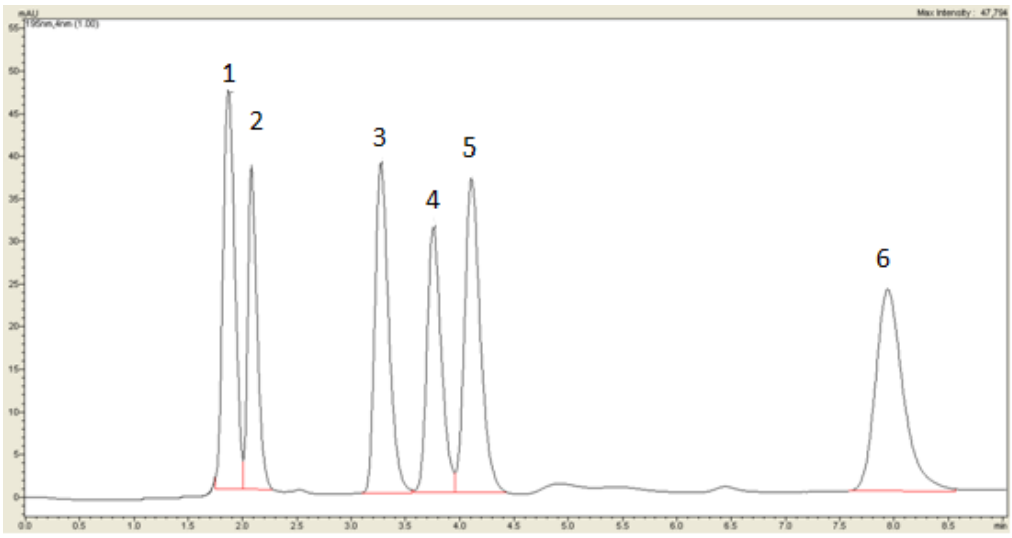


Figure 2. A chromatogram of standard (1: gallic acid, 2: chlorogenic acid, 3: catechin, 4: caffeic acid, 5: epicatechin, and 6: rutin)

three times. A one-way analysis of variance (ANOVA) was applied using SPSS 18.0 (SPSS, 2010). The mean ± SEM was used to express the results.

3. Results and Discussion

pH values, total acidity (%) and total soluble solids (°Brix) of weekly samples were shown in Table 1. While pH had steadily decreased, total acidity had gradually increased during fermentation. pH, total acidity, brix values have significantly changed in the first week. Chemical transformation in yeast fermentation has also significantly affected these values (P<0.05). pH changes in the 1st, 2nd, 3rd in weeks were not found to be statistically significant (P>0.05). Besides, after the 3rd week, the pH tended to decrease significantly (P<0.05) and the pH was observed at close values until the 8th week (P>0.05). In the 8th week, the pH value showed a decrease and the pH value of V8 had 3.38 and it was observed that the vinegar formation was completed. Total acidity of 2nd, 3rd, 4th weeks had increased (P>0.05) but total acidity of 7th and 8th weeks had significantly increased (P<0.05). While the total acidity values of the wine and the juice were close to each other, the value of the vinegar was higher than them (P<0.05). It was considered that the increase of the total acidity value in these stage of fermentation, might be caused by the production of mainly acetic acid and other organic acids, also the stability of the pH value might be caused by a weak acid property of the organic acid. Because, while the pH value was expressed as a negative logarithm of the concentration of dissociated hydrogenions, titratable acidity deals with measurement of the total acid concentration contained within a food, regardless of the effectiveness of the acid, that is, whether it is weak or strong. While the pH values of the wine and the juice were close to each other, the value of the vinegar was lower than them (P<0.05). This situation has also observed in vinegar productions using different fruits (Sadler and Murphy, 2010). Budak (2010) reported that total acidity of apple vinegar samples was 57.2 g L^{-1}. Moreover, total soluble solid of apple juice was 14 (°Brix), this value decreased step by step in the ethanol fermentation (P<0.05). Especially, there was a significant decrease in brix value in the first week (P<0.05). Because, sugar has turned into ethanol by alcohol fermentation (Treck and Teuber, 2002). Total soluble solid of V8 had shown 2.15 °Brix in the end of fermentation. Alcohol value reached its highest value at 3rd week, and this value remained the same in the 4th week. Acetic acid fermentation was started in the 4th week. That's why alcohol value decreased with the initiation of acetic acid fermentation (P<0.05). Since, acetic acid bacteria operates under oxygen, alcohol, suitable temperature conditions for acetic acid fermentation (Guillamon and Mas, 2011). Yeast counted during ethanol fermentation. Acetic acid bacteria counted during acetic acid fermentation (Table 1). In the post-inoculation yeast count was the highest observed at the end of the 1st week. Yeast count and decreased in sugar consumption is balanced with each other. The yeast value entered the stationary phase in the 3rd and 4th weeks. Finally, yeast entered the death phase at 5th week and counting could not be made. Acetic acid bacteria count was determined between 4.55 and 5.80 log kob mL^{-1}.

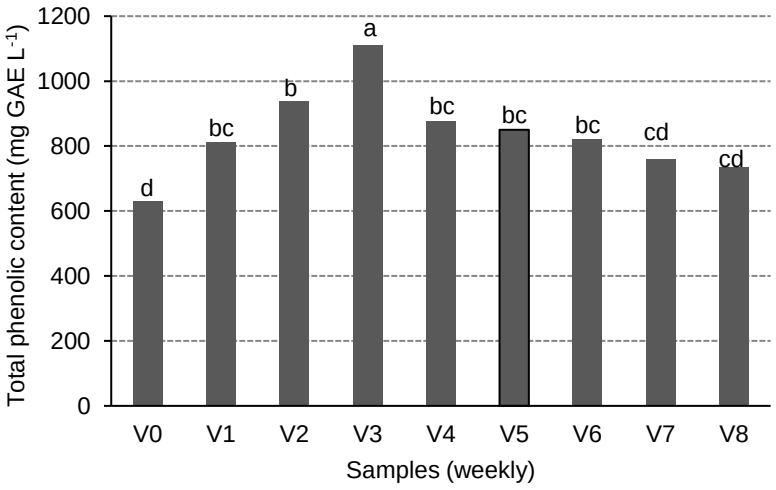
Total phenolic contents (TPC) of samples were presented in Figure 3. Total phenolic substance value increased significantly weekly (P<0.05) and reached the highest level in the 3rd week. Total phenolic substance was 1110.63 mg GAE L^{-1} in V3

Table 1. Chemical properties and phenolic compounds of samples (8 weeks)

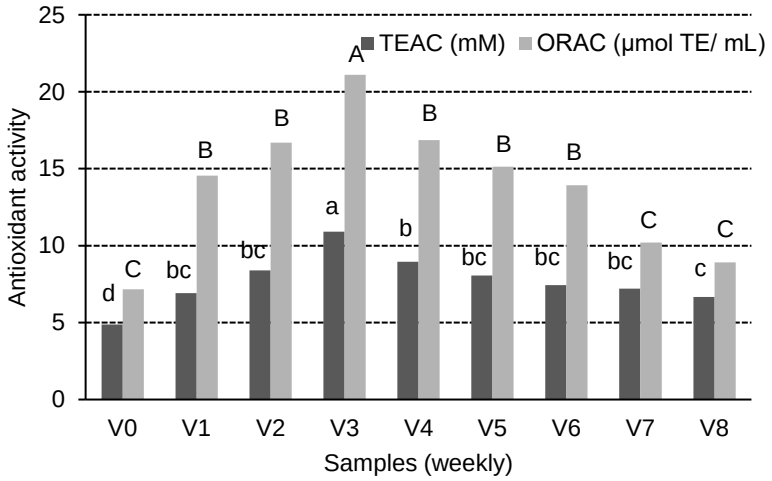
S	pH	TA (%)	TSS	A	Y	AAB	GA	CA	C	E	p-CA
V0	4.38 ±0.04^{a}	1.64 ±0.09^{c}	14.00 ±0.46^{a}		5.56 ±0.08^{b}		7.94 ±1.17^{b}	12.16 ±1.41^{b}	1.24 ±0.33^{a}	0.41 ±0.05^{b}	0.06 ±0.01^{b}
V1	3.91 ±0.08^{b}	2.32 ±0.01^{bc}	6.25 ±0.89^{b}	7.30 ±0.24^{b}	7.14 ±0.09^{a}		9.33 ±1.02^{b}	33.52 ±0.51^{b}	1.69 ±0.11^{a}	1.28 ±0.10^{b}	0.07 ±0.01^{b}
V2	3.90 ±0.07^{b}	2.31 ±0.02^{bc}	4.75 ±0.26^{bc}	8.35 ±0.14^{a}	5.81 ±0.10^{b}		10.36 ±0.76^{b}	35.38 ±0.91^{ab}	2.05 ±0.16^{a}	3.24 ±0.08^{a}	0.12 ±0.01^{ab}
V3	3.67 ±0.09^{bc}	2.33 ±0.01^{bc}	3.45 ±0.20^{cd}	8.59 ±0.15^{a}	4.38 ±0.20^{b}		11.91 ±2.20^{b}	41.05 ±1.05^{a}	2.09 ±0.49^{a}	3.65 ±0.20^{a}	0.16 ±0.04^{ab}
V4	3.53 ±0.05^{cd}	2.23 ±0.03^{bc}	3.60 ±0.12^{cd}	8.55 ±0.15^{a}	4.18 ±0.11^{b}	5.24 ±0.02^{a}	23.69 ±1.35^{a}	46.36 ±2.78^{a}	1.92 ±0.15^{a}	3.55 ±0.18^{a}	0.19 ±0.01^{a}
V5	3.43 ±0.04^{cd}	3.47 ±0.11^{b}	3.25 ±0.43^{cd}	3.45 ±0.16^{c}		5.56 ±0.09^{a}	25.58 ±1.48^{a}	49.71 ±2.93^{a}	1.75 ±0.42^{a}	2.67 ±0.14^{a}	0.17 ±0.03^{ab}
V6	3.47 ±0.05^{cd}	3.87 ±0.03^{b}	2.95 ±0.32^{d}			5.80 ±0.03^{a}	27.22 ±0.99^{a}	45.64 ±1.56^{a}	1.50 ±0.48^{a}	2.79 ±0.31^{a}	0.13 ±0.02^{ab}
V7	3.51 ±0.04^{cd}	4.54 ±0.07^{a}	2.35 ±0.08^{d}			5.10 ±0.05^{a}	24.83 ±2.93^{a}	43.45 ±2.01^{a}	1.59 ±0.42^{a}	2.80 ±0.27^{a}	0.11 ±0.04^{ab}
V8	3.38 ±0.05^{d}	5.19 ±0.09^{a}	2.15 ±0.08^{d}			4.55 ±0,03^{a}	25.68 ±3.39^{a}	45.07 ±2.48^{a}	1.54 ±0.47^{a}	2.66 ±0.48^{a}	0.08 ±0.01^{ab}

S:Samples, TA: Total acidity (%), TSS:Total soluble solids (°Brix), A: Alcohol, Y: Yeast (log kob mL^{-1}), AAB: Acetic acid bacteria (log kob mL^{-1}), GA: Gallic acid (mg L^{-1}), CA:Chlorogenic acid (mg L^{-1}), C: Catechin (mg L^{-1}), E: Epicatechin (mg L^{-1}), p-CA: p-Coumaric acid (mg L^{-1})

Data expressed as mean ± standard error (SEM). a, b, c, d: There is a statistically significant difference between groups in the same column without common letters (P<0.05).



a



b

Figure 3. Total phenolic contents (a) and antioxidant activity (b) of samples (Data expressed as mean ± standard error (SEM). a, b, c, d: There is a statistically significant difference between samples without common letters (P<0.05). Capital letters and lower case letters are evaluated among themselves (P<0.05).

sample. TPC of the V4 sample decreased to 876.25 mg GAE L^{-1} with the start of acetic acid fermentation (P<0.05). After the 4th week, TPC values started to decrease, but no significant decreases were observed (P>0.05). TPC values of V5, V6, V7, and V8 had 850.15, 820.03, 758.56, 734.55 mg GAE L^{-1}, respectively. The weekly analyzed results show that the total phenolic matter values increased during ethanol fermentation (P<0.05) and did not change throughout acetic acid fermentation (P>0.05). This increase could be associated with the release of the phenolic acids bound to sugar or/and organic acid molecules in the juice in the alcohol fermentation (Crozier et al., 2009).

In the literature research conducted so far, although there is no weekly follow-up in apple vinegar process, total phenolic substance results in apple juice, wine and vinegar samples have been determined. It was indicated that the total phenolic content were 3392 (Rababah et al., 2005), 2110-3470 (Wu et al., 2004), 1100-3570 (Podsedek et al., 2000; Liu et al., 2001), 977 mg GAE L^{-1} (Wolfe et al., 2003) in apple juice samples; 730-1343 mg GAE L^{-1} in apple cider produced with different techniques (Budak et al., 2015); 908, 568, 757, 416 mg GAE L^{-1} of apple vinegars produced with different techniques (Budak et al., 2011), 33-284 mg GAE L^{-1} apple vinegars (Du et al., 2019), 357-459 mg GAE L^{-1} (Bakir et al., 2016), 43-495 mg GAE L^{-1} in eleven apple vinegars purchased from local markets in China (Liu et al., 2019).

The differences in the total phenolic content of apple juice can vary according to apple varieties and growing conditions, and this change is reflected in the products produced from apple (apple wine, apple cider vinegar). TEAC ($ABTS^{\cdot}$) and ORAC values of apple samples were presented in Figure 3. ORAC and TEAC values were similar tendency in weekly measurements of apple samples. ORAC and TEAC values increased weekly until the 3rd week (P<0.05). ORAC and TEAC values reached the highest antioxidant value in the 3rd week.

TEAC and ORAC value of V3 (apple cider) had 10.92 mM and 21.11 µmol TE mL^{-1}, respectively. While the decrease in the ORAC value in acetic acid fermentation was significant ($P<0.05$), the decrease in the TEAC value was not significant ($P>0.05$). It has been stated that yeast use and fermentation conditions affect phenolic compounds during ethanol fermentation (Brandolini et al., 2007). Because, phenolic compounds related to sugar are released when yeast uses sugar, and antioxidant activity increases during fermentation (Crozier et al., 2009). Ubeda et al. (2011) reported that ORAC value of balsamic vinegar, apple vinegar, sherry vinegar, persimmon vinegar, red wine vinegar had 40049, 8986, 7879, 1857, and 1462 µmol TE kg^{-1}, respectively. Budak et al. (2011) determined that ORAC values between 3.00 and 5.89 µmol mL^{-1} in apple vinegar samples, while TEAC values between 5.4 and 13.5 mmol L^{-1}. In our study, ORAC values of apple vinegar (V8) had 8.90 µmol TE mL^{-1}.

Gallic acid, catechin, epicatechin, chlorogenic acid, and p-coumaric acid were detected in all samples (Table 1). Contents of catechin, epicatechin and p-coumaric acid were lower than gallic acid and chlorogenic acid content in all samples. Gallic acid content of samples increased weekly until the 4rd week ($P>0.05$). Gallic acid value of V3 and V4 had 11.91 and 23.69 mg L^{-1}, respectively ($P<0.05$). This increases could be associated with the release of the phenolic acids bound to sugar or/and organic acid molecules in the juice, in the alcoholic mediums (Crozier et al., 2009). Differences in gallic acid value were not significant in acetic acid fermentation ($P>0.05$). Chlorogenic acid was the dominant phenolic substance in apple cider and apple cider vinegar samples; especially, V4 and V5 samples had the highest content of chlorogenic acid. Budak et al. (2011) reported that chlorogenic acid of apple cider vinegar sample had 18.67 mg L^{-1} and chlorogenic acid was the dominant phenolic substance in apple vinegar. Catechin, epicatechin, p-coumaric acid content of V8 had 1.54, 2.66 and 0.08 mg L^{-1}, respectively. It has been shown that epicatechin significantly changed 2nd week while coumaric acid significantly changed 4th week ($P<0.05$). The leading polyphenols in apple cider vinegar were chlorogenic acid, caffeic acid, phlorizin, vanilla acid, gallic acid, coumaric acid and ferulic acid (Du et al., 2019). Phenolic compounds in apples changes induced by apple cultivar, breeding approaches, fruit postharvest and transformation into juice. Total and individual polyphenols in apple cultivars and cultivation may have been shown to vary (Volz and McGhie, 2011). As a result of weekly analyzes, we was observed that gallic acid and chlorogenic acid content was the dominant phenolic component in apple cider vinegar.

4. **Conclusion**

This study is the first detailed report determining the weekly change in antioxidant properties and bioactive substances during fermentation of apple cider vinegar. These values reached the highest value as a result of ethanol fermentation and no significant change was observed during acetic acid fermentation. Significant biochemical changes were observed especially until the 4th week of fermentation. As a result, it was observed that the antioxidant and phenolic component values increased with the release of phenolic compounds bound to sugar as a result of using the sugar in the fruit by yeast. It is important for human health to increase the usage area and consumability of apple cider vinegar and to benefit from its functional properties. In addition, being preferred for its sensory properties, apple wine and vinegar is one of the important functional products for health. Determining the weekly change of apple cider vinegar made from apple fruit in terms of antioxidant and phenolic components (especially gallic acid and chlorogenic acid) is important in terms of detecting the change in fermentation steps. This study will shed light on the emergence of new studies especially in fermentation stages.

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RESEARCH PAPER

HORTICULTURAL STUDIES

Effects of Different Pollinators on Fruit Set and Quality Attributes of Texas Almond (*Prunus dulcis* L.) Cultivar

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Abstract

There is a positive relationship between pollination and fruit yield of almonds. Since self-incompatibility is a major problem in almond cultivars, foreign pollinators are generally used in almonds to get high yield and quality. This study was conducted to investigate the effects of different pollinators (inbreed hybrid combinations) on fruit set and pomological characteristics of Texas almond cultivar in 2018 - 2019 growing seasons. Fruit set ratios varied between 3.6% (Texas × A3) and 5.2% (Texas × A4) in the first year of the study and between 8.0% (Texas × A3) and 13.2% (Texas × A2) in the second year. As the average of two years, the lowest fruit length (32.03 ± 2.45 mm) was observed in Texas × A4 combination and the greatest fruit length (35.31 ± 3.05 mm) was observed in Texas × A1 combination, whereas fruit width values varied between 19.37 ± 2.43 mm and 23.79 ± 1.33 mm. Although pollinators influenced fruit mass values, the greatest value (5.73 ± 0.74 g) was observed in Texas × A3 combination. It was concluded based on present findings that use of A2 cultivar as a pollinator in almond orchards established with Texas cultivar may have positive effects on fruit set.

1. Introduction

Turkey with diverse ecological conditions harbors several plant and fruit species (Ercisli, 2004). With such a great genetic diversity, Turkey is placed among precious countries and motherland of almond, apricot, plum, hazelnut-like important fruit species.

Almond (*Prunus dulcis* L.) with quite high nutritional values and various benefits on human health is grown in various parts of the world and in areas where there is no late spring frost in Turkey (Simsek, 2011). It either naturally grows or commercially grown in orchards as culture almond. There are several problems experienced in almond orchards of Turkey and such problems are mostly related to fruit set and yields (Boyaci and Çaglar, 2009). Flower and small-fruit drops are also the common problems of almond orchards.

Successful pollination is the first condition to be set for desired yield levels in fruit trees (Ortega et al., 2004). Receptivity of stigma, survival of ovule and perceiving time for pollen on ovary are the key parameters of a successful pollination and fertilization signaling. Pollens land on a receptive stigma, germinated pollens then generate a pollen tube and ultimately reach to surviving ovary to execute fertilization. Since the seeds are consumed in stone-fruits, all the bloomed flowers should turn into a fruit (100% fruit set) for maximum yield (Boyaci and Çaglar, 2009). Growing and maintenance practices, ecological conditions, type of pollinator and cultivar genetics can directly affect the growth and yield of almond (Brittain et al., 2014; Klein et al., 2015). Self-incompatibility is the common case in majority of almond cultivars. Self-incompatibility is mostly related to S-allele status of the cultivar. Therefore, for optimum yields, S-allele

status of relevant cultivar should be known and the cultivars with different S-allele profiles should be selected in breeding programs. Besides yield, S-allele profiles also generate genetic diversity in almond breeding programs (Gómez et al., 2019). On the other hand, use of different pollinators influence fruit mass, shape and size. Previous researchers investigated the effects of different pollinators on fruit quality attributes of almonds (Dicanta et al., 2000), apples (Akkurt et al., 2019) and cherries (Cirtlik and Beyhan, 2012).

Besides pollinator cultivars, ecological conditions and cultural practices also significantly influence fruit set and yield in almonds. Since different ecological conditions are dominant in every region, even in different parts of the same region, almond cultivars do not exhibit the same yield and quality in every region (Kuden and Kuden, 2000; Yildiz and Perdahci, 2019).

In this study, effects of different hybrid combinations obtained through inbreed hybridizations of Texas almond cultivar on fruit set and some pomological characteristics of almonds were investigated.

2. Material and Method

2.1. Material

Experiments were conducted in collection orchards of Alata Horticultural Research Institute in the years 2018-2019. Texas almond cultivar was used as the parent material. The almond types encoded as A1, A2, A3 and A4 developed and registered at Alata Horticultural Research Institute were used. The flowering times of the mother and father parents partially match. The collection orchard (36°37'12"N, 34°19'40"E) where the experiments were conducted was established in 1993. The orchard has different almond cultivars and there are 3 trees from each cultivar. Orchard soils have silty-loam texture. Trees are about 25 years old. Present almond cultivars are early flowering ones as compared to the other cultivars and mostly used as unripe almond. Meteorological data for flowering, pollination and fertilization periods are provided in Table1.

2.2. Method

Pollens of selected cultivars were gathered from non-burst flower buds at balloon stage. Pollens were rubbed onto emasculated flowers with the aid of a watercolor paint brush and hybridization procedures were performed accordingly. Hybridization was performed on 250 flowers for each hybridization combination. Routine cultural practices (irrigation, soil tillage, pruning) were performed accordingly. At the end of dormancy, 3 kg of 15-15-15 N-P-K fertilizer per tree were applied. Fruits were harvested at harvest maturity and harvested number of fruits were used to get fruit set ratios for different hybrid combinations. Hybridizations were performed on 20.02.2018 and 23.02.2019; harvests were practiced on 10.07.2018 and 13.07.2019. Pomological characteristics including fruit mass, width and length were determined (Gulcan, 1985; Hanine et al., 2016). Measurements were performed on 10 fruits of each hybrid combination. Experimental data were subjected to statistical analyses using SPSS software and significant means were compared with Duncan's multiple range test at $P<0.05$ significance level.

3. Results and Discussion

3.1. Fruit set ratios

Fruit set ratios of the years 2018 and 2019 varied with the pollinators. Fruit set ratios varied between 3.6–5.2% in 2018. Based on pollinators, fruit set ratio was 4.2% in Texas × A1 combination, 4.4% in Texas × A2 combination, 3.6% in Texas × A3 combination and 5.2% in Texas × A4 combination (Table 2).

Table 1. Meteorological data for experimental years

Years		Wind speed (m^{-s})		Precipitation (mm)		Max. temperature (°C)		Min. temperature (°C)	
		February	March	February	March	February	March	February	March
2018	Max.	1.9	4.0	19.00	11.80	21.3	27.3	10.5	15.5
	Min.	0.5	0.8	0.80	0.20	16.1	18.2	4.0	6.7
	Mean	1.0	1.5	1.57	0.44	18.2	21.0	7.3	9.9
2019	Max.	2.6	1.7	146.80	73.0	19.4	22.7	9.9	11.3
	Min.	0.8	0.7	0.20	0.20	11.5	15.2	2.3	0.9
	Mean	1.4	1.3	5.20	2.30	16.4	18.4	6.1	6.9

Table 2. Fruit set ratios of 2018

Combination	Number of pollinated flowers	Number of fruit sets	Fruit set ratio (%)
Texas × A1	250	10	4.0
Texas × A2	250	11	4.4
Texas × A3	250	9	3.6
Texas × A4	250	13	5.2

In 2019, fruit set ratios and number of fruit sets were greater than the year 2018. The lowest fruit set ratio (8.0%) was observed in Texas × A3 combination and the greatest fruit set ratio (13.2%) was observed in Texas × A2 combination (Table 3). Differences in fruit set ratios of the experimental years were mostly attributed to climate parameters. Previous researchers indicated the effects of ecological conditions on fruit set in almond orchards (Kuden and Kuden, 2000; Yildiz and Perdahci, 2019). Present findings well comply with the results of earlier studies.

Pollinators influence fruit set of almonds. Despite the use of a foreign pollinator, fruit sets may vary based on fruit loads and nutritional status of almond trees (Cunningham et al., 2019). Such difference can be noticed in subsequent years of heavy fruit-load years (Tombesi et al., 2017). Medium fruit set ratios were observed in almond breeding programs conducted with different almond cultivars (Acar et al., 2014).

Positive impacts of pollinator cultivars on fruit sets were reported for different species. In a previous study conducted to investigate the effects of different pollinators on fruit set and quality attributes of Canino apricot cultivar, fruit set ratios varied between 18.04-21.70% based on pollinator cultivars (Taha and Sheriff, 2015). Another study conducted with persimmons, “Risoli” and “Moro” pollinators had greater effects on fruit set than open-pollinating persimmons (Yildiz and Kaplankiran, 2013). Present findings show different with the results of earlier studies reporting varying fruit set ratios with the pollinator cultivars and years. The effect of climate and the use of different genotypes can be shown as the reason for this difference.

3.2. Pomological characteristics

During 2018, pomological characteristics such as fruit length and width values of hybrid combinations were found to be significant (Table 3). Fruit lengths of hybrid combinations varied between 32.26±1.71 mm and 33.91±2.37 mm. Fruit length of open-pollinating Texas cultivar was measured as 30.22±1.66. The lowest fruit width (21.85±2.75 mm) was observed Texas × A2 combination and the greatest fruit width (23.79±1.33 mm) was observed in Texas × A4 combination. Fruit mass values also significantly varied with the father parent. While the Texas × A2 combination had the lowest fruit mass with 2.99±0.38 g, Texas × A4 combination had the greatest fruit mass with 3.60±0.42 g. Regarding fruit mass values, all combinations had greater values that open pollination (Table 4).

In 2019, significant differences were observed in fruit length, width and mass values of the hybrid combinations. The lowest fruit length (28.26±2.48 mm) was observed in Texas × A3 combination. The lowest fruit width (19.37±2.43 mm) was observed again in Texas × A3 combination. However, despite the lowest fruit length and width, Texas × A3 combination had the greatest fruit mass with 5.73±0.74 g (Table 5). As compared to open-pollinating Texas cultivar, different pollinators had significant positive effects on fruit length and weight, but insignificant effects on fruit width. Different effects of the same pollinators in different years can be attributed to differences in climate parameters.

Sumbul and Bayazit (2019) reported fruit lengths of different almond cultivars as between 12.81-19.35 mm, fruit widths as between 15.43-27.12 mm and fruit mass values as between 1.55-6.34 g. In

Table 3. Fruit set ratios of 2018

Combination	Number of pollinated flowers	Number of fruit sets	Fruit set ratio (%)
Texas × A1	250	21	8.4
Texas × A2	250	33	13.2
Texas × A3	250	20	8.0
Texas × A4	250	22	8.8

Table 4. Pomological characteristics of hybrid combinations in 2018

Combination	Fruit length (mm)	Fruit width (mm)	Fruit mass (g)
Texas	30.22±1.66 b	24.51±2.83 a	2.94±0.33 c
Texas × A1	32.26±1.71 a	22.85±1.37 ba	3.34±0.33 ba
Texas × A2	32.58±1.71 a	21.85±2.75 b	2.95±0.38 bc
Texas × A3	33.91±2.37 a	23.56±2.38 ba	3.11±0.50 bc
Texas × A4	33.16±2.05 a	23.79±1.33 ba	3.60±0.41 a
Mean	32.45±2.21	23.31±2.32	3.20±0.45

Table 5. Pomological characteristics of hybrid combinations in 2019

Combination	Fruit length (mm)	Fruit width (mm)	Fruit mass (g)
Texas	32.38±1.84 b	22.12±1.68 a	3.66±0.71c
Texas × A1	35.31±3.05 a	23.68±2.66 a	3.26±1.09 c
Texas × A2	33.47±3.26 ba	23.52±3.02 a	4.53±1.00 b
Texas × A3	28.26±2.48 c	19.37±2.43 b	5.73±0.74 a
Texas × A4	32.03±2.45 b	21.91±1.97 a	3.64±0.65 c
Mean	32.37±3.43	22.18±2.76	4.13±1.20

another study conducted on almonds, fruit mass values were reported as between 7.74-3.40 g (Khadivi et al., 2019). In a previous study conducted on adaptation of almond cultivars, fruit mass values were reported as between 1.62-4.15 g, fruit lengths as between 32.30-38.72 mm and fruit widths as between 16.00-26.36 mm (Yildiz and Perdahci, 2019).

4. Conclusion

Present findings on fruit mass, length and widths comply with the findings of previous studies. Partial differences were mostly attributed to differences in cultivars, pollinators, and climate parameters. Pollination problems are mostly resulted from insufficient development of pollens at low temperatures (below 10°C) during the flowering period or inability of pollen to germinate even if they sufficiently developed, unacceptance of pollens by stigma. Even if the pollination was realized, such problems also end up with small fruit sizes, thus reduce quality. On the other hand, besides ecological conditions, cultural practices (irrigation, fertilization, pruning, tree age) and yield potential also significantly influence fruit size. Almond is a significant income-generating fruit both in the world and in Turkey. It was concluded based on present findings that use of pollinator cultivars had significant effects on fruit sets and quality attributes. Present pollinators can be recommended for new almond orchards to be established with Texas cultivar for high yields and quality attributes.

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RESEARCH PAPER

HORTICULTURAL STUDIES

The Effect of Antioxidants on Micropropagation of Avocado by Nodal Segments

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Abstract

Tissue culturing, which is an alternative method to clonal reproduction of avocado (*Persea americana* Mill.) has started to become widespread in recent years. However, the browning of tissue which is caused by oxidation of phenolic compounds is one of the most important factors limiting success for the in vitro reproduction. Therefore, in this study, the effects of different antioxidants (activated charcoal (AC), ascorbic acid (ASA), citric acid (CA) alone or in combination with each other on browning and regeneration of avocado shoots, grown under in vitro conditions, were investigated. In the study, MS (Murashige and Skoog) nutrient medium with 1 mg L^{-1} BAP, 0.1 mg L^{-1} GA_3 and 3% sucrose was used in all treatments. Moreover, antioxidants alone or combined with each other with different concentrations were used in the nutrient media in all treatments except the control. As a result of the research, the lowest browning intensity and the highest survival rate values were shown in the application of 100 mg L^{-1} ascorbic acid (ASA) alone and in the combination of 60 mg L^{-1} ascorbic acid (ASA) and 40 mg L^{-1} citric acid (CA). Furthermore, maximum shoot length (2.8 cm) and maximum leaf number (6.4) in terms of survival rate of the explants, shoot length and number of leaves were determined in MS nutrient medium containing a combination of 60 mg L^{-1} ascorbic acid (ASA) and 40 mg L^{-1} citric acid (CA).

1. Introduction

Avocado (*Persea americana* Mill.) is the most economically important crop in the Lauraceae family. It is widely grown in many tropical and subtropical parts of the world (Morton, 1987). The popularity of avocado has increased over the last few decades and became a very important commercial crop to cultivate recently in many countries of the world (Silva and Ledesma, 2014). Worldwide production of avocado almost doubled in the last 10 years due to the increase in consumption. Thus, the trade of avocado reached over 6.4 million tons in 2018 (FAO, 2018). In 2018, the largest avocado producers were Mexico (2.1 million tons), Dominican Republic (644 thousand tons), Peru (504 thousand tons), Indonesia (410 thousand tons), and Colombia (326 thousand tons) (FAO, 2018).

Commercial cultivation of avocado has been generally accomplished by vegetative propagation (grafted) (Ben-Ya'acov and Michelson, 1995). However, seed-propagated rootstocks showed high genetic variation and trees were not true-to-type. Hence, seedling rootstocks are not suitable for commercial purposes. Clonal rootstocks are vegetatively propagated by the Frolich & Platt double grafting method, and has been used commercially for more than 40 years. However, this method is an expensive, time consuming and laborious (Frolich and Platt, 1972; Ernst, 1999). Micropropagation is an essential part of clonal

propagation and more advantageous than the traditional propagation methods (Thorpe, 2007). However, in vitro techniques are considered as an alternative method for seedling production of avocado, in vitro propagation of avocado is associated with several problems as do other woody plants (Barceló-Muñoz and Pliego-Alfaro, 2012; Hiti-Bandaralage et al., 2017). One of the most important problem is browning, it occurs at the initial establishing stage of in vitro culture of avocado due to leaching of phenolic substances and secondary metabolites from the cut surfaces of explants (Schall, 1987).

Browning of the explant usually depends on the phenolic compounds that are secondary metabolites and the quality of the total phenols in the explant tissues (Ozyigit, 2008). Phenolic compounds frequently cause browning and are well known to be inhibitory to the plant's cellular growth (Monaco et al., 1995). Due to browning, the tissues of the explant turned necrotic and died within a few days after inoculation. Different treatments have been investigated to eliminate the browning problem with varying efficiency from species to species (Saenz et al., 2010). For instance, low temperature and dark incubation, have been used to reduce browning in the explants of avocado (Barceló-Muñoz et al., 1999; Castro et al., 1995) and pear (Poudyal et al., 2008). Besides, antioxidants have been commonly used in tissue culture media to improve cell growth and development and have many roles in the plants physiological processes (Shao et al., 2007). They play an important role in the adsorption of phenolic compounds. Therefore, antioxidants should be considered as one of the most important factors for an effective control of browning (George, 1996). Adding the antioxidants (activated charcoal (AC), ascorbic acid (ASA), citric acid (CA), polyvinylpyrrolidone (PVP) and etc.) to the nutrient medium is among the most commonly used treatment for tissue browning in woody plant species (Ahmad et al., 2013). Using antioxidants in browning control has been demonstrated in several species such as guava (Zamir et al., 2004), mango (Chandra et al., 2003), pomegranate (Singh and Patel, 2016), pear (Poudyal et al., 2008), banana (Munguatosha et al., 2014), and hazelnut (Shirazi et al., 2020). In addition, different antioxidants have been used to reduce browning in avocado tissue culture medium. Several researchers have reported the addition of PVP (Dalsaso and Guevara, 1989; Ahmed et al., 2001; O'Brien et al., 2020) and melatonin (O'Brien et al., 2020) alone in the medium to control browning of cultured explants of avocado. Pre-treatment with different antioxidants is one of the ways to remove phenols or reduce the media browning (Krishna et al., 2008). Pre- treatment of different antioxidants was found to be quite efficient, not only for inhibition of browning, but also for prevention of leakage of phenolic compounds into avocado (Castro et al., 1995; Wessels et al., 1996), guava (Ahmad et al., 2016), longan (Hong-bin, 2008) and mango (Krishna et al., 2008). Therefore, various antioxidant compounds were selected in this study.

According to previous published literature, there is still no exact treatment to control of the avocado nodal segment completely. Based on that, the objective of the study was to investigate the effects of various antioxidants (AC, ASA, CA) and their combinations (ASA+CA, AC+ASA, AC+CA) on the browning of nodal segments and the survival rate of the explants, shoot length and number of leaves in avocado.

2. Materials & Methods

The experiment was carried out in the Tissue Culture Laboratory in Batı Akdeniz Agricultural Research Institute in Antalya province in Turkey in August, 2020.

2.1. Plant material

Shoots having at least five nodal segments were collected from 3-4 years old grafted plants grown in the glasshouse. Explants were taken in early August of 2020 and before sterilization, all leaves were removed from the shoots.

2.2. Surface sterilization

Shoots were washed under tap water for 30 minutes, after that, they were submerged with 70% (v/v) ethanol for a minute followed by rinsing with sterile distilled water three times in a laminar air-flow cabinet. Finally, the twigs were treated with 5% (v/v) sodium hypochlorite with three drops of tween-20 for 3 minutes and then rinsed three times with sterile distilled water. The sterilized explants were cut into 1-2 cm nodal segments with axillary buds aseptically.

2.3. Culture conditions and media

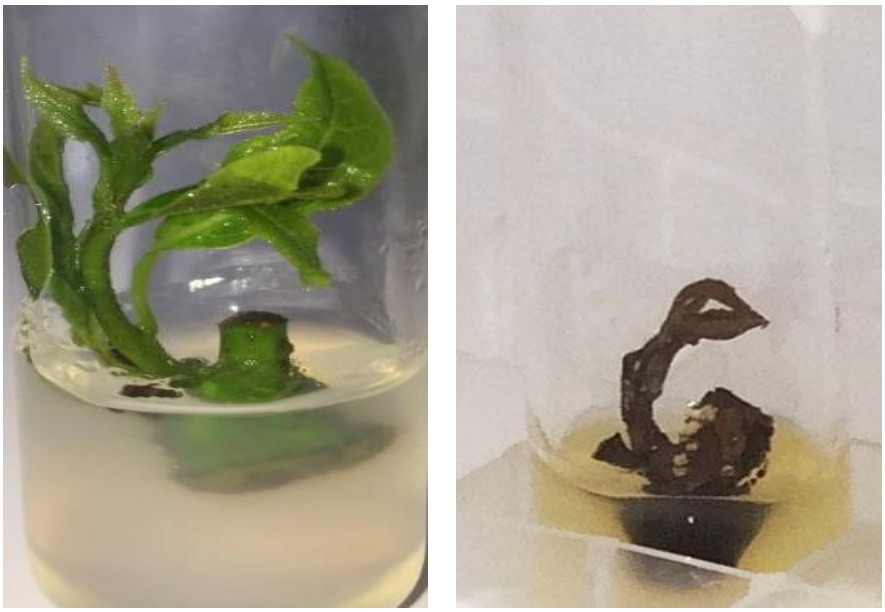
As a nutrient medium, Murashige and Skoog (MS) basal medium was used in the experiment. Also, gibberellic acid (GA_3) at 0.1 mg L^{-1} and benzylaminopurine (BAP) at 1 mg L^{-1} were added to the MS basal medium in all treatments. In addition, various antioxidants (AC, ASA and CA) with different concentrations and combinations were added to the medium (Table 1).

Besides, 3% sucrose (w/v), 7% agar (w/v), and 100 mg L^{-1} myo-inositol were included to media. The pH of the medium was adjusted to 5.7 before the addition of agar and autoclaved at 121°C for 20 min under 1.0 atm. After surface sterilization, nodal segments were cultured on 15 ml semi-solid medium as 1 explant per test tube. Explants were cultured on initiation media for four weeks and then were transferred to sub-culture media. Cultures

Table 1. The concentration and combination of antioxidants used in the study

AC (mg L^{-1})	ASA (mg L^{-1})	CA (mg L^{-1})	ASA+CA (mg L^{-1})	AC+ASA (mg L^{-1})	AC+CA (mg L^{-1})
0	0	0	0	0	0
150	50	20	30+20	1000+50	1000+20
250	100	40	60+40	500+100	500+40
500	150	60	90+60	250+150	250+60
1000	200	80	120+80	150+200	150+80

AC: activated charcoal, ASA: ascorbic acid and CA: citric acid



a b

Figure 1. Green, healthy and vigorous shoot obtained in the basal medium with the 60+40 mg L^{-1} combination of ASA+CA (a) and dead shoots due to the presence of phenolic compounds in the control treatment (b).

were incubated in a growth room at 25±2°C with a 16-h photoperiod for eight weeks.

2.4. Investigation criteria

2.4.1. Browning intensity: Browning intensity was observed four weeks after culturing explants in the initiation media. The number of died explants due to the phenolic compounds were evaluated for each treatment. Browning intensity was evaluated on an arbitrary browning scale ((++++) = intense browning, (+++) = moderate browning, (++) = low browning, (+) = very low browning) (Singh and Patel, 2016).

2.4.2. Survival rate of the explants (%): Survival rate was recorded four weeks after the sub-culturing of explants. The number of green, healthy and vigorous shoots were counted for each treatment and the survival percentage were calculated.

2.4.3. Shoot length (cm): The average shoot length was recorded four weeks after sub-culturing. Shoot length was measured from the node to the tip of the shoot at the time of subculturing.

2.4.4. Number of leaves: The number of leaves was recorded four weeks after sub-culturing. The total number of leaves were counted per shoot and the percentage was calculated.

2.5. Data analysis

The experiment was established according to completely randomized design with three replicates and 4 explants for each replicate. The data were subjected to analysis of variance using the SAS software version 9.00. The least significant difference (LSD) method was used to test the difference between treatments and $p \leq 0.05$ was considered statistically significant.

3. Results & Discussion

The effects of different antioxidants alone or in combination on browning intensity are shown in Table 2. It was found that the use of all antioxidants both alone and together reduces the intensity of browning according to the control treatment. Increasing the concentration of some antioxidants used alone in the experiment decreased the intensity of browning, while some increased the browning intensity (Figure 1 a, b). For instance, it was found that when the concentration of AC exceeds 500 mg L^{-1}, the intensity of browning increased. It is thought that this situation occurs when AC increases the uptake of plant growth regulators in the nutrient medium. Unlike AC, the intensity of browning decreased as the concentrations of other antioxidants in the

Table 2. The effects of different antioxidant concentrations and combinations on browning intensity, survival rate of the explants, shoot length and number of leaves

Treatments	Concentration (mg L^{-1})	Browning intensity	Survival rate of the explants (%)	Shoot length (cm)	Number of leaves
AC	0	++++	16.67 b*	1.4 b	3.2 b
	150	++	58.33 a	1.5 ab	3.4 ab
	250	++	58.33 a	1.6 ab	3.5 ab
	500	++	66.67 a	1.7 a	3.6 a
	1000	+++	41.67 ab	1.4 b	3.2 b
$LSD_{5\%}$			26.259	0.215	0.372
ASA	0	++++	16.67 c	1.4 c	3.2 d
	50	+++	41.67 bc	1.9 b	4.9 b
	100	+	83.33 a	2.6 a	6.0 a
	150	++	75.00 ab	2.4 a	5.7 a
	200	++	66.67 ab	1.8 bc	4.4 c
$LSD_{5\%}$			31.070	0.417	0.453
CA	0	++++	16.67 b	1.4 c	3.2 b
	20	+++	41.67 ab	1.6 bc	3.4 b
	40	++	75.00 a	2.2 a	5.3 a
	60	++	66.67 a	2.0 ab	5.1 a
	80	++	66.67 a	1.5 c	3.4 b
$LSD_{5\%}$			37.136	0.384	0.479
ASA+CA	0	++++	16.67 b	1.4 d	3.2 d
	30+20	+++	41.67 b	1.8 c	4.5 c
	60+40	+	83.33 a	2.8 a	6.4 a
	90+60	++	75.00 a	2.2 b	5.2 b
	120+80	++	75.00 a	1.9 bc	4.8 bc
$LSD_{5\%}$			28.765	0.318	0.616
AC+ASA	0	+++	16.67 c	1.4 c	3.2 c
	1000+50	+++	41.67 b	1.7 bc	3.2 c
	500+100	++	75.00 a	1.9 ab	4.1 b
	250+150	++	66.67 a	2.2 a	5.2 a
	150+200	++	58.33 ab	1.6 bc	3.5 bc
$LSD_{5\%}$			23.487	0.433	0.662
AC+CA	0	++++	16.67 b	1.4 c	3.2 d
	1000+20	+++	41.67 ab	1.8 ab	4.1 b
	500+40	++	66.67 a	2.1 a	5.0 a
	250+60	++	58.33 a	1.6 bc	3.6 cd
	150+80	+++	41.67 ab	1.8 ac	4.0 bc
$LSD_{5\%}$			26.259	0.384	0.492

(++++) = Intense browning, (+++) = Moderate browning, (++) = Low browning, (+) = Very low browning.
* Represent significant differences ($p<0.05$) among treatments.

experiment increased. For example, increasing concentrations of ASA and CA reduced the intensity of browning. In the use of antioxidants with each other, the best results in terms of browning intensity were found in 60+40 mg L^{-1} combination of ASA and CA. In combinations of AC with other antioxidants (AC+ASA and AC+CA), when the concentration of AC was decreased and the concentration of ASA and CA was increased, browning intensity reduced. When the research findings were evaluated in terms of browning, it was determined that ASA is the best antioxidant when using antioxidants alone and using the ASA+CA combination is the best antioxidant treatment when using them together. The results of this study were similar to some previous researches. In these studies, it has been reported that the application of AC, ASA, CA together to avocado explants gave better results than the application alone in terms of browning intensity (Nel et al., 1983; Pliego-Alfaro and Murashige, 1987; O'Brien et al., 2020). Moreover, in the study conducted by Munguatosha et al. (2014), it was stated that adding 100 mg L^{-1} ASA in the nutrient media decreased the browning in bananas. Also, in the study performed by Ndakidemi et al., (2014) about *Brachylaena huillensis* (Asteraceae), it was reported that the best results were obtained in terms of browning intensity when 200-250 mg L^{-1} ASA was added to the medium.

The effects of using different antioxidants alone or in combination with each other on the survival rate of the explants are given in Table 2. The survival rate increased compared to the control in all antioxidant treatments. It is clearly seen in Table 2 that there is a relationship between the survival rate and browning intensity. It has been determined that as the density of browning increased, the survival rate decreased. When AC is used among the antioxidants, the highest survival rate was determined at the concentration of 500 mg L^{-1} with 66.67%. When another antioxidant, ASA, was used

alone, the best result was obtained at a concentration of 100 mg L^{-1} with 83.33%, followed by 150 mg L^{-1} with 75% and 200 mg L^{-1} with 66.67%, respectively. As in ASA, when used above a certain concentration in CA too, the survival rate decreased. The highest survival rate in CA was 75% at a concentration of 40 mg L^{-1}. When antioxidants were used together, the highest survival rate was found at 60+40 mg L^{-1} concentration from ASA + CA combination. This was followed by 500+100 mg L^{-1} AC+ASA combination with 75% and 500+40 mg L^{-1} AC+CA combination with 66.67%. As a result of our findings, the highest survival rate was determined with 83.33% as a result of using ASA alone and ASA+CA together. The findings in the present study were similar to previously published results. In a study carried out by O'Brien et al. (2020), it was reported that the survival rate in avocado increased in the media containing 100 and 250 mg L^{-1} AA. In the study conducted by Ahmad et al. (2016), it was found that using AC, ASA and CA alone increased the survival rate in guava.

The effects of using different antioxidants alone or in combination with each other on shoot length are given in Table 2. As can be seen in Table 2, it has been found that shoot length varied between 1.4-1.7 cm when using AC alone. Moreover, in the use of ASA, the highest shoot length was obtained as 2.6 cm with 100 mg L^{-1} concentration, followed by 2.4 cm with 150 mg L^{-1} and 1.9 cm with 50 mg L^{-1}. Besides, when using CA alone the highest shoot length was 2.23 cm with 40 mg L^{-1}. When antioxidants were used together, the best results in terms of shoot length were found with a combination of 2.8 cm and 60+40 mg L^{-1} ASA+CA. In other combinations, the best results were obtained at 2.2 cm with 250+150 mg L^{-1}, AC+ASA and 2.1 cm with 500+40 mg L^{-1} AC+CA. As a result of our findings, the highest shoot length was obtained from the media containing ASA when antioxidants were used alone and ASA+CA combination when used together. In a study conducted by Al-Mayahi et al. (2016) on persimmon, it was reported that the highest shoot length was obtained by combining ASA with salicylic acid (SA). In the study conducted by Jakhar et al. (2019) on guggul (*Commiphora wightii*), the highest shoot length was obtained in the medium where 150-200 mg L^{-1} AC were added, different from our results.

When the effect of the treatments on the number of leaves was examined, it was found that the use of all antioxidants alone or together with each other was found to be statistically significant as in the shoot length, except for the use of AC alone, (Table 2). In addition, it is clearly seen in Table 2 that there is a relationship between shoot length and the number of leaves, as well as the relationship between browning density and the survival rate. The number of leaves has been found to be between 3.2 and 3.6 when using AC alone. The best result in terms of leaf number of ASA, another antioxidant, was obtained as 6.0 at a concentration 100 mg L^{-1}, followed by 5.7 with 150 mg L^{-1} and 4.9 with 50 mg L^{-1}. The effect of CA on the leaf number changed between 3.4-5.3. In the use of antioxidants with each other, the best treatment for the number of leaves as well as the number of shoots and the survival rate was obtained in the combination of ASA+CA at a concentration of 60+40 mg L^{-1}. In other antioxidant combinations, the highest number of leaves was found as 5.2 in the combination of 250+150 mg L^{-1} AC+ASA and 5.0 in 500+40 mg L^{-1} AC+CA. When the results were examined in terms of number of leaves, the best results were obtained in 100 mg L^{-1} ASA used alone and 60+40 mg L^{-1} ASA+CA in combination with each other. Al-Mayahi et al. (2016) reported that the highest number of leaves of persimmon was obtained with the combination of ASA+SA.

4. Conclusion

As a result of the study, it was observed that browning of survival rate of the explants, shoot length and number of leaves in avocados can be prevented by adding antioxidants to the plant nutrient media. The best results, in order to prevent browning, were obtained by the addition of 100 mg L^{-1} ASA and the combination of 60+40 mg L^{-1} ASA+CA to the nutrient medium and statistically significant differences were found in these treatments compared to the control group. Moreover, the best result in terms of shoot length and leaf number was obtained from the 60+40 mg L^{-1} ASA+CA combination. In future studies, it is recommended to try different antioxidant combinations in different plant nutrient media to prevent browning in avocado plants under *in vitro* conditions.

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