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Effects of Boron and Cold Stress on Germination of Almond Pollen in Vitro Culture

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Abstract

This study was carried out in order to determine the effects of boron and cold stress on pollen germination of almond hybrids in vitro culture, two different media including the absence and presence of boron (0 mg/l, and 100 mg/l) in 10% sucrose and 1% agar medium at 24°C in dark conditions. The results showed that different treatments had a significant effect on the germination percentage. The highest pollen germination percentage in H24 and S8 almond hybrids was recorded as 98.35 % and 97%, 34% in vitro culture medium containing 100 ppm boric acid, 10% sucrose +1% agar, whereas these amounts were 87.56% and 89.46% in the basic medium without the presence of boric acid, respectively. In conclusion, in vitro pollen germination of almond was inhibited in the medium culture without boron and in cold stress, while it was increased in the medium with boron. Thus, boron can increase the almond yield.

Keywords: Boric acid, Frost damages, Pollen viability, *Prunus dulci* L..

Introduction

It is known that most almond cultivars in the world, and all the major commercial cultivars, are self-incompatible. Hence, each cultivar must be grown with another cultivar to ensure cross pollination. The commercial part of the fruit is the kernel. Pollination and ovule fertilization are of critical importance to obtain optimal yields, and typically require the joint planting of at least two cultivars with cross-compatible and simultaneously blooming and the presence of honeybee as a pollinator to transfer the pollen between cultivars (Socias i Company *et al.,* 1992; Kester and Gradziel, 1996). Therefore, kernel setting in almonds depends on the transfer of viable, compatible pollen by honey bees between healthy flowers. Thus, it is very important to know the factors that affect pollen viability (Mussen and Montague, 2004).

Pollen germination and pollen tube growth are prerequisites for fertilization and seed development. Due to involvement of the pistil tissue in the nature, physiological and biochemical, investigations on pollen germination and pollen tube growth *in vivo* are rather difficult. Therefore, *in vitro* germination techniques have been used extensively on pollen germination. Hence *in vitro* pollen germination rates are considered the best indicator of pollen viability (Shivanna *et al.,* 1991). Most researchers' advise to use sucrose solution as a pollen germination media (Stanley and Linskens, 1974; Martinez-Gomez, *et al.,* 2002; Acar *et al.,* 2010; Imani *et al.,* 2011). Boron is involved in many processes, including sugar transport, cell wall synthesis and maintenance, membrane integrity, and RNA, indole acetic acid (IAA) and phenol metabolism (Loomis and Durst 1992, Dordas and Brown, 2000). Its precise role has not been elucidated. Recently, it

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was demonstrated that boron is essential in pollen germination and tube formation. It has a vital function in fertilization of flowering crops as well. The use of boron in pollen *in vitro* culturing of most species often leads to improvement of germination and tube growth of pollen (Wang *et al.,* 2003b). Nyomora *et al.* (2000) showed that foliar-applied boron reduced bursting of pollen tubes during in vitro germination. The addition of B (100 mg/l) to the culture media significantly increased *in vitro* germination and pollen tube growth from trees sprayed with 0.8 kg B/ha, decreased germination in pollen from trees sprayed with 1.7 or 2.5 kg B/ha and had little effect on pollen tube growth from trees sprayed with 1.7 or 2.5 kg B/ha.

Seasonality changes create various stresses. These stresses affect vegetative and crop achievement. Among these stresses, spring frost is one of the important factors that affect the productivity of temperate zone fruits. Almond is damaged with late spring frost, due to early flowering (Kester and Gradziel, 1996). It has been reported that almond cultivation in Iran and also in the most regions of the world is affected by adverse climatic factors such as drought, soil salinity and spring frost (Rodrigo 2000). The almond is resistant to low temperatures in the winter, but low temperatures in the spring frost is lethal to most reproductive organs during the blooming period. It has been shown that even the cold resistant cultivars can be damaged by low temperatures (Anderson and Seeley, 1993). The periods of major frost risk are from in the beginning of the blooming period to the active (shoot or fruit growing) growth period (Proebsting 1985; Ballard *et al.,* 1990). The minimum temperature in which almond cultivars can resist in various phenological stages may define its adaptation to specific agro-ecological zones. The temperature at which flower buds are injured depends primarily on their stage of development. Buds are most durable during the winter when they are fully dormant. As they begin to swell and expand into blossoms, they become less resistant to freeze injury (Charrier *et al.,* 2015; Saudreau *et al.,* 2009). Therefore, in temperate climates, losses due to frosts during the blooming period are more significant than those due to low winter temperatures. Resistance to frost varies among buds of different cultivars of the same species.

Cold resistance in flower buds may be the result of several factors including; structural, physiological and morphological features (Ashworth *et al.,* 1985). Factors such as genotype, flower developmental stage, formation of ice, moisture content and nutritive status of pistil, have been reported to be linked to the sensitivity or resistance of flowers to spring frost (Rodrigo, 2000). Critical temperature indices for almond have been determined for different stages of phenological development (Kester and Gradziel, 1996). Freezing temperatures can seriously damage the plant tissues. The effects of spring frost on reproductive organs of almond are highly variable and depend on the characters of both the freezing stress and the plant material status. The different responses to frosts observed among plant genotypes, tissues of the same plant and different seasons have led to the study of mechanisms of injury (Anderson and Seeley, 1993; Rodrigo, 2000).

Although studies have been conducted on the effects of chemical material on pollen germination and pollen tube growth of almond (Kester and Gradziel, 1996; Nyomora *et al.,* 2000; Mussen and Montague, 2004; Tosun and Koyuncu, 2007), very few have been carried out on the interaction of boron and cold stress on *in vitro* pollen germination in almonds.

The objective of this study was to determine the effects of boron on the pollen germination rate of almond hybrids and the interaction of boron and cold stress on *in vitro* pollen germination of almond.

Materials and Methods

Pollen collection

Branches with unopened flowers were pruned off from trees of 24 almond hybrids (Flip Ceo as male parent and Fragness as female parent) growing in the orchard of Horticultural Science Research Institute (HSRI) in Karaj, Iran. Pollen grains were collected in large quantities from these pruned branches, 24 hours following pruning from freshly opened blossoms at room temperature (20–25°C) (Imani *et al.*, 2011). The collected pollen grains before culturing were located in a chamber (432 L; ASLA paratos Cientificos, Madrid Spain). This programmable chamber model is equipped with a heat – cold unit working in the – 20°C to 30°C – ±0.3°C precision. Five termopar probes connected to a data logger (LI-100; LI-COR, Inc., Lincoln, Neb) were placed near the samples.

Frost temperature treatment

Air temperature in the chamber was maintained at 7° C for 50 minutes, and then programmed to decline by 3°C per hour until the desired frost temperature was reached. The frost temperature was maintained for 30 minutes and then increased up to 7°C by 3°C/ h. Frost (-3°C) was applied to the collected pollen. The rate of frost damages was evaluated 24 hours after the frost treatment.

Pollen germination

To determine the effects of boron and cold stress (-3°C) on pollen germination of almond hybrids by their parents in vitro condition, two different medias, one with boron and one without boron, in 10% sucrose and 1% agar medium was prepared.

Pollen grains were scattered uniformly onto the medium in petri dishes. The petri dishes were covered and placed in a growth chamber and incubated at 24±2°C in darkness for 24 hours. The percentage of pollen germination and pollen tube length was determined under a light transmission Nikon type-2 microscope. Pollen grains were recorded as germinated when the pollen tube was equal to or longer than the diameter of the pollen grain (Henny, 1977). Data on pollen germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen grains per field of view (Imani *et al.*, 2011).

Statistical analysis

The statistical analysis was performed using Microsoft Excel (2007) and SAS software (SAS Institute Inc, 1990) and means were compared using Duncan's Multiple Range Test (DMRT).

Results

The effect of boron and cold stress on pollen germination of almond is shown in Fig.1. The highest pollen germination percentage of 'H24'(Fig. 2) and 'S8 'was recorded as 98.35 % and 97.34% in vitro culture medium containing 100 ppm boric acid, 10% sucrose +1% agar, whereas these amounts were 87.56% and 89.46% in the basic medium and in the absence of boric acid, respectively (Fig. 4).

On the other hand, a considerable difference in the ability to germinate was observed among the hybrids (Figs. 1 and 3). Different media showed significantly different effects with regard to their germination percentage of almond pollen grain following the in vitro culture (Figs. 1 and 3). The pollen germination was extremely inhibited in media without boric acid (Figs. 1 and 3).

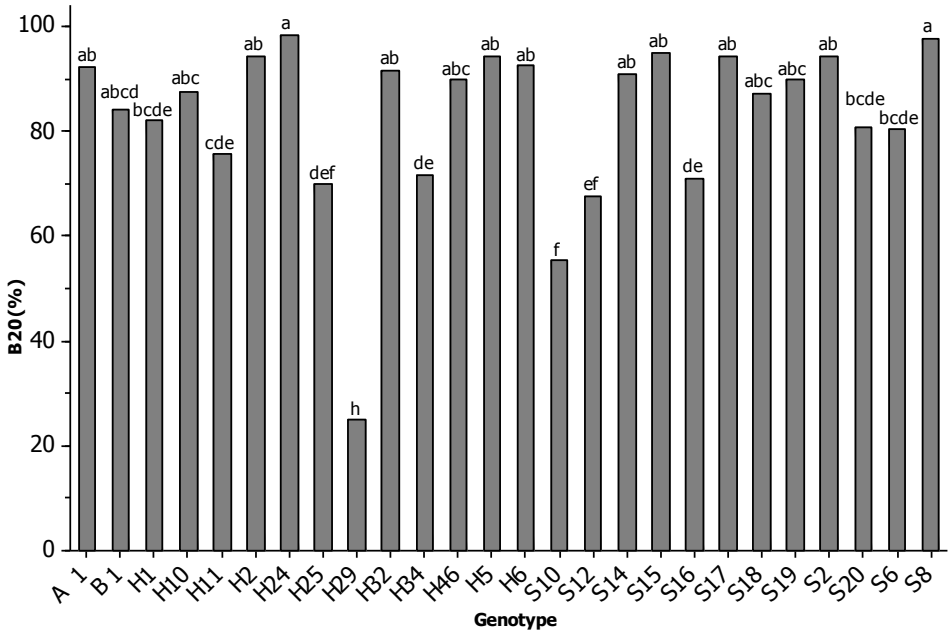

Fig. 1. Pollen germination rate in vitro culture media containing boron (100 mg/l), 10% sucrose and agar1% without cold stress. Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).

B20 (%): Percentage of pollen germination in media containing boron (100 mg/l), 10% sucrose and agar1% without cold stress

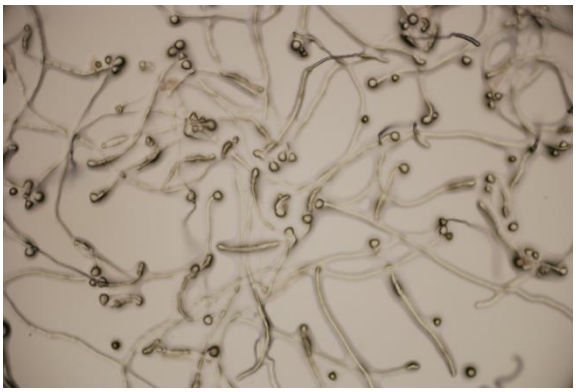

Fig. 2. Pollen germination rate in in-vitro culture media containing boron (100 mg/l), 10% sucrose and agar1% without cold stress for 'H24 ' hybrid**.**

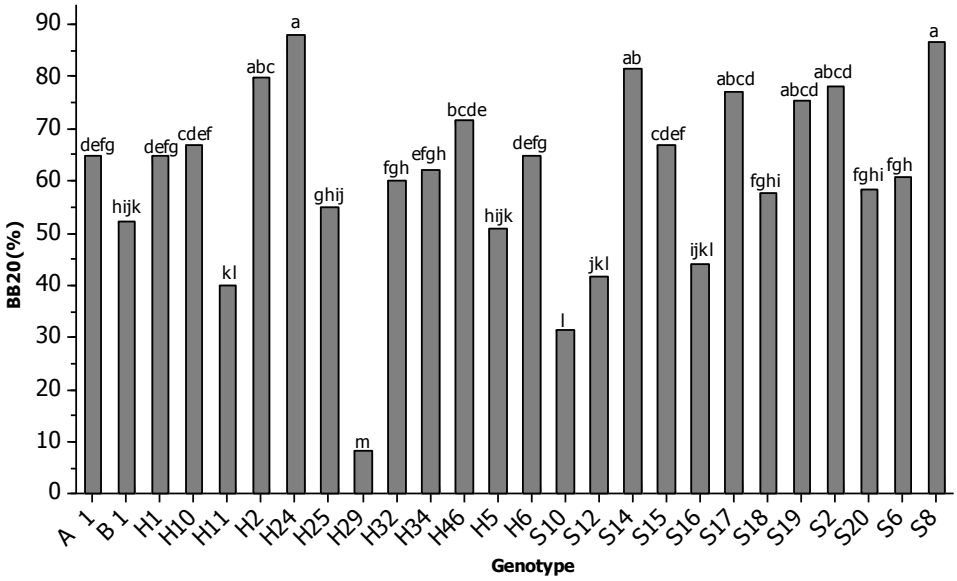

Fig. 3. Pollen germination rate in in-vitro culture media containing 10% sucrose and agar1% without cold stress.
Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).
BB20 (%): Percentage of pollen germination in media containing 10% sucrose and agar1% without cold stress

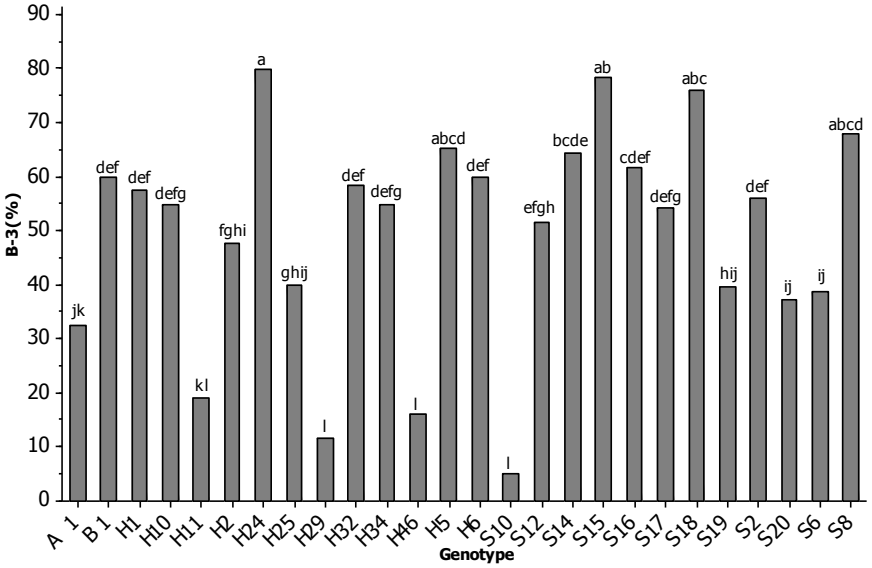

Fig. 4. Pollen germination rate in in-vitro culture media containing boron (100 mg/l), 10% sucrose and agar1% with cold stress (-3°C).
Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).
B-3(%): Percentage of pollen germination in media containing boron (100 mg/l), 10% sucrose and agar1% with cold stress (-3°C).

The highest pollen germination for 'H24'(Fig.5) and 'H15'almond hybrids was recorded 80.23% and 78%, respectively. The lowest germination percentage was recorded 7.5% for 'S10' hybrid in 100 mg/l boric acid,

10% sucrose and 1% agar medium with cold (-3°C) stress (Figs. 4 and 6).

The results in Fig. 7 showed that the highest pollen germination for 'H24' and 'H15' almond hybrids was recorded 75.12% and 67.13%, respectively The lowest germination percentage was 0.05% for 'S10' hybrid in vitro culture media containing 10% sucrose and 1% agar with cold (-3°C) stress. In media containing 10% sucrose and agar 1% with cold stress (-3°C) (Fig. 8). The germination percentage for 'S10'hybrid (Figs. 4 and 6) in the media containing boron (100 mg/l), 10% sucrose and agar1% with cold stress (-3°C) was 7.5%.

Pollen germination measured on culture media containing 10% sucrose and agar % without (with) cold stress affected by the absence and presence of boron (0 mg/l, and 100 mg/l) are shown in Fig. 8. According to the results, the pollen germination rate was lower in the in vitro culture media containing 10% sucrose and agar1% with cold stress (-3°C) for S10 hybrid. On the other hand, the pollen germination percentage in culture media containing 10% sucrose and agar 1% not only was affected by boron and cold stress but also by the almond genotype (Figs. 9-12).

Fig. 5. Pollen germination rate in media containing boron (100 mg/l), 10% sucrose and agar1% with cold stress (-3°C) for 'H24' hybrid

Fig. 6. Pollen germination rate in media containing boron (100 mg/l) , 10% sucrose and agar 1% with cold stress (3°C) for, S10' hybrid.

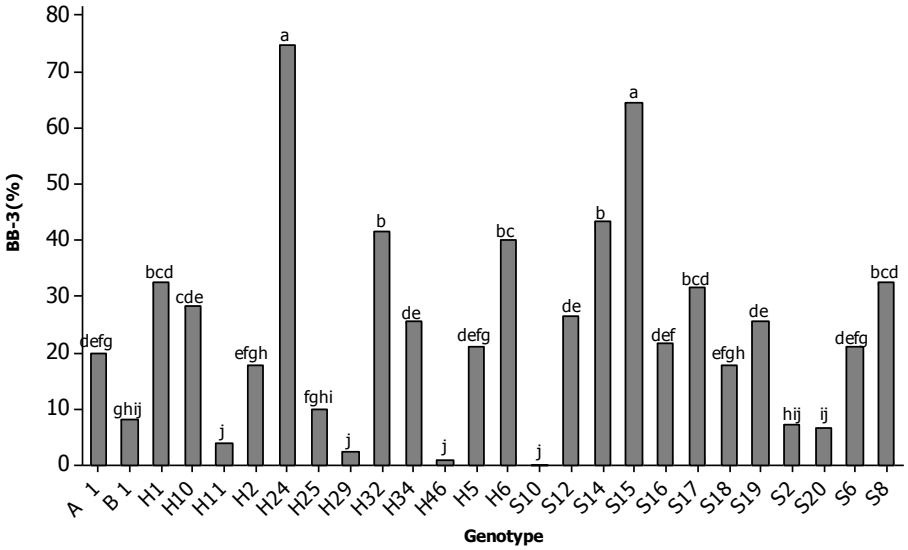

Fig. 7. Pollen germination rate in culture media containing 10% sucrose and agar1% with cold stress(-3°C).
Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).
BB-3(%): Percentage of pollen germination in 10% sucrose and agar1% with cold stress (-3°C).

Fig. 8. Pollen germination rate in culture media containing 10% sucrose and agar 1% with cold stress (-3°C) for S10 hybrid

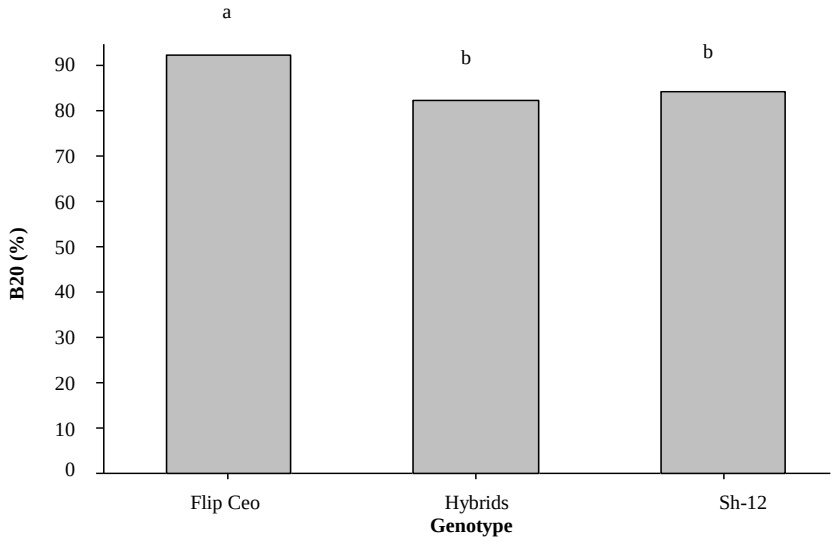

Fig. 9. Mean of pollen germination percentage in containing boron (100 mg/l), 10% sucrose and agar1% without cold stress in parents and hybrids.

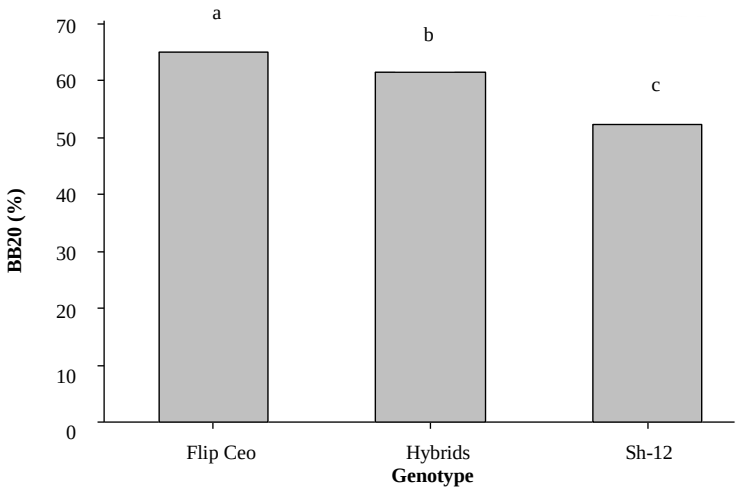

Fig. 10. Mean of pollen germination percentage in containing boron (100 mg/l), 10% sucrose and agar1% without cold stress in parents and hybrids.

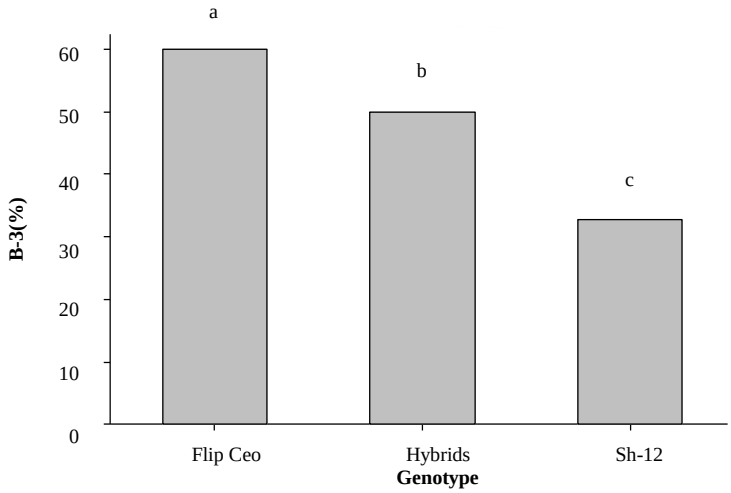

Fig. 11. Mean of pollen germination percentage in containing boron (100 mg/l), 10% sucrose and agar 1% without cold stress in parents and hybrids.

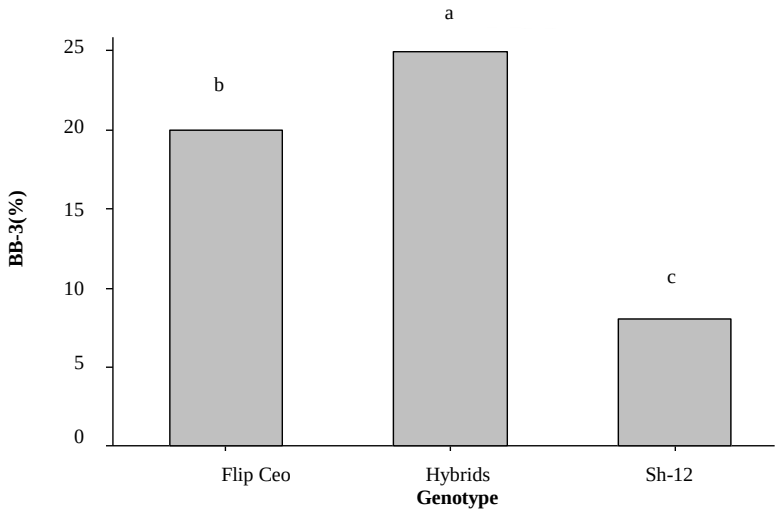

Fig. 12. Mean of pollen germination percentage in containing boron (100 mg/l), 10% sucrose and agar 1% without cold stress in parents and hybrids.

Discussion

The results of pollen germination measured in the culture media containing 10% sucrose and agar % without (with) cold stress in the absence and presence of boron (0 mg/l, and 100 mg/l) are shown in Figs. 1-8. According to Brewbaker and Majumder (1961), boric acid is known to be crucial for pollen germination and tube growth, and it is required at concentrations of 100 ppm in order to affect almond cultivars (Kester and Gradziel, 1996; Martinez-Gomez *et al.,* 2002). The role of boron at optimal concentrations affects the flowering and fruiting process in almonds (Nyomora and Brown, 1997). Similar results have been reported on the positive effects of supplemented boric acid on sucrose medium for increasing the germination rate of stone fruits (Vitagliano and Viti, 1989). These results correlate to the results of Gupta and Mutry (1985) which showed an increase in *Vicia faba* L. pollen germination and tube growth in concentration (0.5-1.0 ppm) of boric acid, gibberellic acid, indole acetic acid (in a basic sucrose) and agar medium. Also, according to the report of Acar *et al.* (2010), *in vitro* pollen germination of pistachio trees was greatly inhibited by increased gibberellic acid concentration, while it was increased by boron in the germination medium. As shown in Figs. 1 and 3, all treatments had different effects on the percentage of pollen germination, regardless of the genotype. The positive effects of boric acid on the pollen germination percentage and germ-tube elongation were variable and dependent on material concentration and cultivars.

It is clear that the highest pollen germination was found in ‘H24‘(Fig.5) and ‘the lowest germination percentage was found in the ‘S10‘hybrid (Figs. 4 and 6). Similar results were reported on the positive effects of supplemented boric acid on sucrose containing medium, where the germination rate of pistachio pollen was increased (Brown *et al*., 1994; Acar *et al*., 2010). This may be due to sugar transport, cell wall synthesis and maintenance, membrane integrity, and RNA, indole acetic acid (IAA) and phenol metabolism by boron (Loomis and Durst 1992, Dordas and Brown, 2000).

In this research, it was shown that pollen germination containing boron (100 mg/l), 10% sucrose and agar1% without cold stress in Flip Ceo (male parent), Fragness (female parent) and hybrids was 92.33%, 84.31% and 82.45%, respectively (Fig. 9). Pollen germination in 10% sucrose and agar 1% without cold

stress in Flip Ceo (male parent), Fragness (female parent) and Hybrids was 65%, 52.56% and 61.41%, respectively (Fig.10). The pollen germination rate in vitro culture media containing boron (100 mg/l), 10% sucrose and agar1% with cold stress (-3°C) for 'Flip Ceo' (male parent), Fragness (female parent) and Hybrids was 60.13%, 32.61% and 49.86%, respectively (Fig.11). However, the pollen germination percentage in the in vitro culture media without boron (0 mg/l), 10% sucrose and agar 1% with cold stress (-3°C) for 'Flip Ceo'(male parent), Fragness (female parent) and mean of hybrids was 20.13%, 8.10.61% and 24.93%, respectively (Fig.12).

The limited pollen germination in media under cold stress (-3°C) without of boric acid suggest that this element be related to nutrient uptake or pollen metabolism.

The addition of 100 mg/l boric acid to a medium in comparison to the control greatly enhanced pollen germination. Our result was similar to studies completed by Khanal (2010) on pollen viability of tomato and Demotes-Mainard *et al.* (1995) on pollen viability of wheat.

Conclusions

In conclusion, in vitro pollen germination of almond was inhibited in the medium culture without boron and in cold stress (-3°C), while it was increased in the medium culture with boron. The highest pollen germination was found in the 'H24" (Fig. 5) and the lowest germination percentage was found in the 'S10'hybrid.

Acknowledgments

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