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Preliminary evaluation of artificial pollination in pistachio using pollen suspension spray

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Abstract

Pistachio trees (*Pistacia vera* L.) are dioecious and pollination is necessary in order to get filled nuts. The effects of suspension media containing agar (A) and boric acid (B) combined with either 0.05% or 0.15% (w/v) pollen (P) used for spray pollination on pollen grain viability were investigated in pistachio (*Pistacia vera* L.) cv. Owhadi. We also evaluated the effects of spray pollination on fruit set, fruit and kernal quality. The pollen grain viability was measured by germination rates. It was determined that the pollen grain has the ability of germination in suspension media and the pollen grain germination percentage significantly increased by the application of the media containing agar and boric acid as compared with other media. Application of medium containing agar (A) and 0.01 % boric acid (B1) combined with 0.15% (w/v) pollen (P2) produced better fruit set than suspension media without boric acid. The medium containing agar and 100 mg L^{-1} boric acid combined with 0.05% (w/v) pollen increased fresh and dry weight of kernel. Moreover the pollination treatments affected kernel nutrient elements concentration so that highest concentration of the kernel P, Ca, Mg, Cu and Fe were obtained with spray pollination method. Our finding showed that the spray pollination may be the effective method in pistachio trees.

Keywords: Agar, Boric acid, Fruit set, Pollen.
Abbreviations: A: Agar, B: Boric acid, P: Pollen.

Introduction

The pollination is one of the most important factors in management of pistachio orchards. The blooming period of male and female trees does not overlap and pistillate flowers are usually unable to receive pollen grain (Ozeker et al., 2006). It is mainly related to the time difference between flowering of male and female trees. Besides, the pollination may not be satisfactory since male trees are not planted in the suitable ratio and in appropriate direction in orchards. Furthermore, pollen quality of the pollinator is low in the most orchards. This situation results in reduction of the production and increase of blank fruits. In the case of insufficient pollination, blank nut forms in which only pericarps develop. Since the yield is reduced, this creates an economic problem (Kaşka, 1994). Artificial pollination can be applied as a temporary solution. Some efforts were done on this matter (Ak, 1992; Ayfer and Kuru, 1990; Caglar and Kaşka, 1994; Kuru, 1994). It has been reported that in some countries such as Iran, Syria and Tunisia, artificial pollination had good result when natural pollination is not sufficient (Caglar and Kaşka, 1994). It was demonstrated that artificial pollination could be as much effective as natural pollination (Ayfer and Kuru, 1990). However, the effective period for artificial pollination is limited, and the success of hand pollination is dependent on the environmental factors such as wind and rainfall. Furthermore, the hand pollination is a labor-intensive process resulting in high labor costs. As an alternative technique, the spray pollination using aqueous pollen grain solutions is expected to reduce labor and costs in fruit tree cultivation. Therefore, several efforts have been made to establish spray pollination methods. In a spray pollination of a peach tree, a 10% sucrose solution and a wetting agent were used, but this yielded poor fruit set (Mizuno et al., 2002). In kiwifruit, a practical technique was successfully established through the development of a pollen grain suspension medium (Hopping and Simpson, 1982). More recently, a liquid pollen grain suspension medium thickened with agar was also developed for kiwifruit (Yano et al., 2007). The time needed for spray pollination was less than half of the time needed for hand pollination. Furthermore, the amount of the pollen grain required for the spray pollination was about one-third of that needed for the hand pollination for japanese pear (Sakamoto et al., 2009). Furthermore the pollen grain enrichment by additional nutrient elements such as boron, calcium and zinc at pollen grain suspension solutions is possible. Although genetic factors are responsible for most of the pollenizer's potentiality, other variables, including mineral nutrition of the plant, may influence pollen quality and its subsequent performance (Wells et al., 2008). The requirement of boron for proper pollen germination and tube growth has been demonstrated in both *in vitro* and *in vivo* experiments (Nyomora et al., 2000; Jayaprakash and Saria, 2001; Wang et al., 2003). Boron is an essential element for plant growth. It's involved in various physiological phases, from bud break to fruit ripen. It influences flowering (its lack can cause dropping of entire flower clusters), pollen viability and production. Boron has been reported to be involved in such diverse processes as nucleic acid metabolism, cell division, sucrose biosynthesis and translocation and membrane functions (Meli et al., 2009). The application of boron in pistachio trees caused an increase of fruit set and a qualitative improvement of the kernel (Meli et al., 2009). Low boron levels in flowers reduce fertility by damaging pollen formation and affecting the growth of the pollen tube. Low boron levels can also have post-insemination effects that affect embryogenesis, leading to seed abortion and fruit

malformation (Sotomayor et al., 2010). It is generally known that it is difficult to maintain pollen grain viability in solution (Ohno et al., 1964). Boron compounds, such as boric acid and sodium borate (borax), can be effective in maintaining pollen grain viability. At concentrations of 10 – 100 mg L^{-1}, boron was shown to stimulate *in vitro* pollen grain germination and promote the growth of the pollen tubes in *Amaryllis hybrida* and *Pyrus communis* (Visser, 1955; Stanley and Lichtenberg, 1963). Almond pollen viability *in vivo* pollen germination and tube growth were enhanced by foliar-applied B. More effect of applied B on *in vivo* growth appeared as pollen tubes progressed toward the ovary. For *in vitro* germination, foliar-applied B reduced bursting of tubes, and addition of B to the culture media significantly increased almond pollen germination and pollen tube growth (Nyomora et al., 2000). However the spray pollination in the peach (Mizuno et al., 2002), date (Awad, 2010), Japanese pear (Sakamoto et al., 2009) and kiwifruit (Yano et al., 2007) was carried out successfully but there is no report of using the pollen grain suspension medium for the pollination of nuts. The objective of this study is investigating the possibility of the spray pollination in pistachio cv. Owhadi and determining the effect of the spray pollination on pistachio fruit cropping.

Results

Effects of the suspension media on pollen grain viability

The effect of germination mediums on the pollen germination was evaluated and expressed as the percentage of germinated pollen grains (Fig. 1). The pollen germination percentage by the spray method was lower than the control, irrespective of the type of suspension medium used. In medium containing agar, pollen germination was not affected by boric acid concentration. The highest pollen germination (81.25%) was obtained with control (Hand pollination) and followed by 100 and 200 mg L^{-1} boric acid in media contained 0.15% (w/v) pollen, respectively (Fig. 1).

Effects of the spray pollination on fruit set and fruit quality

As it is shown in Table 1, the percentage of primary fruit set decreased with "P1" and "P2" of suspension medium compared to control, whereas there were not significant differences between other treatments compared to control. The percentages of secondary and terminal fruit set were decreased in all treatment in comparison with control. In the spray method, the highest terminal fruit set was observed with "P2 + A + B1" medium however, no had significant difference with "P1 + A + B1", "P1 + A + B2" and "P2 + A + B2" media. The media containing boric acid gave higher percentages of fruit set than the media without boric acid, although these differences were not significant. According to the obtained data, fruit drops were lower in natural pollination method (86.40). In the spray method, the lowest fruit drops were obtained with "P2 + A + B1" medium (91.51) and the most was in "P1 + A". As is shown in Table 1 split percentage decreased by using spray pollination method. The highest split percentage was observed with control and the lowest was in"P1 + A". Also the spray pollination method produced more blank nuts than the natural pollination "P1 + A + B2" and "P1 + A + B1" that did not have significant difference with control. According to our results, no significant differences were observed between treatments and control in relation to kernel percentage, nut fresh weight, nut length and width and kernel length; although the nuts dry weight was decreased with P1 and P2 treatments. The highest (0.55 g) and the lowest (0.40 g) kernel dry weight was obtained with "P1 + A + B2" treatment and control. The nut thickness decreased in P1 treatment compared with control.

Effects of the spray pollination on kernel quality

Measured elements concentration of kernel increased with spray pollination methods compared to control exception to Fe and Cu (Table 3). The medium combined with 0.15% pollen showed higher nutrient elements concentration than the medium combined with 0.05% pollen. The highest P, Ca and Mg concentration was obtained with medium containing agar (P + A) and agar and boric acid (P + A +B). Carbohydrate and oil percentage is shown in Table 3. According to our results, no significant differences were observed between treatments and control in related to carbohydrate percentage. In suspension media, the highest oil percentage was observed with medium without boric acid and the lowest was observed with medium containing boric acid. The oil percentage were significantly decreased in "P1 + A + B2" and "P2 + A + B2" treatments compared with control.

Discussion

The pollen germination percentage in the spray method was lower than the control which may be due to the osmotic balance in the medium. Golan-Goldhirsh et al. (1991) studied germination of pistachio pollen in liquid medium and reported that pre hydration of pollen in a saturated atmosphere for 10 h was necessary to obtain maximum in vitro germination. Imbibitions of pollen in water resulted in rapid leakage of solutes in the medium and decreased germination. The germination pollen increased with applying agar or agar and boric acid combination. Sukhvibul and Considine (1993) reported that the germination of *Anigozanthos manglesii* pollen was affected by concentration of agar. It is well known that boron induced pollen germination *in vitro* (Acar et al., 2010). Many aspects of the physiological role of boron in plants are poorly understood but boron may play a role in both initial fruit set and retention of fruit (Wells et al., 2008). Boron added in the form of boric acid, is also essential for the *in vitro* culturing of pollen from most species, for example, it is well appreciated that the elimination of boric acid from the culture medium often leads to tube bursting (Holdaway-Clarke and Hepler, 2003). Our finding showed that the percentage of terminal fruit set decreased with spray method compared to control; it may be due to the damage to the plasma membrane of pollen grains. Our finding was in agreement with Awad (2010) on date palm plant. He reported that the fruit set decreased using the spray pollination method. Other reasons of reduction of the fruit set in present study may be related to stigmatic surface on pistachio flower. It was observed that the supplementary pollination using pollen suspension was more successful on kiwifruit than on plums, largely because of the greater stigmatic surface area on kiwifruit flowers facilitated pollen capture (Hopping and Jerram, 1982b). Hopping and Jerram (1982b) tested the effect of suspension medium for Japanese plum and bronze plum pollens and reported that the addition of 0.01 % boric acid to the suspension medium increased pollen tube elongation but not percentage germination. Boric acid is known to be crucial for pollen germination and tube growth and it is required at concentrations of 100 mg L^{-1} for

Table 1. Effects of suspension media and pollination methods on fruit set, split nut, blank nut and kernel percentage.

Pollination method	Suspension medium[b]	Fruit set (%)[c]			Split nut (%)	Blank nut (%)	Kernel (%)
		Primary	Secondary	Terminal			
Spray	P1	49.66 d[d]	4.57 cd	2.70 c	21.66 def	65.00 a	55.07 a
Spray	P2	57.33 cd	4.83 cd	3.00 c	13.33 ef	62.00 a	49.11 a
Spray	P1 + A	76.00 a	3.78 d	2.50 c	25.00 de	53.66 ab	54.99 a
Spray	P2 + A	61.33 abcd	5.11 cd	3.27 c	10.00 f	57.33 ab	52.54 a
Spray	P1 + A + B1	72.33 abc	6.47 bc	4.20 bc	28.33 cd	30.66 c	47.36 a
Spray	P2 + A + B1	66.33 abcd	8.30 b	5.63 b	41.66 bc	41.00 bc	49.31 a
Spray	P1 + A + B2	72.00 abc	6.38 bcd	4.15 bc	45.00 b	29.66 c	56.08 a
Spray	P2 + A + B2	58.33 bcd	6.34 bcd	4.10 bc	30.00 cd	50.00 ab	52.09 a
Control		75.00 ab	14.66 a	10.20 a	71.66 a	22.33 c	55.06 a

Control: Open pollination, P1: 0.05% Pollen, P2: 0.15% Pollen, A: 0.1% Agar, B1: 0.01% boric acid, B2: 0.02% boric acid. All suspension media included 10% sucrose. Different letters within a column indicate significant differences by LSD Test.

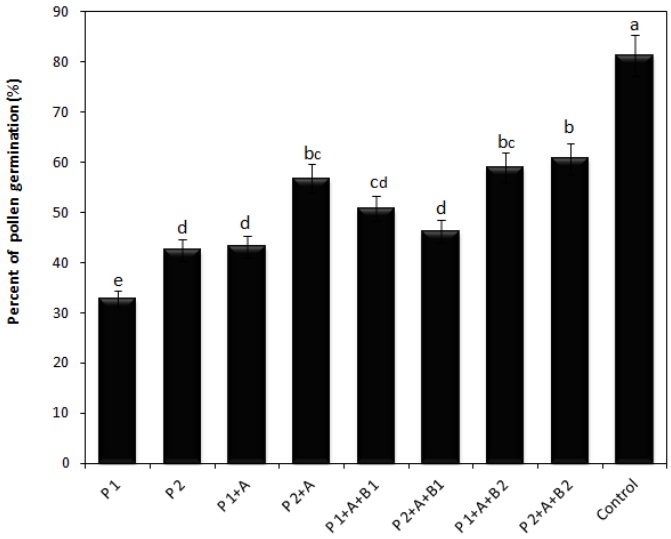

Fig 1. Effects of the suspension media on the pollen germination *in vitro* condition. Means having different letter(s) are significantly different according to LSD at 5% level. Control, P1: 0.05% Pollen, P2: 0.15% Pollen, A: 0.1%Agar, B1: 0.01% boric acid, B2: 0.02% boric acid. All suspension media included 10% sucrose.

most species. It is known that, supplemented boric acid on sucrose medium had increased germination rate of pistachio pollen (Acar et al., 2010). This result confirms the role of this element especially with sucrose in improvement of pollen receptivity on the stigma in pollen viability. Meli et al. (2009) reported that boron solved in sucrose solution increased the fruit set, kernel dry weight, carpological parameters and kernel yield rate in pistachio trees. The kernel dry weight was increased with "P1 + A + B2" treatment compared with control. The agar component is contained Ca (0.5%) and Mg (0.1%) elements, therefore it can be concluded that agar had osmotic and nutrition effects on pollen germination and or fruit quality. The nutrient elements concentration by the spray method was higher than the control which may be due to decreased fruit set in this medium. Baninasab et al. (2007) reported that the fruiting status has significant influence on the amount of nutrient that is available in various organs of pistachio trees. Increased nutrient concentrations in the medium containing 0.15% of pollen may be due to a high proportion of these elements in pollen and elements leakage with pollen leaching in to aquatic environment. In conclusion, according to this study, it can be postulated that artificial pollination in pistachio using pollen suspension spray is possible. A full physiological and histological coverage for evaluating pollen suspension spray in pistachio is required.

Materials and methods

Plant material

The present study was carried out on Owhadi cultivar at pistachio orchards of Chatrud region; about 35 km to the north of Kerman in Iran. Pollen was obtained at the beginning of blooming from inflorescences collected from male trees. Inflorescences that had some flowers with dehiscent anthers were removed from the male trees, transferred to the laboratory, and placed on paper in constant room conditions (25˚C and 35% RH). Collected pollens was cleaned by passing it through a 100 µm mesh sieve and putted in a bottle. In *in vitro* pollen germination experiment, hand pollination was done out by soft brush that was considered as control and in spray pollination experiments in the field, open pollination was considered as control.

In vitro pollen germination

The media used for the experiment were as follows: (i) 10 % sucrose + 0.05% pollen grain (P1), (ii) 10 % sucrose + 0.15% pollen grain (P2), (iii) 10 % sucrose + 0.05% pollen grain + agar (P1 + A), (iv) 10 % sucrose + 0/15 % pollen grain + agar (P2 +A), (v) 10 % sucrose + 0.05 % pollen grain + agar + 0.01% boric acid (P1 +A + B1), (vi) 10 % sucrose + 0/15 % pollen grain + agar + 0.01% boric acid (P2 +A + B1),

Table 2. Effects of suspension media and pollination methods on fruit quality.

Pollination method	Suspension medium[a]	Nut					Kernel				
		Weight (g)		Size (mm)			Weight (g)		Size (mm)		
		Fresh	Dry	Length	Width	Thickness	Fresh	Dry	Length	Width	Thickness
Spray	P1	0.75 ab	0.46 b	1.67 a	1.14 a	1.05 b	0.70 ab	0.43 ab	1.44 a	0.75 b	0.75 b
Spray	P2	0.75 a	0.46 b	1.77 a	1.19 a	1.08 ab	0.81 ab	0.46 ab	1.54 a	0.82 ab	0.86 a
Spray	P1 + A	0.83 a	0.53 ab	1.76 a	1.20 a	1.07 ab	0.87 ab	0.51 ab	1.55 a	0.81 ab	0.84 a
Spray	P2 + A	0.89 a	0.61 ab	1.81 a	1.22 a	1.09 ab	0.89 ab	0.52 ab	1.55 a	0.83 ab	0.82 a
Spray	P1 + A + B1	0.84 a	0.54 ab	1.75 a	1.17 a	1.06 ab	0.69 ab	0.42 ab	1.45 a	0.84 ab	0.83 a
Spray	P2 + A + B1	0.83 a	0.53 ab	1.70 a	1.10 a	1.07 ab	0.75 ab	0.44 ab	1.53 a	0.83 ab	0.81 ab
Spray	P1 + A + B2	1.07 a	0.73 a	1.74 a	1.22 a	1.15 a	0.91 a	0.55 a	1.59 a	0.89 a	0.88 a
Spray	P2 + A + B2	0.87 a	0.53 ab	1.66 a	1.17 a	1.08 ab	0.87 ab	0.48 ab	1.59 a	0.84 ab	0.85 a
Control		1.06 a	0.71a	1.64 a	1.10 a	1.05 b	0.68 b	0.40 b	1.45 a	0.83 ab	0.83 a

Control: Open pollination, P1: 0.05% Pollen, P2: 0.15% Pollen, A: 0.1% Agar, B1: 0.01% boric acid, B2: 0.02% boric acid. All suspension media included 10% sucrose. Different letters within a column indicate significant differences by LSD Test.

Table 3. Effects of suspension media and pollination methods on kernel quality.

Pollination method	Suspension medium[a]	Oil (%)	Carbohydrat (%)	Nutrient concentrations				
				P (%)	Ca (%)	Mg (%)	Fe (mg/kg)	Cu (mg/kg)
Spray	P1	53.73 a	12.81 a	0.25 c	0.08 b	0.10 b	15.86 a	4.33 a
Spray	P2	53.69 a	15.59 a	0.26b c	0.13 a	0.10 b	20.73 a	5.40 a
Spray	P1 + A	52.13 ab	9.89 a	0.26b c	0.13 a	0.10 b	19.53 a	6.06 a
Spray	P2 + A	51.36 abc	13.01 a	0.42 a	0.15 a	0.13 a	20.20 a	5.80 a
Spray	P1 + A + B1	49.21 bc	12.93 a	0.24 c	0.14 a	0.11 ab	17.06 a	5.40 a
Spray	P2 + A + B1	49.95 bc	12.07 a	0.38 a	0.09 b	0.13 a	21.60 a	6.55 a
Spray	P1 + A + B2	48.71 c	10.89 a	0.28 bc	0.14 a	0.09 b	21.60 a	5.63 a
Spray	P2 + A + B2	44.62 d	12.664 a	0.35 ab	0.12 a	0.11 ab	18.83 a	5.66 a
Control		52.01 ab	12.39 a	0.27 bc	0.06 b	0.09 b	15.45 a	4.16 a

Control: Open pollination, P1: 0.05% Pollen, P2: 0.15% Pollen, A: 0.1% Agar, B1: 0.01% boric acid, B2: 0.02% boric acid. All suspension media included 10% sucrose. Different letters within a column indicate significant differences by LSD Test.

(vii) 10 % sucrose + 0.05% pollen grain + agar + 0.02 % boric acid (P1 + A+ B2), (viii) 10 % sucrose + 0.15 % pollen grain + agar + 0.02 % boric acid (P2 + A + B2). All media contained 10 % (w/v) sucrose to prevent explosion of the pollen grains. The collected pollen grains were suspended in media and 0.2 mL of each pollen grain suspension were spread on the surfaces of agar plates (1% (w/v) agar and 15% (w/v) sucrose), and incubated at 25 °C for 24 h in dark conditions. Four plates were used for each suspension. Control was cotained 15% (w/v) sucrose and 1% (w/v) agar and 100 mg L^{-1} boric acid (H_3BO_3). After incubation, the germination rates were assessed under a microscope. The pollen grains which produced a tube equal to their own diameter were counted as germinated. The germination percentage was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains per field of view (Acar et al., 2010).

Spray pollination experiments in the field

The field pollination experiments were performed in 2011 in pistachio orchards of Chatrud, Kerman, Iran. Twenty seven mature pistachio trees ('Owhadi'), selected in the field were considered as blocks on April 8th. Five shoots as sample were randomly selected in each tree. To prevent open pollination, female trees were isolated by using cotton bags before blooming. The experiment consisted of nine treatments in which natural pollination was used as controls in the determination of fruit set and pistachio nuts quality. Treatments application time was done when the female flowers opened and stigma became receptive between 9 to 12 am of April 12th, 14th and 16th, 2011. The pollen grains were prepared as described above. The spray pollination with the pollen suspensions was carried out using a hand sprayer. The pollen treated clusters were covered with cotton bags until April 23th.

Evaluation of the suspension media on fruit set and fruit quality

The number of produced fruits per cluster was counted in the three stages from the time of pollination to the harvesting time, to determine the percentages of primary, secondary and terminal fruit set. Harvesting was done at 13th September/2011 when the hulls separated easily from the shell. The clusters of each five shoots were collected in the cotton bags for analysis. After harvesting the nut size (mm), nut fresh weight (g), nut dry weight (g), kernel size (mm), kernel fresh weight (g), kernel dry weight (g), split nuts percentages, blank percentages and kernel percentages (kernel dry weight/nut dry weight × 100) were determined.

Evaluation of the suspension media on kernel quality

The powder was ashed in a muffle oven at 550 ± 25°C. The resulting white ash was then dissolved in 10 mL of 2N HCl and adjusted to volume of 100 mL for determination of macronutrients. The potassium and calcium content were determined by flame photometer. The spectrophotometric method was used for phosphorous determination. P, Ca, Mg, Fe and Cu concentrations was measured on kernel. The power was ashed in a muffle furnace (600°C, 4-5 h), the obtained ash was dissolved, resuspended in 1-2 mL of 1 NHCl and made up to 50 mL with distilled water. Phosphorus was determined colorimetrically using the molybdovanadate reagent; absorbance was read at 400 nm (Spectronic 70,

BAUSCH and LOMB). Atomic absorption spectrometry (Varian A-300, Varian Techtron Pty Ltd, Australia) was used to determine Mg, Fe and Cu (acetylene-oxygen flame), and Ca (acetylene nitrous-oxide flame). For carbohydrate measurement, 100 mg of powder were extracted three times in hot 80% ethanol. Total soluble sugars were determined using the anthrone reagent method (Schaffer et al., 1985). For oil measurement, the unshelled pistachio samples were ground to 0.6–0.8 mm in a Waring Laboratory type blender. Extraction of oil was carried out with petroleum ether in a Soxhlet apparatus (at 45–50˚C) for 8–9 h. The solvent was removed in a rotary vacuum evaporator. The oil content was determined as the difference in weight of dried samples before and after extraction (American Oil Chemist's Society, 1989).

Experimental design and statistical analysis

A randomized completely block design (RCBD), with three replicates (trees) was used. Analysis of variance (ANOVA) was performed using the SAS softwares. If ANOVA determined that the effects of the treatments were significant ($P \leq 0.05$), then the means were compared with the least significant difference (LSD) test.

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