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Evaluation of Breeding Strategies for Pistachio Male Genotypes Based on Flowering Index, Pollen Traits, and Female Yield Elements

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A R T I C L E I N F O

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A B S T R A C T

This study was carried out in the Khatam, Yazd province, Iran from the beginning of 2014 to the end of 2015. Phenological traits and pollen properties of some male pistachio genotypes 'A1', 'A2', 'A3', 'A4', 'A5', 'A6', 'A7', 'A8', 'A9', 'N1', 'Mh1', 'G1', 'M1', 'M2', and 'F1' were surveyed. To find the effect of pollen source on the nut traits of the resulting seeds as well as the initial fruit set, Xenia and Metaxenia were evaluated. Moreover, pistillate flowers of the 'Akbari' cultivar were crossed with different pollen sources. Results showed that pollen germination of male pistachio genotypes was various. Among all of the pistachio male genotypes, the highest pollen germination percentage was obtained from 'M1' (92.66%) followed by 'Mh1' (86.66%) and 'G1' (86.00%). There were also significant differences between cluster and pollen weight of different pollen sources. Based on our findings, among the studied genotypes, 'M1' had the longest pollen tube 391.6 (µm), as well as the maximum pollen tube growth rate of 39 (µm h^{-1}). The longest flowering periods were recorded to be 10 days in 'M1' and 7 days in 'Mh1', while the shortest period was 4 days in genotype 'A5' and 'A3'. A significant correlation was found between pollen grain characteristics and initial fruit set, pistachio ounce, nut blanking, and as well malformed fruits. Based on the results pollens of genotype 'M1' decreased the nut blanking and malformed fruits in 'Akbari' cultivar, pollens of genotype 'Mh1' improved the pistachio ounce, and genotype 'G1' increased the initial fruit set.

Introduction

Pistachio is the most grown fruit tree and adapted to semi- and -arid regions in the largely agricultural area of Iran (Eslami et al., 2019; Raoufi *et al.,* 2020b). The genus *Pistacia* L. belongs to the Anacardiaceae family comprising 83 genera and 860 species (Raoufi *et al.,* 2020a). Pistachio is a deciduous, dioecious, and wind-pollinated subtropical fruit tree (Acar and Ak, 2001; Raoufi *et al.,* 2021) that originated from Iran (Roozban *et al.,* 2006; Mahmoudi and Odivi, 2014; Raoufi *et al.,* 2020c). Dioecious means the pistillate and staminate flowers are produced in large clusters on different trees. Apetal pistachio female flowers attract no honey bees to facilitate pollination; thus, pollination is normally performed by the wind (Sharifkhah et al., 2020). In male trees, with a more vigorous growth habit flower opening starts from the end of March. For the optimal management of pistachio orchards, the right number and type of male trees considered to be planted between female trees is a highly important factor because of proper pollination. (Barzamini and Fotouhi Ghazvin, 2017; Mahmoudi and Odivi, 2014). For commercial production of pistachio, approximately one male tree is needed for eight to 11 females (Maranto and Crane,

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1982; Mahmoudi and Odivi, 2014), however, in addition to the insufficient number of male trees, the quality of pollen in most orchards is not good and the flowering of male and female trees does not overlap, which is called dichogamy (Ozeker *et al.*, 2006). In such conditions, the effective pollination period is limited; therefore, more homogamous male pistachio trees are needed (Ozeker *et al.*, 2006). To achieve the maximum fruit set, most of the pollen grains should remain viable in the *in vivo* condition for two or three days after the male flower anthesis (Acar, 2004; Crane and Maranto, 1989). In the case of insufficient pollination, blank nut forms, this phenomenon is called blanking in which only fruit pericarps develop (Kaska, 1994). The phenology of male trees, as well as the pollen grain of pistachio, has been already well-studied. These studies have introduced pistachio male genotypes, cultivars, and individual hybrids (Kader, 1982; Martinez and Herrero 1994; Atli *et al.*, 1995; Cruzan and Barrett 1996; Acar 2004; Afshari *et al.*, 2008). They have also reported different pollen characteristics, such as pollen germination percentage, pollen germination rate, amount of pollen production, pollen weight, as well as pollen tube growth, which may provide some information on pollen performance (Cruzan and Barrett, 1996). Moreover, pollen viability, pollen tube growth, and morphological characterization of pollen grains have been defined as important characteristics of male plants (Poloti and Luza, 1988; Cruzan and Barrett, 1996). Acar and Ak (2001) reported a higher germination rate (approximately between 72- 94%) in pistachio pollens. Furthermore, genetic improvement of the male tree can modify the characterization of pollen grains. Therefore, breeding programs such as the selection of pollinators are needed to improve the potential of new pistachio orchards (Bolat and Pirlak, 1999; Martinez and Herrero, 1994). Additionally, the morphology of the pollen grain, containing its shape, pores, size, and exine pattern has been proposed for the classification and identification of male pistachio cultivars (Fogle, 1977). In addition, some studies have announced that the size and shape of pollens are different and unstable within species and cultivars (Talie and Imani, 1998). Similarly, the effects of a particular pollen source on seed (xenia) or fruit (metaxenia) traits in several nut crops have been well-known. In the literature, pollinator's agent has been reported to be effective in fruit setting index, increasing nut and kernel weight, and decreasing blank percentage (Rahemi and Mojadad, 2001). Xenia and metaxenia have been reported in almond, fig, chestnut, pecan, hazelnut, and walnut (Marquard, 1988; Kumar and Das, 1996; Rahemi and Jafari, 2008; Golzari *et al.*, 2016). Riazi *et al.* (1996) reported that open pollination increased kernel weights in three pistachio cultivars. However, since the effect of pollen source on nut traits of pistachio has been less studied in detail, the current work aimed at examining the correlations between pollen grain characteristics and pistachio yield elements.

Materials and Methods

Plant material

The present study was carried out in Khatam county, Yazd province, Iran from the beginning of 2014 to the end of 2015. In the first year (2014), we standardized fertilizing, pruning, foliar application, irrigation, pest, and weed management conditions for different male genotypes. Pollen grains were collected from fifteen different mature male genotypes of pistachio trees, including ‘A1’, ‘A2’, ‘A3’, ‘A4’, ‘A5’, ‘A6’, ‘A7’, ‘A8’, ‘A9’, ‘N1’, ‘Mh1’, ‘G1’, ‘M1’, ‘M2’, and ‘F1’. Male clusters were removed from these trees in the bulk stage and transferred to the laboratory and dried in shady condition around 20-22 degrees centigrade (°C) for 3 days. Pollen that was shed overnight was sieved and collected under laboratory conditions and preserved in freeze condition at -20 degrees centigrade (°C).

Pollen collection and growth medium

In vitro germination was assessed with a simple medium containing 15% sucrose, 1% agar, and 0.1% boric acid (Ebrahimzadeh *et al*., 2021; Karimi and Zeraatkar, 2015). First, pollen grains were sown on a medium with a clean brush. Next, the medium was preserved for 6 hours at 25 degrees centigrade (°C) in dark conditions to let us measure the pollen growth parameters.

Pollen diameter, in vitro pollen germination, and pollen tube growth

The pollen germination parameter was assayed by direct microscopic observation (10×). For each male pistachio genotype, germination was recorded at three petri dish points after 6, 16, and 24 hours of culture. A pollen grain was considered germinated when the pollen tube length was at least equal to or greater than the grain diameter. Germination percentage was also determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains per field of view. Measurements of pollen diameter and pollen tube length were recorded directly by an ocular micrometer fitted to the eyepiece of the microscope (Fig. 1).

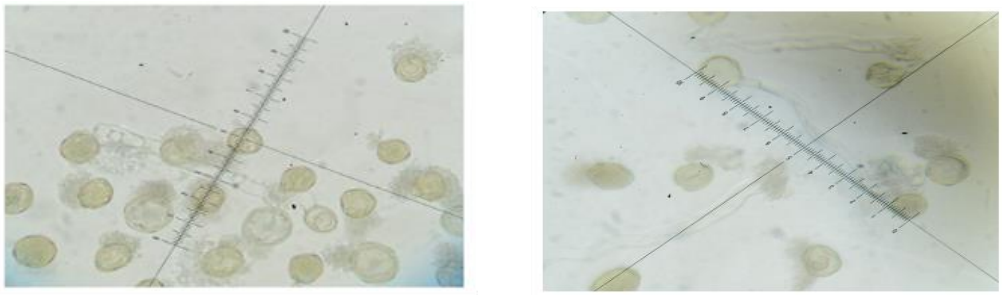

Fig 1. Pollen tube growth in vitro in two pistachio male genotypes (M2 (left), and M1 (Right) for up to 24 h pollen germination.

Pollen tube growth rate

The rate of pollen tube growth (μm h^{-1}) was measured 16 and 24 hours following tube emergence.

Controlled pollination

In the ‘Akbari’, all in 'on year' for bloom; the female clusters were first enclosed with double cheesecloth bags to exclude unwanted pollination before anthesis in the green tip stage. The stigma in pistillate flowers on the selected branches was then pollinated by hand at the first stage of full bloom (Mahmoudi and Odivi, 2014). The bags were removed 10 days after pollination and the fruit set was recorded after 3 weeks. Blanking nuts, malformed nuts, as well as the number of shelled nuts per ounce were determined for every 6 replicates, as discussed later.

Pistachio yield elements

final fruit set (percentage of blossoms which end up forming fruits), blanking nuts (determined by dividing the number of seedless nuts by the total number of fruits), data on the percentage of malformed nuts, as well as the number of shelled nuts per ounce, were determined by the number of pistachio nuts per ounce (28.3495 g).

Statistical analysis

Experiments were carried out as a completely randomized design (CRD) and each treatment had 6 replications. The data were statistically analyzed using (ANOVA) for in vitro pollen germination testing and

investigation of cluster and pollen weight per genotype, respectively. The significance of differences among mean values was determined by Duncan's multiple range tests at P≤0.01 and P≤0.05. Data analysis was done by SAS software (9.2).

Results

Cluster and pollen grain weight

Genotypes differed significantly in their cluster and pollen weight (P≤0.05) (Table 1). Cluster weight for various male genotypes ranged from 2.5 (g) for 'A2' to 4.2 (g) for 'G1' and 'N1'. Furthermore, pollen grain weight for different pistachio male genotypes ranged from 266.3 (mg) for 'N1' to 77 (mg) for 'A4'. Pollen grain quantity or pollen production of genotypes 'N1', 'M1', and 'F1' were also found to be significantly higher than the other genotypes (P≤0.05). On the contrary, the lowest amount for pollen grain weight index was observed for genotypes 'A4', 'A6', 'A3', and 'A5'.

Table 1. Compersion of cluster weight (gr) and pollen grain weight (mg) in male pistachio genotypes.

Genotypes traits	G1	N1	A6	A8	Mh1	A3	M1	F1	A5	A9	A7	A4	M2	A1	A2
cluster weight (gr)	4.2^{a}	4.2^{ab}	4.2^{ab}	3.9^{abc}	3.9^{abcd}	3.9^{abcd}	3.8^{abcd}	3.0^{de}	3.4^{abcd}	3.3$^{bc}_{de}$	3.1^{cde}	3.1^{cde}	3.1^{cde}	3de	2.5^{e}
Pollen grain weight (Mg)	182^{c}	266.3^{a}	81^{f}	142.3^{d}	185.6^{c}	104.6^{ef}	233.6$_{b}$	263.7^{a}	130.6^{de}	182^{c}	130.3^{de}	77^{f}	124de	117de	139.6^{d}
Pollen grain diameter (µm)	60.1^{ab}	61^{a}	46^{f}	50.1^{de}	60.1^{ab}	47.6^{ef}	62.8^{a}	49.8^{de}	47.6^{ef}	57.5$^{b}_{c}$	52.3^{d}	45.6^{f}	57.1^{bc}	56.5^{c}	58^{bc}

Different letters indicate significant differences using Duncan's multiple range test at a 5% level *

Pollen germination

Fifteen genotypes of male pistachio were evaluated in terms of the percentage of their pollen germination in *vitro* (Fig. 2). The percentage of pollen germination ranges from 0.00% ('A4' and 'A6') to 44.00% ('M1') after 6 hours of culture; after 16 hours of culturing, this range was from 50.00% ('A4, 'A5', 'A6', 'F1') to 70.00% ('M1'); and finally, after 24 hours of culture, it was from 51.00% ('A5') to 92.66% ('M1'). Moreover, based on the reported results, pollen germination started 6 hours after culturing in *vitro* conditions in all genotypes, except for 'A4' and 'A6'.

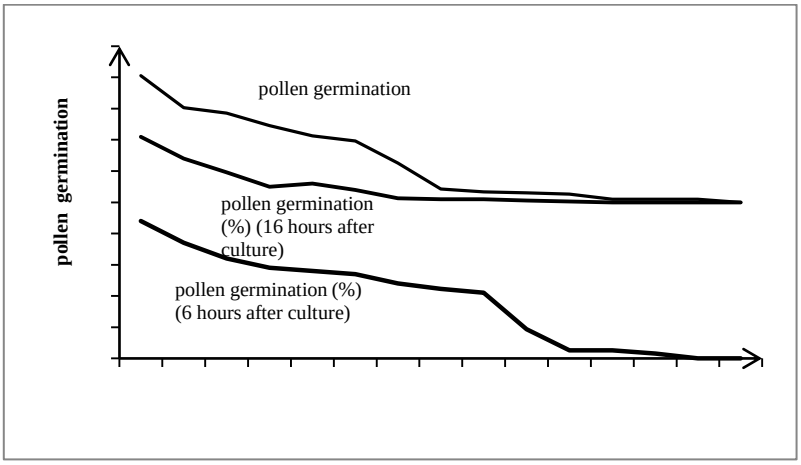

Fig. 2. Pollen germination percent in different genotypes (6, 12, and 24 h after pollen culture)

Pollen Diameter

As regards the average values, pollen grains diameter was approximately similar in pollen weight index. Pollen grain diameter for different pistachio male genotypes ranged from 62.8 (µm) for 'M1' to 45.6 (µm) for 'A4'. The sequence of pollen shape

growth in vitro in two pistachio male genotypes 'M1' and 'M2' for up to 24 hours is shown in Fig. 1.

Pollen tube length

According to the obtained results, mean tube length (µm) was affected by different pistachio male genotypes 6 hours after the emergence of the pollen tube ($P \leq 0.05$) (Table 2). Pollen tube length for different pistachio male genotypes ranged from 53.8 (µm) for 'M1' to 37.5 (µm) for 'A6'. However, 16 hours after the emergence of the pollen tube, the mean tube length was not affected by different pollen sources. Moreover, we noticed that 24 hours after the emergence of the pollen tube, the 'M1' genotype had the highest tube growth of 391.6 (µm).

Table 2. pollen tube length (µm) of male pistachio genotypes up to 24 h of emerging

Genotypes	Mean tube length (µm)		
	After 6 h	After 16 h	After 24 h
M1	58.3a	79.1a	391.6a
Mh1	54.1a	70.8a	329.1bcd
G1	50ab	75 a	337.5bc
N1	45.8ab	79.1a	358.3ab
A1	50ab	70.8a	366.6ab
A2	50ab	70.8a	345.8bc
A9	50ab	70.8a	350abc
A7	50ab	66.6a	358.3ab
M2	50ab	58.3a	350abc
A8	54.1a	70.8a	354.1ab
F1	54.1a	66.6a	325bcd
A3	54.1a	70.8a	325bcd
A5	50ab	62.5a	341.6bc
A4	54.1a	66.6a	308.3cd
A6	37.5b	50a	287.5d

* Different letters indicate significant differences using Duncan's multiple range test at a 5% level.

Pollen tube growth rate

About the mean values in the first 16 hours after emergence, the pollen tube growth rate of various pistachio male genotypes did not differ (Table 3). However, 24 hours after the emergence of the pollen tube, the maximum growth rate was recorded for 'M1' genotype 39 (µm h^{-1}).

Table 3. Pollen tube growth rate (µm h^{-1}) of male pistachio genotypes up to 24 h of emerging

Genotypes	Mean tube growth speed (µm h^{-1})	
	After 16 h	After 24 h
M1	2.08a	39.0a
Mh1	1.6a	32.2bc
G1	2.5a	32.8bc
N1	2.91a	34.8abc
A1	2.08a	36.9ab
A2	2a	34.3abc
A9	2.08a	34.8abc
A7	1.6a	36.4ab
M2	1.2a	36.4ab

A8	1.2a	35.4abc
F1	1.2a	32.2bc
A3	1.6a	31.7bc
A5	0.8a	34.8abc
A4	1.2a	30.2c
A6	0.4a	29.6c

* Different letters indicate significant differences using Duncan's multiple range test at a 5% level

Assessment of phenological traits (Flowering period length)

According to our results, the flowering of the male pistachio genotypes mostly occurred between March 26th and April 13th (during 18 days). Among the assayed genotypes, the longest flowering period was recorded to be 10 and 7 days in genotypes 'M1' and 'Mh1', respectively. On the other hand, the shortest flowering period was 4 days in genotype 'A5' and 'A3' (Fig. 3). Also, our results introduced 'Mh1' as a late-flowering genotype. The flowering of 'Mh1' male pistachio genotypes occurred between April 6th to April 15th, Since the flowering time of 'Akbari' female cultivar was occurred during the second and third weeks of April, 'Mh1' can be more homogamous with the 'Akbari' female cultivar.

Fig. 3. Flowering period length in survived male pistachio genotypes.

Controlled pollination results

Fruit set

Based on the results, Akbari's fruit set was affected by different pollen sources ($p \leq 0.01$) (Table 4). For example, compared with open pollination, Akbari's fruit set increased by 19.54% using a 'G1' pollen source. Moreover, the fruit set of 'Akbari' pollinated with 'M1' and 'Mh1' increased by 16.5 and 15.03%, respectively. Contrarily, compared with open pollination, 'Akbari's fruit set decreased by 1.12% using the pollen of 'A4' (Table 4).

Table 4. Effect of pollen sources on fruit setting and nut characteristics of pistachio female Akbari cvs.

Male Genotypes	Female Akbari cvs ♀			
	Fruit set (%)	Blanking (%)	Ounce	Malformed Fruit (%)
M1♂	77.5b	3.3e	21.6abc	0.4cd
Mh1♂	76.5b	6ab	20.5d	0.4bcd
G1♂	79.5a	3.7cde	21.6abc	0.4d
N1♂	77.2b	3.8cde	21.3bcd	0.4bcd
A1♂	77b	4.5bcde	21.6abc	0.5abcd
A2♂	66.7cd	6.1ab	22.3a	0.6ab
A9♂	67.5c	5.3abcd	22ab	0.5abcd
A7♂	66.2cd	4.8abcd	21.6abc	0.6ab
M2♂	66.2cd	3.5de	21.1cd	0.5abcd
A8♂	66d	5.3abcd	22.4a	0.62ab
F1♂	66d	6.6a	22.2a	0.5abcd
A3♂	66d	5.9ab	22ab	0.62ab
A5♂	66.2cd	5.4abc	22.4a	0.5abc
A4♂	65.7d	6.6a	22.5a	0.6ab
A6♂	66.2cd	6.62a	22.4a	0.65a
Open pollination♂	66.5cd	5.07abcd	22.4ab	0.62ab

* Different letters indicate significant differences using Duncan's multiple range test at a 5% level

Nut blanking

In the present study, we observed the significant effect of pollen sources on nut blanking. According to results, using 'M1', as a pollinator, reduced the nut blanking by 34.91% (Table 4) compare to open pollination, 'Akbari' s nut blanking increased by 30.57% using the pollen of 'A6'.

Pistachio ounce

Pistachio ounce was also significantly ($P \leq 0.01$) affected by the type of pollen in a way that the 'Akbari' cultivar pollinated with 'Mh1' produced fruits with the best ounce (8.48% higher than open pollination treatment) (Table 4). A 0.44% decrease was also observed in pistachio ounce in 'Akbari' cultivar pollinated with 'A4' in comparison with the open pollination treatment.

Malformed fruit

'Akbari' cultivar pollinated with 'G1' produced significantly fewer malformed fruits than other pollen sources (Table 4). Compared with open pollination treatment, pollen of the 'G1' pollinator reduced the malformed fruits by 35.48%. On the contrary, in the 'Akbari' cultivar, pollen of 'A6' significantly increased malformed fruits (4.82%) more than open pollination treatment (Table 4).

Correlation between pollen grain characteristics and nut traits

In the current research work, we found the following significant correlations; between pollen grain diameter and initial fruit set (0.76^{**}), between pollen grain diameter and nut blanking (-0.69^{**}), between pollen grain diameter and pistachio ounce (-0.70^{**}), and between pollen grain diameter and malformed fruits (-0.77^{**}) (Table 5). Based on the result, a positive association was observed between blanking (0.77^{**}) and the percentage of pollen germination within the first 6 hours of the culture medium (Table 5). On the other hand, in the first 6 hours in the culture medium, negative correlations were found between the nut blanking and the percentage of pollen germination (-0.66^{**}), the percentage of pollen germination and pistachio ounce

(-0.71**), and the percentage of pollen germination and malformed fruits (-0.70**). Moreover, the Pearson correlation results showed that as the percentage of pollen germination increased, 'Akbari's fruit set increased by .80 and .89% within the first 6 and 24 hours of culture medium, respectively, and the percentage of pollen germination (Table 5). Also, the correlation trend between the malformed fruits and the percentage of pollen germination was similar to the initial fruit set. Additionally, strong positive correlations were found between the tube growth rate and 'Akbari's fruit set in the first six h of the culture medium (0.77**). For this cultivar, there was no significant correlation (-0.29 n.s) between the initial fruit set and tube growth rate within the first 24 hours in the culture medium. Contrarily, using the Pearson correlation formula, we found a significant negative correlation (-0.58*) between 'Akbari's nut blanking and its pollen tube growth rate in the first 6 hours in the culture medium. Finally, the rate of pollen tube growth showed no significant correlation with neither of the following variables; pistachio ounce (-0.34 n.s), abnormal fruits (-0.46 n.s), and the fruit set (0.43 n.s). The only variable in which the rate of pollen tube growth had a significant negative correlation was nut blanking (-0.76**) (Table 5).

Table 5. Pearson correlation coefficients between pollen grain characteristics and nut traits of pistachio Akbari cultivar

Male Genotypes	Female Akbari cvs			
	Fruit set (%)	Blanking (%)	ounce	Malformed Fruit (%)
Pearson Correlation Coefficients with pollen diameter	0.76	-0.69	-0.70	-0.77
Significant	**	**	**	**
Pearson Correlation Coefficients with pollen germination% up to 6h	0.77	-0.66	-0.71	-0.70
Significant	**	**	**	**
Pearson Correlation Coefficients with pollen germination% up to 16h	0.80	-0.48	-0.56	-0.71
Significant	**	n.s	*	**
Pearson Correlation Coefficients with pollen germination% up to 24h	0.89	-0.54	-0.57	-0.77
Significant	**	*	*	**
Pearson Correlation Coefficients tube growth rate up to 16h	0.77	-0.58	-0.44	-0.60
Significant	**	*	n.s	n.s
Pearson Correlation Coefficients tube growth rate up to 24h	0.29	-0.75	-0.30	-0.35
Significant	n.s	**	n.s	n.s
Pearson Correlation Coefficients pollen tube length up to 24h	0.43	-0.76	-0.34	-0.46
Significant	n.s	**	n.s	n.s

Means followed by the same letters are not significantly different (P≤0.01 and P≤0.05). Prob > |r| under H0: ρ=0

Discussion

Among all the studied genotypes, the highest pollen germination percentage was obtained from 'M1' (92.66%) followed by 'mh1' (80.33%) up to 24 hours. Pollen germination percentages of the male genotypes as the main factor have been also reported by other researchers (Martinez and Herrero, 1994; Atli *et al.*, 1995; Cruzan and Barrett, 1996; Acar, 2004; Afshari *et al.*, 2008). It has been well-documented that in pistachio fruit trees, protandry in which male flowers spread their pollen before female

ones becoming receptive is a common phenomenon (Ak, 1992). Previous research indicated that the flowering period of male trees was shorter than female's, in other words, to produce the suitable yield, the flowering time of both male and female flowers need to be the same (Ak, 1992). Moreover, based on our results, the longest flowering period was recorded 10 days in 'M1'. In the present work, we also managed to introduce 'Mh1' as a late flowering genotype, which can be more homogamous with the ''Akbari' female cultivar. 'M1' is a genotype with medium-flowering and long-blooming periods. Since in the present study, we confronted weak pollination overlap between male and female trees and given that most male pistachio genotypes, so this is comfortable genotypes for late flowering female genotypes as well as 'Mh1'. According to previous on pistachio male cultivar selection 'Ask' and 'Gazvin' were introduced from Israel-based phenological considering (Ak, 1992). Several of new pollen sources have been evaluated as part of the pistachio breeding program conducted by Parfitt (1997). These pollen sources were evaluated for the quantity and quality of their pollen grains, the length of their flowering pollen period, and their pollen durability. Also, our findings of the present study showed that pollen grain weight for different pistachio male genotypes ranged from 266.3 (mg) for 'N1' to 77 (mg) for 'A4'. Based on our findings, the 'M1' genotype had the longest pollen tube 391.6 (μm) as well as the maximum pollen tube growth rate of 39 (μm h^{-1}) among the studied genotypes. Significant correlations were found between pollen grain characteristics and initial fruit set, pistachio ounce, nut blanking as well malformed fruits. In this study, results of examining the effect of pollen type on fruit set and nut characteristics are in agreement with results reported by Osman *et al.* (1974); Mehlenbacher and Smith (1993); Javadi and Gheshlaghi (2006). Unlike our findings, Riazi *et al.* (1996) showed that open pollination produced heavier nuts on three pistachio cultivars. This implied that compared with open pollination, pistachio ounce improved by using the pollen of 'Mh1', 'N1', 'A1', and 'M1'. Based on our results, in the 'Akbari' cultivar, pollens of 'M1' decreased the nut blanking and malformed fruits, while pollen of 'G1' increased the initial fruit set. Rahemi and Mojadad (2001) also proved the pollinator agents' effects on fruit setting index. They also verified the effect of these agents on increasing nut and kernel weight and decreasing the percentage of nut blanking.

Conclusions

In conclusion, we found significant correlations between pistachio pollen traits and yield elements. At present we can make a relatively accurate prediction by calculating pollen grain traits on pistachio nut and fruit. Finally, 'Mh1', as a late flowering genotype and 'M1' as a medium flowering, can be more synchronized with 'Akbari' female pistachios as well as other late-flowering female cultivars.

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