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Self-incompatibility in the Iranian Almond Cultivar 'Mamaei' Using Pollen Tube Growth, Fruit Set and PCR Technique

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Abstract

Self-incompatibility has been studied by using controlled pollination, pollen tube growth and PCR methods in the Iranian almond 'Mamaei.'. Pollen tube growth and fruit set following self and cross-pollination treatments were evaluated. The percentage of initial and final fruit set was determined for each treatment at 30 and 60 days after controlled pollination. Pollen germination and pollen tube growth were assessed by fluorescence microscopy at different times after self and cross pollination. Results showed that the percentage of the final fruit set was 0% after self-pollination, while values of 16.34%, 17.22%, 19.12%, and 21.15% were determined after cross-pollination with 'Azar', 'Rabie', 'Shahrood-21', and 'Sefied' cultivars as pollen sources, respectively. After 192 hours, observation of pollen tube growth showed that the percentage of reached pollen tubes at the style base from cross-pollination was significant but there were not any reached pollen tubes from self-pollination. According to the results of controlled pollination and pollen tube growth 'Mamaei' is self-incompatible. *S*-RNase assay was used to confirm these results. PCR amplification of genomic DNA from 'Mamaei' with EM-PC2consFD and EM-PC3consRD primers revealed the presence of two DNA fragments of sizes around 850 bp and 1250 bp on agarose gels. The size of the smaller fragment is similar to that of S_{25} almond RNase, while the size of the other fragment is different from all S_1-S_{30} RNase alleles. *S*-genotype can be regarded as $S_{25}S^x$, with S_x being a new *S*-RNase allele.

Keywords: Controlled pollination, fluorescence microscopy, *Prunus dulcis*, *S*-RNase alleles.

Introdution

Almond (*Prunus dulcis* L.), as a species of genus *Prunus*, is one of the most important cultivated fruit trees in Iran (Rahemi 2002; Talaei 2006). Fertilization is essential for almond commercial production but most almond cultivars are self-incompatible (Socias i Company, 1990; Kester and Gradziel, 1996). Self-incompatibility in almond is gametophytic and controlled by a single S-locus with multiple codominant alleles (Dicenta and Garcia 1993). The main regions of almond production in Iran are Chaharmahal va

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Bakhtiari, Azerbaijan, Khorasan, Esfahan, Fars, Yazd and Kerman provinces. The most cultivated Iranian almond cultivars are 'Mamaei', 'Sefied', 'Rabie', 'Shokufe', 'Azar', and 'Sahand' (Imani *et al*., 1996, Moradi *et al*., 1999; Moradi and Mousavi 1999; Rahemi 2002). 'Mamaei' is one of the main Iranian almond cultivars and 65% of almond orchards were established with this cultivar in Chaharmahal va Bakhtiari province (Moradi and Mousavi, 1999). Self-incompatibility has traditionally been assessed by recording fruit set following controlled pollination in the field and also by observing pollen tube growth following the self-pollination of flowers in the laboratory (Socias i Company and Felipe, 1977, Kester *et al.*, 1994, Lopez *et al*., 2004). In recent years, molecular methods based on PCR and length polymorphism in the two introns have been developed to accelerate the determination of *S*-genotypes in almond (Tamura *et al.*, 2000; Channuntapipat *et al.*, 2001, 2003). Degenerate primers were designed based on the S-RNase conserved regions of *Prunus* species (Sonneveld *et al.*, 2003, Sutherland *et al.*, 2004, Ortega *et al*., 2005). The different combinations of these primers were used to identify S-genotypes in almond and consensus primers designed from sequences of C2 and C3 regions that covered the second intron successfully discriminated the S-alleles in almond (Sutherland *et al.*, 2004, Ortega *et al*., 2005). Thus, the study of self-incompatibility and determination of the S-genotype in almond cultivars is useful for orchard design and for parental choice inbreeding programs (Ortega *et al*., 2005 and 2006, Mousavi *et al*., 2011a, b).

The objective of this study was to study the pollination and self-incompatibility of the commercial Iranian almond Cultivar 'Mamaei' by using controlled pollination, pollen tube growth and PCR methods.

Materials and Methods

Plant Material

This study was carried out on 12-year-old 'Mamaei' trees that were growing in a almond orchard at Emamiye Station (32° 30′ N, 50° 58′ E, elevation 1900 m) during 2006-2007 years in Saman region, 40 km southeast of Shahrekord in Chaharmahal va Bakhtiari Province, Iran.

Evaluation of self-incompatibility

The self-incompatibility of "Mamaei" was evaluated in three different methods: (1) fruit set after self and cross pollination, (2) observation of the pollen germination on stigma and pollen tube growth along the style by fluorescence microscopy, and (3) S-alleles identification using PCR technique with the consensus primers for the second intron of *S*-RNases.

Fruit set at orchard

The evaluation of the self-pollination on fruit set was carried out on completely randomized blocks design (CRBD) with three replications on the same almond trees (cv. 'Mamaei') in two consecutive years (2006-2007). From each tree, four branches with average of 100 flower buds at the balloon stage within the middle third of the canopy were randomly selected around the trees and bagged with thin-cloth bags to prevent insect pollination. The bags were removed after three weeks and the percentage of initial and final fruit set was determined for each treatment at 30 and 60 days after pollination. (Ortega *et al*., 2007). In addition, initial and final fruit sets were evaluated on 'Mamaei' by cross-pollination by using pollen sources from 'Azar', 'Rabie', 'Shahrood-21', 'Sefied' and 'Mamaei' cultivars in order to select the best pollinizer.

Pollen tube growth:

From each treatment, 80 flower buds in the Popcorn stage (D stage) were selected and emasculated. After 24 hours, the pistils were self and cross pollinated in

field condition. The pistils were collected 1, 2, 4, 6 and 8 days after self and cross pollination and fixed in FAA solution and prepared for fluorescence microscopy (Imani et al., 1998; Dicenta et al., 2002; Lopez et al., 2004). The pollen germination and pollen tube growth were assessed on stigma and in different parts of style by UV fluorescence microscopy at 24, 48, 96 and 192 hours after self and cross pollination.

S-allele identification using PCR technique

DNA extraction:

Genomic DNA was extracted from young leaves of 'Mamaei' cultivar using the CTAB extraction method based on Doyle and Doyle (1987) that was described in Sonneveld *et al*. (2001) and modified by Ortega and Dicenta (2003).

PCR amplification:

To amplify the second intron of almond S-RNases, the consensus primers EM-PC2consFD (5´-TCA-CMA-TYC-ATG-GCC-TAT-GG-3´) and EM-PC3consRD (5´-AWS-TRC-CRT-GYT-TGT-TCC-ATT-C-3´) were used (Sutherland et al. 2004). The PCR reaction and cycling conditions were described for almond (Sutherland *et al*., 2004, Ortega *et al*., 2005). DNA from 15 almond cultivars previously genotyped was included as reference for the almond *S*-RNase alleles *S1-S29* and S_f (Ortega *et al*., 2005). PCR products of 'Mamaei' separated in a 1.5% TAE agarose gel alongside PCR products of the reference cultivars and the 1Kb DNA ladder and stained with ethidium bromide. Finally, amplification product sizes in 'Mamaei' were compared with reference alleles in reference cultivars (Table 1) and DNA ladder 1Kb plus (Invitrogen, Carlsbad, CA, USA).

Table 1. S-genotype in almond cultivars as reference alleles.

Cultivar	S-genotypes	Origin	Cultivar	S-genotypes	Origin
Ardechoise	S_1S_{10}	France	La Mona	$S_{23}S_{25}$	Spain
Avellanera Gruesa	$S_{22}S_{26}$	Spain	*Marcona A.D.*	$S_{11}S_{12}$	Spain
CEBAS-I	S_4S_{13}	Spain	Primorskyi	S_5S_9	Ukrain
Cristomorto	S_1S_2	Italy	Ramillete	S_6S_{23}	Spain
Ferragnes	$S1S_3$	France	Rumbeta	$S_{11}S_{21}$	Spain
Fina del Alto	$S_{28}S_{29}$	Spain	Titan	S_8S_{14}	USA
Fournat de Brezenaud	$S_{24}S_{27}$	France	A2-198	S_fS_f	Spain
IXL	S_7S_8	USA			

Statistical analysis

All the statistical analysis was performed with the SAS programme (SAS Institute, 2000). Prior to analysis, percentage values of fruit set were transformed by calculating the arc sin value of the square root. Mean values were analyzed by Duncan's multiple range test.

Results

Fruit set evaluation

The results of variance analysis of pollen source on fruit set showed that the initial and final fruit sets had significant difference among pollen sources and year (Table 2). According to results in 2007, the percentage of initial fruit set at 30 days after pollination in 'Mamaei' cultivar was 0% after self-pollination while values of 22.40 %, 25.88%, 27.96%, and 29.60% were determined after cross-pollination with 'Azar', 'Rabie', 'Sefied' and 'Shahrood-21' cultivars as pollen source, respectively (Fig. 1). The percentage of final fruit set at 60 days after pollination was 0 % after self-pollination but was determined 16.34%, 17.22%, 19.12%, and 21.15% after cross- pollination with 'Azar', 'Rabie',

'Shahrood-21', and 'Sefied' cultivars as pollen source, respectively (Fig. 2).

Table 2. Analysis of variance of Pollen source on fruit set after self- and cross-pollination (%)

SOV	df	MS	
		Initial fruit set (%)	Final fruit set (%)
Rep	2	0.469	0.119
Year	1	215.42**	264.21**
Pollen source	4	883.49**	429.37**
Interaction effect	4	13.79**	16.55**
Error	18	0.299	0.078

**: Significant differences at 1% level

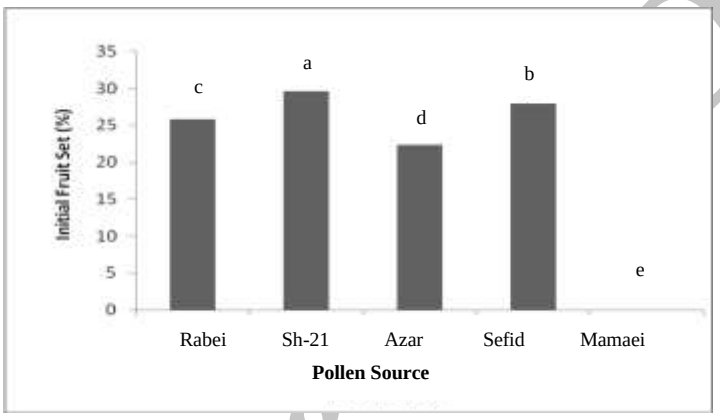


Fig.1. Percentage of initial fruit set for the different Pollen sourcesfollowing self and cross pollination of 'Mamaei' almond in 2007

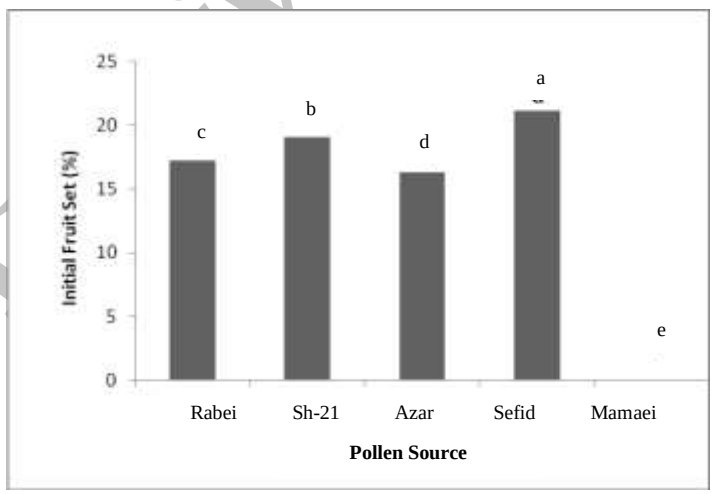


Fig.2. Percentage of final fruit set for the different Pollen sources following self and cross pollination of 'Mamaei' cultivar in 2007

Pollen tube growth

The results showed that there were significant differences among cultivars for pollen germination and pollen tubes growth in different parts the style from self and cross-pollination (Table 3). The results showed that there was a significant difference in the percentage of pollen germination from self- and cross-pollination in 'Mamaei' cultivar (Fig.2). Although there was a difference in the percentage of pollen germination from self-

and cross-pollination, pollen germination from self-pollination was normal (88.60 %). The pattern of pollen germination was very similar for all pollen sources in all cultivars (Fig.2). Results of variance analysis showed that the effect of pollen sources was significant for the percentage of pollen tubes in the deferent parts of the style (Table 3). Figure 3 (a, b, c and d) showed the pollen germination and pollen tub growth from cross pollination. After 48 hours, percentage of pollen tubes in 1/4 of style was significant from self and cross-pollination among cultivars. Reached pollen tubes in this style part from self pollination was less than cross-pollination (Table 4). After 96 hours, the percentage of reached pollen tubes from cross-pollination in 3/4 of style had significant differences (Table 4). According to the results, the percentage of reached pollen tubes at the style base from cross-pollination had not significantly varied among cultivars after 192 hours, but there were no reached pollen tubes from self-pollination (Table 4).

Table 3. Analysis of variance of pollen germination and pollen tube growth indifferent parts of style after self- and cross-pollination (%) in 2007

SOV	df	MS			
		Germination (%)	48 h(1/4 style)	96 h(3/4 style)	192 h(base)
Replication	2	0.37	1.27	0.22	6.26
pollen source	4	15.91**	860.28**	75.42**	48.48**
Error	8	0.172	5.267	0.246	6.887

Significant differences at 1% level

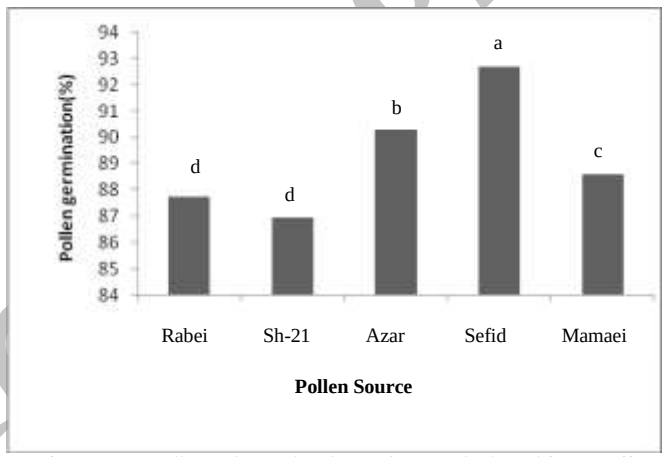
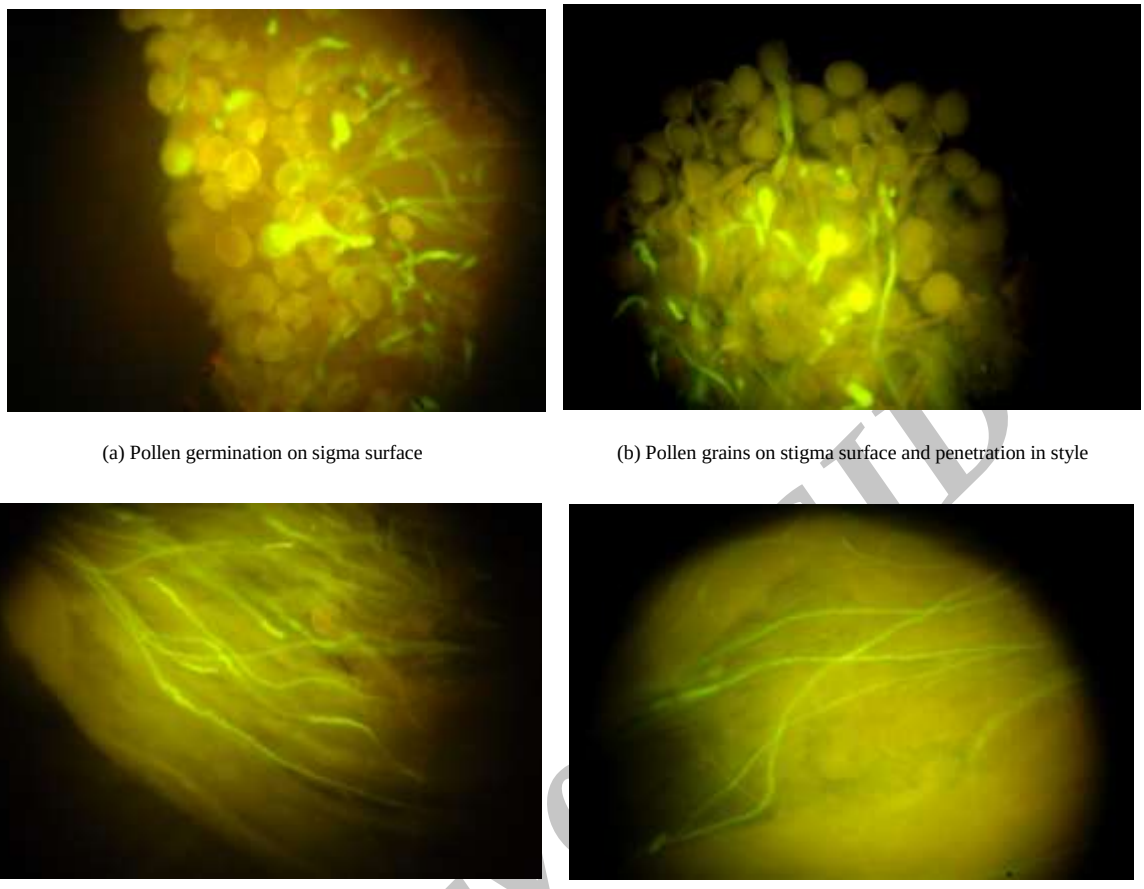


Fig.3. Percentage of germinated pollen grains on the stigma of 'Mamaei' almond for the different Pollen sources following self- and cross- pollination in 2007

Table 4. Mean values for pollen tubes growth in different parts of style in 'Mamaei' for the different pollen sources following self and cross pollination in 2007

Cultivar	Pollen tube growth in different parts of style (%)		
	at 1/4 of style(48 h)	at 3/4 of style(96 h)	at base of style(192 h)
Azar	59.00c	11.10b	15.07a
Mamaie	29.67d	3.72e	0.00 b
Rabie	65.67b	5.57d	12.52a
Sefid	73.67a	12.67a	15.58a
Shahrood-21	64.33b	8.47c	15.85a

Means in columns with the same letters are not significantly different using Duncan's multiple range test (P = 0.05)



(a) Pollen germination on sigma surface

(b) Pollen grains on stigma surface and penetration in style

(c) Pollen tube growth in 1/4 of style at 48 hours after pollination

(d) Pollen tube growth in 3/4 of style at 96 hours after pollination

Fig.3. Pollen germination and pollen tube growth on stigma and style of almond cv. 'Mamaei' at different time hours

S-allele identification using PCR

PCR amplification of genomic DNA from 'Mamaei' with the consensus primers for the second intron of *S*-RNases, EM-PC2consFD and EM-PC3consRD, revealed the presence of two DNA fragments of sizes around 850 bp and 1250 bp on agarose gels (Fig. 4). The size of the smaller fragment (850 bp) is similar to that of S_{25} almond RNase, while the size of the other fragment is different from all S_1-S_{30} RNase alleles previously characterized with these primers (Fig.4 and Table 1).

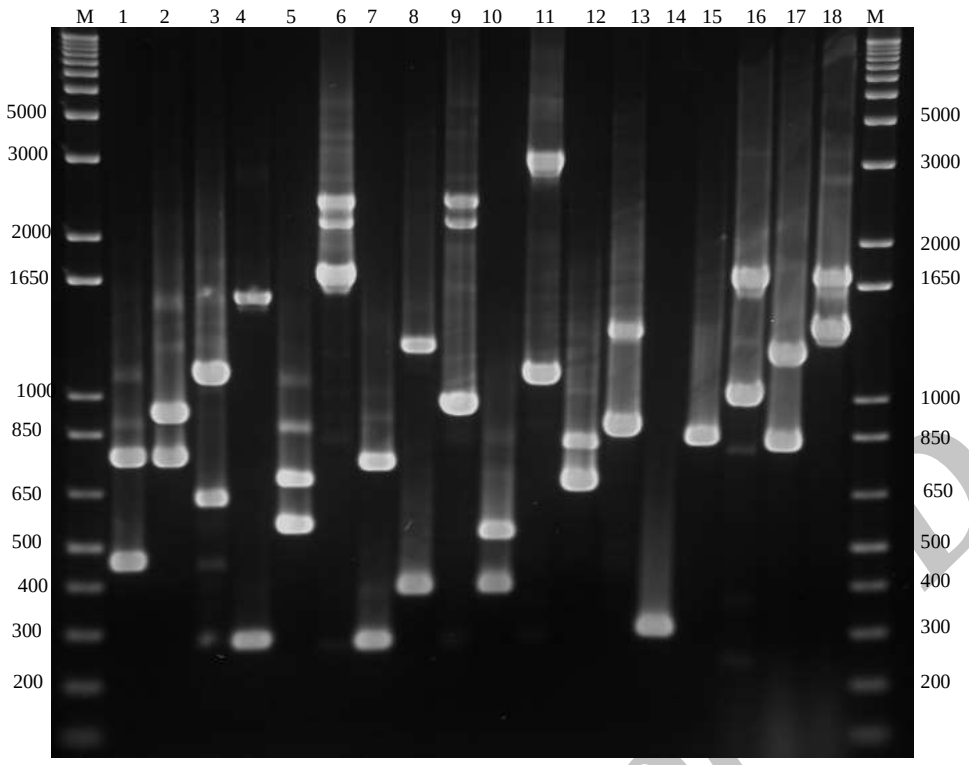


Fig.4. PCR products obtained with consensus primers EM-PC2consFD and EMPC3consRD for 'Mamaei'almond using reference cultivars. (M :1Kb Plus DNA Ladder (Invirogen), 1: Cristomorto, 2: Ferragnes, 3: CEBAS-I, 4: Primorski, 5: Ramillete, 6: IXL, 7: Ardechiose,8: Marcona, 9: Titan, 10: Rumbeta, 11: Avellanera Gruesa, 12: Lamona, 13:Fornat de Brez., 14: Fina del Alto, 15: A2-198, 16: Sefied, 17: Mamaei, 18: Rabie)

Discussion

According to the results, fruit sets showed the significant effect of pollen sources and year, as well as the interaction between these variables as excepted and previously reported by other investigators (Socias i company *et al*., 1976; Kodad and Socias i company, 2008). Moradi *et al*. (1999) previously reported that pollen sources had significant differences on initial and final fruit set in almond cvs. 'Sefied' and 'Mamaei.' that our results confirmed their results. The results showed that there were significant differences among cultivars for pollen germination and pollen tubes growth in different parts the style from self and cross-pollination. Observation of pollen tube growth by fluorescence microscopy showed a restricted growth of the incompatible tubes. Pimenta *et al*. (1983) reported that self-incompatibility in 'Nonpareil' was due to the prevention of pollen tube growth in different parts of style. The decrease in the number of pollen tubes at different style levels followed the normal reduction pattern for pollen tubes growing through the style (Hormaza and Herrero, 1996). According to the results, the percentage of reached pollen tubes at the style base from cross-pollination had not significantly varied among cultivars after 192 hours but there were not any reached pollen tubes from self-pollination (Table 4). Imani *et al*. (1999) reported that compatible pollen tubes reached the style base after 136 hours under orchard conditions while incompatible pollen tubes (few in numbers) reached 3/4 of style at the same time. A few incompatible pollen tubes reached the base of style after 170 hours. PCR products obtained for 'Mamaei'DNA with consensus primers EM-PC2consFD and EMPC3consRD showed two different bands with sizes around 850 bp and 1250 bp. Our results confirmed the existence of a new *S*-RNases in the 'Mamaei' cultivar. These primers successfully dis-

criminated and identified S-genotypes in almond cultivars that were previously reported by other investigators in almond (Ortega *et al*., 2005; Mousavi *et al*., 2011), sweet cherry (Sonneveld *et al*., 2003) and apricot (Zhang *et al*., 2008). Thus, according to the control pollination and pollen tube assays, 'Mamaei' is self-incompatible. Thus, its *S*-genotype can be regarded as $S_{25}S_x$, with S_x being a new *S*-RNase allele. The new *S*-RNase allele (S_x allele) in 'Mamaei' verified using sequencing technique and named S_{43} allele by Ortega *et al*. (2011). S-genotypes in 'Mamaei' ($S_{25}S_{43}$), 'Azar' (S_1S_3) 'Rabie'(S_7S_{27}), 'Shahrood-21' (S_1S_7) and 'Sefied' (S_7S_{38}) cultivars were reported by other investigators (Mousavi *et al*., 2011a, Ortega *et al*., 2011; Valizadeh and Ershadi, 2009). Nut and kernel characteristics and flowering time in these cultivars were reported by Mousavi and Moradi (1999) and Mousavi *et al*., (2010).

Conclusions

All methods used for determining the incompatible relationships in 'Mamaei,' such as pollen tube growth, fruit set and PCR analysis of the S-RNase, confirmed the self-incompatibility in this cultivar. These results emphasized that the combination of both classical and molecular methods was necessary to study and evaluate self-(in) compatibility in almond cultivars. According to these results and identified S-genotypes in 'Mamaei' ($S_{25}S_{43}$), 'Azar' (S_1S_3) 'Rabie' (S7S27), and 'Shahrood-21' (S1S7) and 'Sefied' (S_7S_{38}) cultivars, we recommend to use 'Rabie' and 'Shahrood-21' cultivars with overlap flowering, as they are the best pollinizers for 'Mamaei' cultivar in new orchard design.

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