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The consonant rebound of inbreeding and fluorescent microscope inspections beside *S*-genotyping of 93 apple (*Malus domestica* L.) cultivars

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Abstract

Objective: It is unavoidable to identify the level of self-compatibility of cultivars (CVs) in heterozygote species, including apples for different purposes such as monoclonal orchards, fuggy climate regions, and particularly in breeding programs. This research aimed to investigate the presence of self-compatibility in the apple CVs/genotypes under study by measuring the FSP at field conditions, PTG under fluorescent microscopy, and *S*-genotyping.

Methods: A sequential seven-year field screening for self-compatibility levels of 93 apple CVs was performed through three inbreeding methods. Nine flowering shoots were assessed by counting the flowers and fruitlet numbers at four biological growth stages compared to the free-pollinated shoots. Further inspections were achieved on the pollen tube growth (PTG) trend of the inbred ovaries, taken from the cuttings obtained at field and room conditions, related to the 33 CVs with higher levels of self-compatibility.

Results: The field assessment led to identify 47 CVs, carrying different levels of self-compatibility. The recorded fruit set percent overlapped with the pollen tube number and PTG rates. It was confirmed that the self-pollinated varieties Morabbaei, Mashhad, and Bel du Pontoise, and the promising genotype IRI6, which gained the highest score (5) for PTG till the ovary center, were assumed as fully self-compatible. On the other hand, molecular *S*-genotyping use of the allele-specific primers on 25 CVs led to the detection of some self-incompatibility alleles, which were previously reported in the other apple CVs, while in some others only a unique allele was observed.

Conclusion: Morabbaei, Mashhad, and Bel du Pontoise cultivars, and also the promising genotype IRI6, were recognized as self-compatible genotypes suitable for turbulent climates. Although *S*-genotyping detected some self-incompatibility alleles, however, more research is needed to attribute the direct relation of the studied characteristics with the detected *S*-alleles.

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Introduction

Apple cultivars (CVs) (*Malus*×*domestica* Borkh.) demonstrate mostly gametophytic type of self-incompatibility, needing the presence of foreign pollens for zygote formation (Kon *et al.* 2000; Socias i Company and Alonso 2004). The preliminary multiannual field research on 93 CVs in the National Collection of Commercial Apples, Iran was conducted by Hajnajari (2008). Nine flowering shoots per CV were isolated by tissue-non tissue bags before bud burst, and hand self-pollinated at full bloom. Annually, the relative fruit set percent (FSP) was assessed in successive biological stages of 10 days after bloom (DAB), June drop, and ripening time for seven years. The gradient of self-compatibility rate was registered in 47 apple CVs (Hajnajari 2008). The field studies revealed that early native CVs Mashhad, Mashhad Nouri, Sheikh Ahmad, crab apples Zinati, Morabbaei, as well as the promising IRI6 genotype, were fully self-compatible. The imported CVs Bel du Pontoise, Top Red Delicious, Orlean, and Red Rome Beauty also showed high levels of self-compatibility (Hajnajari and Moradi 2014). Microscopic investigations have been performed to verify the concordance of the FSP data with the sequels of pollen-stigma interaction through the rate of pollen tube growth (PTG) in style unto the ovary. The limited pollinizer attendance, however, improves fruit size, shape, and tree performance of the self-compatible apple CVs (Benedek and Nyeki 1996).

Breeders continue to release novel apple CVs by applying biological techniques. Inbreeding through hand pollination treatments has been frequently achieved to reveal the genetic potential of apple CVs for self-compatibility (De Witte *et al.* 1996; Jana 2001; Hegedűs 2006). Pollen-stigma interaction has been widely used to study the rate of PTG into style till ovary as a valid index of self-compatibility in pear, apricot, sour cherry, hazelnut, and almond in addition to apples by fluorescence microscope investigation (Marcucci and Visser 1987; Lansari and Iezzoni 1990; Mehlenbacher 1997). Self-(in) compatibility investigations were then oriented toward molecular methods, determination of ribonuclease (S-RNases), and cloning of the individuated DNA fragments by the use of PCR products' analysis in different rosaceous species (Burgos *et al.* 1998; Badenes *et al.* 2000; Alburquerque *et al.* 2002; Halász *et al.* 2005; Jie *et al.* 2005). Yoder *et al.* (2009) studied the influence of temperature and liquid lime sulfur of fish oil on PTG, to perceive the synchrony of pollen tube

activity with FSP in a few apple CVs using coloration and fluorescence microscopy. A positive direct relation of PTG and penetrated PTs (pollen tubes) up to the style base with the FSP outcomes was found for the first time. In a study. The influence of different apple pollinizers on fruit set and PTG was compared, concluding the rate of pollen coverage on stigma and PTG is related to the pollinizer type (Jahed and Hirst 2017). Milatović and Nikolić (2007) distinguished the self-compatibility level of 22 out of 36 apricot CVs by fluorescence microscopy.

Alston (1996) reported that the self-incompatibility level in apples is controlled by a system of *S*-allele's gene, expressed differently as semi-self-compatible and self-incompatible. The results determined the dominant mechanism of the biological process regarding stigma acceptance, pollen germination potential, and PTG leading to fruit formation. Self-pollination has caused fruit disorders which reduced seeds per fruit, fruit weight, and FSP (De Witte *et al.* 1996). Hajnjari and Moradi (2014) mentioned further inbreeding defects such as fruit heteromorphy, carpel irregularity, seed wrinkling, and vast peel russeting. According to Kellerhals and Wirthner-Christinet (1996), the double pollination treatment inhibited PTG even by the selected pollinizer, except for a limited self-compatibility in the Gala variety. Within the self-pollinated apple CVs Spur Red Delicious, Anna, Aziza, Red Baron, EC161286, and EC161287, the highest and lowest FSP, 5.76 %, 2.09 %, was attributed to Red Baron and EC161286, respectively (Jana 2001). Poor nutrition, defective tree pruning, low presence of visitors, and inadequate climate will aggravate pollen production and pollination rate and consequent fruit fall, plus the pollen or the mother cell sterility (Matsumoto 2014).

The responsible *S1* and S*11* alleles for self-incompatibility were detected by FEI EGHPEN based on RNase activity (Bošković and Tobutt 1999). Certal *et al.* (1999) declared S-RNase absorption occurs along the PTG route. S-RNase absorption by compatible pollen arises in gametophytic self-incompatibility where S-RNase accumulation inhibits the PTG. The *S*9 allele was detected in the self-compatible variety Delicious by PCR-RFLP (Matsumoto *et al*. 1999). In the self-fertile CVs due to S-RNase gene silencing, the gene expression was observed in the absence of S-RNase proteins as the responsible compound of *S*-gene construction in the ovary. In the case of self-incompatibility, fertilization, and regulation of fruit production are expressed at least by two genes that conform to PTG in the ovary. In another study, the PCR analysis on more apple CVs showed that the self-incompatibility alleles *S1*, *S3*, and *S9* prevailed over *S4*, *S6*, *S8*, *S16*, *S22*, *S23*, and *S26* (Broothaerts *et al.* 2004a). Based on polygenic control of fruit production, a group of S-RNase genes in the ovary and the group of haploid genes known as SFB, are expressed in pollen tubes. Self-(in) compatibility was defined by the acceptance or rejection of the products of these genes (Halász and Hegedűs 2006). Hegedűs (2006) claimed no fully self-compatible apple was identified hitherward

and it is presumably the reason to move toward transgenic methods. Levels of self-compatibility were reported in apple CVs Empire, Gala, Golden Delicious, and Fuji (Broothaerts *et al.* 2004b). Pollens of tetraploid apples inducing self-compatibility demonstrate their relative incompatibility (Adachi *et al.* 2009). Long *et al.* (2010) individuated *S1*, *S2*, *S3*, *S4*, *S5*, *S7*, *S9*, *S10*, *S11*, *S19*, *S20*, *S21*, *S23*, *S24*, *S26*, *S33*, *S44*, *S45*, and *S46* alleles working on 157 apple CVs, using 19 pair of primers. The recent finding of MdDof6 26, detected in the apple genome, was recognized as responsible for resistance to biotic and abiotic stresses, with high activities at the molecular level causing flower development and accelerated PTG (Yang *et al.* 2018). Determining the varietal self-compatibility level is highly important in parent selection, both in the scion CV and rootstock breeding (Hajnajari 2018). Apple cultivation in areas with uneven climates during the flowering period impedes the bees from visiting the flowers causing fruit fall. Self-compatible CVs may fruit in the absence of the pollinizer (Garratt *et al.* 2023). Self-compatible apple CVs are less prone to late spring frost with tragic and harmful damage to the crops in the Plateau of China (Ru *et al.* 2023) and high-latitude regions of South Korea (Lee *et al.* 2023). The best solution against climatic turbulence that impedes bee flight and the visits for the synchrony of apple blooming and pollinating (Wyver *et al.* 2023), seems the use of the self-compatible CVs. For the last, the apple industries that are firmly interested in maintaining the organoleptic characteristic of the fruit prefer monoclonal orchards with no need for pollinizers.

The apple production industry moves to the plantation of monoclonal orchards where the pollinizer absence facilitates pruning, fruit harvest, and sorting or packaging operations, all directed to decrease the production costs. Breeders try to reveal the genetic potential of the CV's self-compatibility level to determine the number of pollinizers per unit area.

This research aimed to investigate the presence of self-compatibility in the apple CVs/genotypes under study by measuring the FSP at field conditions, PTG under fluorescent microscopy, and *S*-genotyping.

Materials and Methods

Fruit set

The present research was performed on 33 apple CVs, nine-year-old trees pruned in the spindle onto MM111, grown in the new National Commercial Apple CV Collection, established in the Horticulture Research Station of KamalShahr, Karaj, Iran. The preselected plant material with different levels of self-compatibility at field conditions consisted of three subgroups of different origin, i.e. eight native CVs Morabbaei, Sheikh Ahmad, Payzeh Mashhad, Mashhad, Ardebil1, Ardebil2, Ahar2, and Golab

Ishahan, 18 imported cultivars Delicious, Golden Delicious, Top Red Delicious, Golden Smoothee, Golden Spur, Yellow Spur, Yellow Transparent, Empire All Red, Bel du Pointoise, Starkan Roge, Hi Early, Northern Spy, Red Rome Beauty, Granny Smith, Ganny Beauty, Glockenapfel, Wealthy, and Prim Gold as well as seven promising genotypes IRI1, IRI2, IRI3, IRI4, IRI5, IRI6, and Englisi Shiraz. To attain reliable results of the PTG rates by the fluorescent microscopic study, the field self-pollination treatments for the FSP estimation were repeated in the same season in other years for coordinated comparisons regarding the self-compatibility level of the CVs under study. Three artificial inbreeding treatments included hand self-pollination, shaking the isolated shoots at full bloom, and merely isolated shoots alongside the free-pollinated plants as the control, each with three replicates per CV. The flower buds per shoot were counted and isolated by tissue-non tissue bags, at the button stage (T1). The flower buds of the 33 CVs were gradually collected at bloom beginning to prepare the pollens. The labeled vials containing the pollens were transferred to the CVs collection site to set up the three defined self-pollination treatments, counting the number of inbred flowers. The treated shoots were assessed for FSP at subsequent phenological stages consisting of 10 days after the bloom end (T2), June drop (T3), and the fruit physiological maturity (T4). High genetic variability related to the ripening time of the studied CVs from very early Golab Isfahan to very late Granny Smith implied accurate daily assessments based on a detailed timetable. The experiment was arranged as factorial based on a completely randomized design. After analysis of variance, means were compared by Duncan's multiple range test using SPSS software.

Fluorescent microscopy

Microscopic investigation for PTG was carried out on the inbred ovaries of 24 apple CVs with different FSP levels obtained from the field self-pollinated treatments. Also, a forced flowering at room conditions was applied on 3-5 cuttings per CV, carrying 10-15 flower buds. The list contained native CVs Morabbaei, Mashhad, Ardebil1, Ardebil2, and Golab Ishahan, the mported CVs Delicious, Golden Delicious, Golden Smoothee, Golden Spur, Yellow Transparent, Empire all Red, Bel du Pointoise, Starkan Roge, Hi Early, Northern Spy, Red Rome Beauty, Glockenapfel, Wealthy, and Prim Gold, as well as the collected chance seedlings across the country as IRI2, IRI4, IRI, IRI6, and Englisi Shiraz. The ovaries (12-15 samples per CV), were immersed in the fixator solution containing acetic acid, 95% ethanol, and formaldehyde in a ratio of 1:3:1. Also, "8 N" NaOH, and aniline blue, $K_3PO_{4+}3H_2O$, was applied for tissue softening and coloration, respectively. Thereafter, the inbred ovaries were transferred to the Lab of Agricultural Biotechnology Research Institute of Iran (ABRII) to assess the PTG under fluorescence microscopy. The scoring schedule quantified the

single parts of PTG through the style till the ovary. PTG was rated by scoring single segments of style from no germination (0), primary penetration into stigma (1), initial part (2), middle parts (3 and 4), and full penetration into the center of the ovary (5) (Figure 1). Each score represents the mean length of the PTs assessed visually for each of the five styles. In total, 25 observations were carried out for each inbred ovary. The 22 self-pollinated ovaries related to the same number of CVs were ranked for the PTG index. Due to the difficulty of recognizing the advancement of a single PT, the color of the formed callus trajectory determined the PTG along the style.

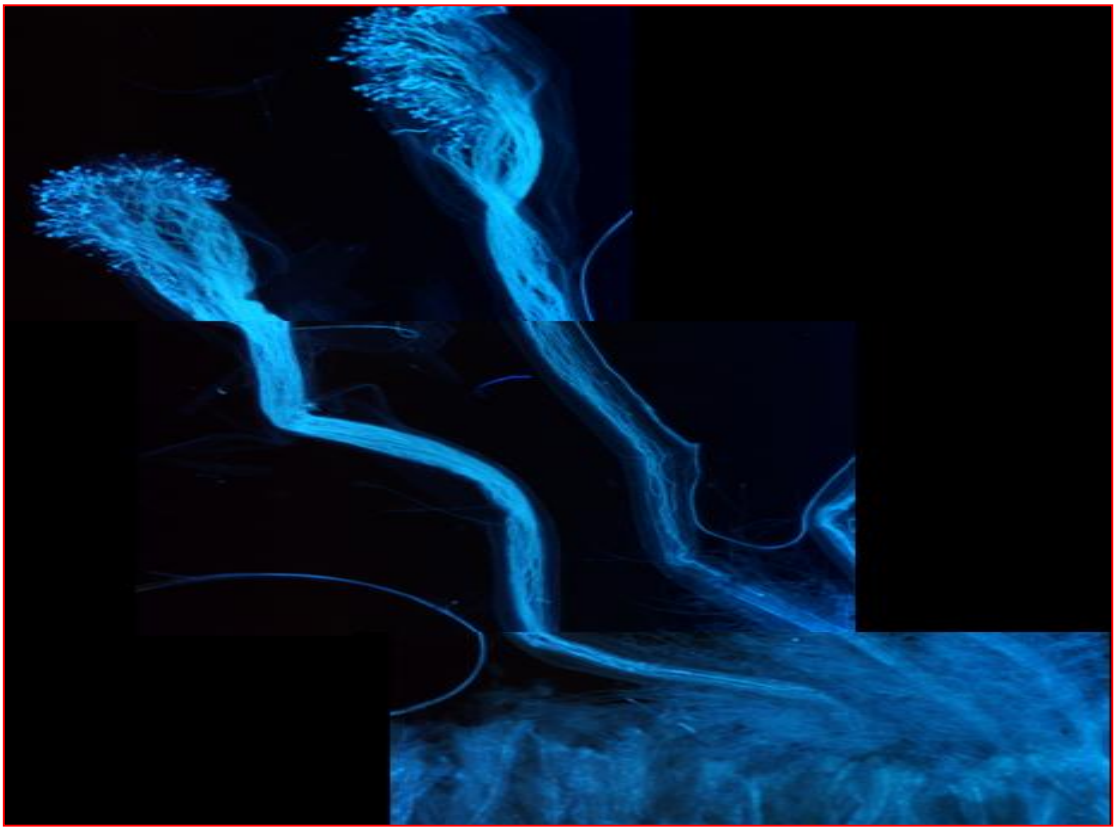

Figure 1. Classification of the pollen tube growth stages from stigma surface till ovary in the self-compatible apple cultivars.

S-genotyping

The collected healthy young leaf samples of the CVs, assigned with high levels of self-compatibility at field conditions, were used for DNA extraction by the CTAB-based method for molecular *S*-genotyping (Gharesheikhbayat *et al.* 2015). These CVs were eight native CVs Morabbaei, Sheikh Ahmad, Payzeh Mashhad, Mashhad, Ardebil1, Ardebil2, Ahar2, and Golab Ishahan, 18 imported CVs Delicious, Shisheh-Tabriz, Top Red Delicious, Golden Smoothee, Golden Spur, Yellow Spur, Yellow Transparent, Empire All Red, Bel du Pointoise, Starkan Roge, Hi Early, Northern Spy, Red

Rome Beauty, Granny Smith, Ganny Beauty, Glockenapfel, Wealthy, and Prim Gold, as well as seven promising genotypes IRI1, IRI2, IRI3, IRI4, IRI5, IRI6, and Englisi Shiraz

To determine the self-incompatibility alleles, a pair of commonly used primers (ASPF3+ASPR3S) with an annealing temperature of 50° C at the initial phase (Table 1) and successively 11 pairs of specific primers, were used as shown in Table 2.

Results

The mean comparisons of the FSP attained by hand-pollination treatments at June drop (T3), put in evidence that the native CVs had the highest self-compatibility potential including Morabbaei, Ahar2, and Mashahd with 92.53%, 87.5%, and 70.48% FSP, compared to the free pollinated control with 88.86%, 66.67%, and 66.67%, respectively. On the contrary, the inbred treatments resulted in minor FSP in other CVs such as Bel du Pointoise (59.26%), IRI6 (46.56%), and Top Red Delicious (37.5%), which demonstrated higher FSP compared to the related controls as 88.86%, 66.67%, and 66.67%. (Figure 2).

The PT fragments grown variously along the inbred ovary tissues of the 22 apple CVs under fluorescence microscopy are presented in Table 3 and Figure 4. The ranges of PTG are defined by the difference of min and max average values of the replicates for each genotype (Table 3) demonstrating also the inter-varietal fluctuations concerning self-(in)compatibility levels. The recorded PTG, observed as a thick hank, inside the ovary was found highly synchronistic with the relative FSP values in the case of the CVs Morabbaei (92.53%), Mashhad (70.48%), Golden Spur (55%), Delicious (25.21%), IRI6 (46.56%), and Bel du Pointoise (59.26%) at the T3 stage. More in detail, the min and max PTG were scored as 4 and 5, respectively, in the Morabbaei cultivar, which conforms to the results of the highest FSP at physiological maturity (T4), a surprising rate that was also slightly superior to the control. This native crab cultivar at T3 culminated the self-compatibility potential (92.53%) among the studied CVs, overlapping the PTG scored at T4 as the most approximate to the ovary in all 25 observations. The early copious cropping CVs Mashhad and Golden Spur demonstrated scores between 3 and 4 (Figure 1). The promising genotype IRI6 and the very old varieties Delicious and Bel du Pointoise showed the min and max PTG index of 4 and 5,

Table 1. Characteristics of common primers applied for the self-compatible apple cultivars.

Number	Common primers	5'—3'(sequence)
1	ASPF3-F	(M13)-CAATTTACGCAGCARTATCAG
2	ASPR3S	CAAAGASHGACCTC AACYAATTS

Table 2. The allele-specific primers that were used in the self-compatible apple CVs.

Self-incompatibility allele	Sequence	Primer	Band size (bp)
S1	5"-TGT-AAG-GCA-CCG-CCA-TAT-CAT-A-<C>-3"	MdS1SpF	734
	5"-CAA-CCT-CCA -ATT-CAG -TCA-ATG -<A>-3"	MdS1SpR	
S2	5"-AAC-ATG-AAT-CGA-AGT-GAA-TTA-TTT-<A>-3"	MdS2SpF	489
	5"-AGG-TTT-GGT-TCC-TTA-CCA-T-<G>-3"	MdS2SpR	
S3	5"-GGC-GAA-AAT-TAA-ACC-GGA-GAA-GA-<A>-3"	MdS2SpF	292
	5"-CCT-CTC-GTC-CTA-TAT-ATG-GAA-ATC-A-<C>-3"	MdS3SpR	
S4	5"-ATT-GCA-AGA-CAA-GGA-ATC-GTC-GGA-<G>-3"	MdS4SpF	533
	5"-AGA-AAT-GTG-CTC-TGT-TTT-TAT-C-<G>-3"	MdS4SpR	
S5	5"-GGT-CAA-ACC-CAC-GGC-GTC-TC-<A>-3"	MdS5SpF	1447
	5"-ATT-CAG-TTA-TCC-CAT-TCT-TC-<G>-3"	MdS5SpR	
S9	5"-CCA-CTT-TAA-TCC-TAC-TCC-TTG-TaG-<A>-3"	MdS9SpF	522
	5"-TCA-ATT-TCC-TTC-TGT-GTC-CTG-AAT-<T>-3"	MdS9SpR	
S10	5"-CCA-AAC-GTA-CTC-AAT-CGA-A-<G>-3"	FTC12a	203
	5"-TCC-CGT-GTC-AAT-AAT-CTC-C-<C>-3"	MdS10SpR	
S11	5"-AAA-TAT-TGC-AAG-GCG-CCG-<C>-3"	MdS11SpF	678
	5"-TTT-CAA-TAT-CTA-CCA-GTC-TCC-GG-<C>-3"	MdS11SpR	
S19	5"-GCC-TTC-AAA-CAA-GAA-TGG-AC-<C>-3"	MdS19SpF	481
	5"-TCA-ATA-TCC-ACC-AAT-GAC-CTG-T-<T>-3"	MdS19SpR	
S20	5"-GTT-GTG-GCC-TTC-AGA-CTC-<G>-3"	MdS20SpF	882
	5"-GGC-CAA-CTA-CTT-TTA-TTT-TTC-AT-<C>-3"	MdS20SpR	
S21	5"-AAG-TAA-TTG-CCC-GAT-AAG-GAA-CAT-<C>-3"	MdS21SpF	584
	5"-AGT-TTA-TGA-AAT-GTT-CTC-CGC-TGT-<A>-3"	MdS21SpR	
S23	5"-AAG-AAT-ACA-ACC-ATT-ACG-CCT-CAG-<C>-3"	MdS23SpF	450
	5"-ATT-GTT-GGT-ACT-AAT-GCT-TAT-GGC-<G>-3"	MdS23SpR	
S24	5"-ATG-GCT-CCT-GTG-CGT-CTT-CC-<C>-3"	MdS24SpF	421
	5"-CGT-CAT-CCG-TGT-ATA-GGG-CAA-C-<T>-3"	MdS24SpR	
S26	5"-TCC-ATC-AAA-CGT-GAC-TTC-TCA-<T>-3"	MdS26SpF	423
	5"-ATC-CTT-CAG-CAT-CCT-GAT-TC-<G>-3"	MdS26SpR	
S31	5"-TGA-CCC-AAA-ATA-TTG-CAA-GGC-CG-<C>-3"	MdS31SpF	556
	5"-TTT-CAA-TAT-CTA-CCA-GTC-TCC-GG-<C>-3"	MdS31SpR	
S44	5"-GCA-TGG-TAG-GAC-CTG-ACC-CAA-GT-<A>-3"	MdS44SpF	561
	5"-TCT-CAA-CCA-ATT-GAG-TCG-TCG-TAC-<C>-3"	MdS44SpR	
S45	5"-CCA-GAA-GGT-TGC-AAG-ACA-CAG-AAA-<T>-3"	MdS45SpF	641
	5"-AGT-TTT-GGT-GCC-TTA-TTG-TTG-GTA-<C>-3"	MdS45SpR	
S46	5"-CCC-AAC-GTG-CTC-GAT-CGA-AC-<A>-3"	MdS46SpF	212
	5"-TCA-ATT-TCC-TTC-TGT-GTC-CAG-AAT-<C>-3"	MdS46SpR	

affirming maximum elongation into the ovaries and indicating the full level of self-compatibility even with very few (1 or 2) PTs (Figure 3, Table 3). These mentioned CVs had the highest growth of pollen germination on stigma and the highest number of PT flourished along the style till ovary as well, coordinated with the best FSP in the orchard. The same concordance was recorded in other CVs such as Empire all Red, Yellow transparent, and natives Ardebil1 and Englisi Shiraz, between the lowest pollen germination and PTG scores and FSP results of the self-pollination treatments. So true that the microscopic observations evidenced an almost total lack of pollen germination (0) or a poor rate of PTG shutting down just at style apex (1), even though a few rare tubes lengthened near the ovary, all confirming the low levels of self-compatibility (Figure 3, Table 3).

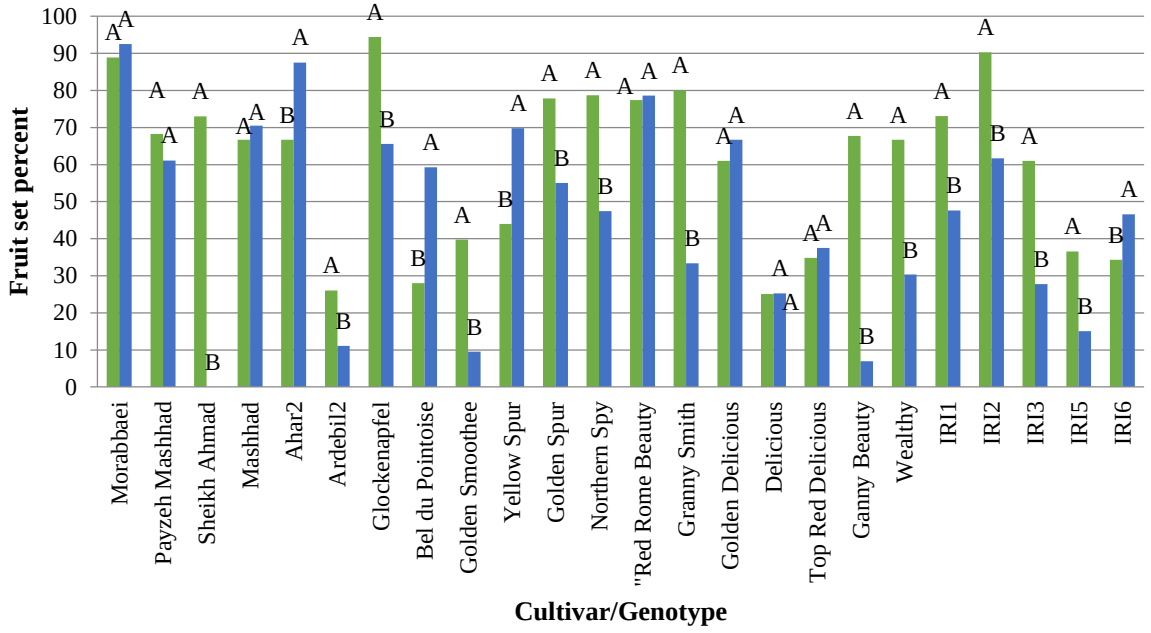

Figure 2. Comparison of fruit set percent in apple cultivars at the June drop stage related to control; Blue: Hand pollination, Green: Open pollination (control).

The max PT penetration till the ovary center (5) was recorded in IRI6 with an FSP of 46.56% and for Morabbaei marked by 92.53% at T3 followed by IRI2 and Starkan Roge with the identical FSP of 61.67%, where the mean PTGs were 2 and 3, respectively (Figure 1). On the contrary, the germinated pollens of Ardebil1 and Englisi Shiraz stopped at the stigma surface (0) (Figure 4).

Table 4 shows that the correlation coefficient between FSP and the PTG index was positive and significant at the 1% probability level for the 15 apple CVs. Based on this result, the fluorescence signals during microscopic investigation raised by the PT lengthening along the style and ovary can provide a confident estimate of the self(in) compatibility level of apple CVs. In supplementary re-screening of pre-selected apple CVs at field conditions, Hajnajari and Moradi (2014), determined the level of self-compatibility of 47 apple CVs as well as a promising chance seedling IRI6 detected as fully self-compatible based on the FSP though with noted fruit disorders caused by inbreeding pressure.

In the present PCR-based experiment, the *S2*, *S3*, and *S4* alleles were the most frequent, using a specific set of primers. The S-genotyping of the collected apple germplasm (and among them 23 apple CVs), revealed the old CVs as self-incompatible carrying alleles *S9, S24, S26,* and *S2*, indicating the prevalence of this group of alleles over others.

Table 3. The indexes of pollen tube growth into ovary in the self- (in) compatible apple cultivars under the fluorescence microscope.

Cultivar	Origin	Fruit set (%)	Max. tube growth index	Min. tube growth index	Range	Pollen tube growth index based on 25 observations per cultivar	S-alleles
Ardebil1	Iran	16.67	2	0	2	0	
Ardebil2	Iran	11.11	4	1	3	2	***S1S3***
Bel du Pointoise	France	59.26	5	0	5	4	***S3S4***
Delicious	U.S.A	25.21	5	4	1	5	***S3S?***
Empire all Red	U.S.A		0	0	0	0	***S3S?***
Englisi Shiraz	Iran		2	1	1	1	***S2S3S4***
Glockenapfel	Switzerland	65.56	3	0	3	2	***S3S?***
Golab Isfahan	Iran	61.11	4	0	4	2	***S2S3***
Golden Delicious	U.S.A	66.67	5	0	5	4	***S2S3***
Golden Smoothee	U.S.A	9.53	4	0	4	1	***S2S3***
Golden Spur	U.S.A	55	5	3	2	4	***S3S5***
Hi Early	U.S.A		4	0	4	1	***S3S?***
IRI2	Iran	61.67	2	0	2	1	***S2S3***
IRI4	Iran		5	2	3	3	***S5S19***
IRI5	Iran	15.03	4	0	4	1	***S2S3***
IRI6	Iran	46.56	5	0	5	4	***S3S?***
Mashhad	Iran	70.48	5	3	2	4	***S1S?***
Morabbaei	Iran	92.53	5	4	1	4	***S3S?***
Northern Spy	U.S.A	47.41	5	0	5	3	***S3S?***
Prim Gold	U.S.A		4	0	4	3	***S3S?***
Red Rome Beauty	U.S.A	78.57	1	0	1	1	***S3S4***
Starkan Roge	Russia		3	1	2	2	***S3S5***
Wealthy	U.S.A	30.3	2	0	2	1	***S3S?***
Yellow Transparent	Russia		1	0	1	0	***S3S?***

Discussion

While a complete PTG (4) occurred in the CVs Morabbaei, Mashhad, IRI6, Golden Spur, Golden Delicious, and Bell due Pointois, the old cultivar Delicious showed an accentuated number of fully extended pollen tubes, touching the ovary core and so having the best score (5). Based on these analogical results, the fluorescence signals during microscopic investigations raised by PT lengthening along the style and ovary can provide a confident estimate from the self(in) compatibility

level of the apple CVs. Similar results were reported in other stone fruit species such as apricots (Milatović and Nikolić 2007). Jahed and Hirst (2017) declared a relationship between stigma coverage rate by pollen grains and the rate of tube penetration into the style of apples and its consequent influence on fruit set.

Overall, the CVs carrying a unique allele of self-(in) compatibility such as Mashhad (*S1*), Payzeh-Mashhad (*S3*), IRI6 (*S3*), IRI3 (*S10*) had a higher FSP than the control. Similarly, *S3* was the sole allele detected in the Ardabil1 and Ahar2 cultivars with a medium FSP in which PTG stopped, extending mostly under the stigma of the inbred ovaries. Such harmony amongst FSP, PTG, and *S*-alleles stands for the existence of a dominant biological system in perfect equilibrium. IRI1 showed a good FSP of 47.62%, a high rate of PTG, carrying also *S3* and *S5* self-incompatibility alleles. Instead, a *S19* allele was detected in Top Red Delicious, with a high FSP of 37.5. Interestingly, the obsolete cultivar Delicious demonstrated the max PTG, as well as elevated FSP through inbreeding treatments compared to the control (Table 1). This was agreed with the results of Rom and Carlson (1987) on self-compatibility. The reason is that the seeds of the cultivar Delicious were used as a selected maternal parent to produce uniform apple trees in the United States (Rom and Carlson 1987), and in the seed rootstock improvement program in Iran (Hajnajari 2019). The 9-year-old trees of six apple CVs onto the improved seed rootstocks, obtained from the self-compatible Morabbaei led to form uniform tree canopies with no significant difference in size and shape due to homozygous dominance in the self-compatible CVs (Hajnajari 2018).

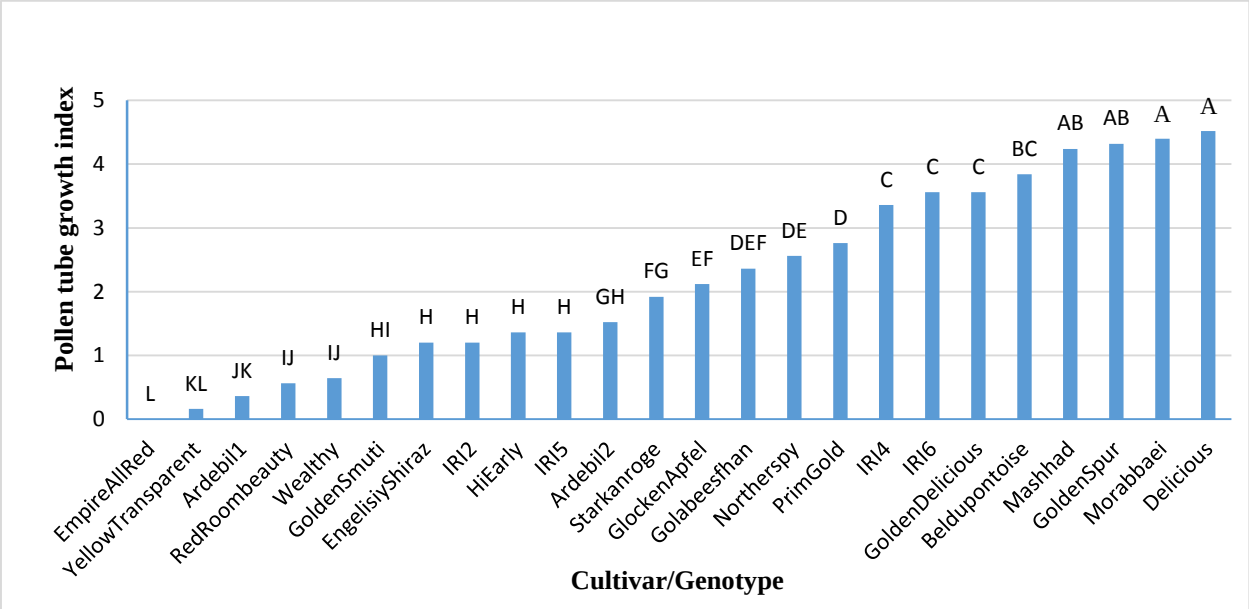

Figure 3. Comparison of pollen tube growth (PTG) indexes of apple genotypes in the hand-pollinated ovaries with different levels of self- (in) compatibility; The pollen tube length was scored visually based on penetration into stigma and style till ovary center. The PTG becomes visible through callus formation by the injury brought to the tissues.

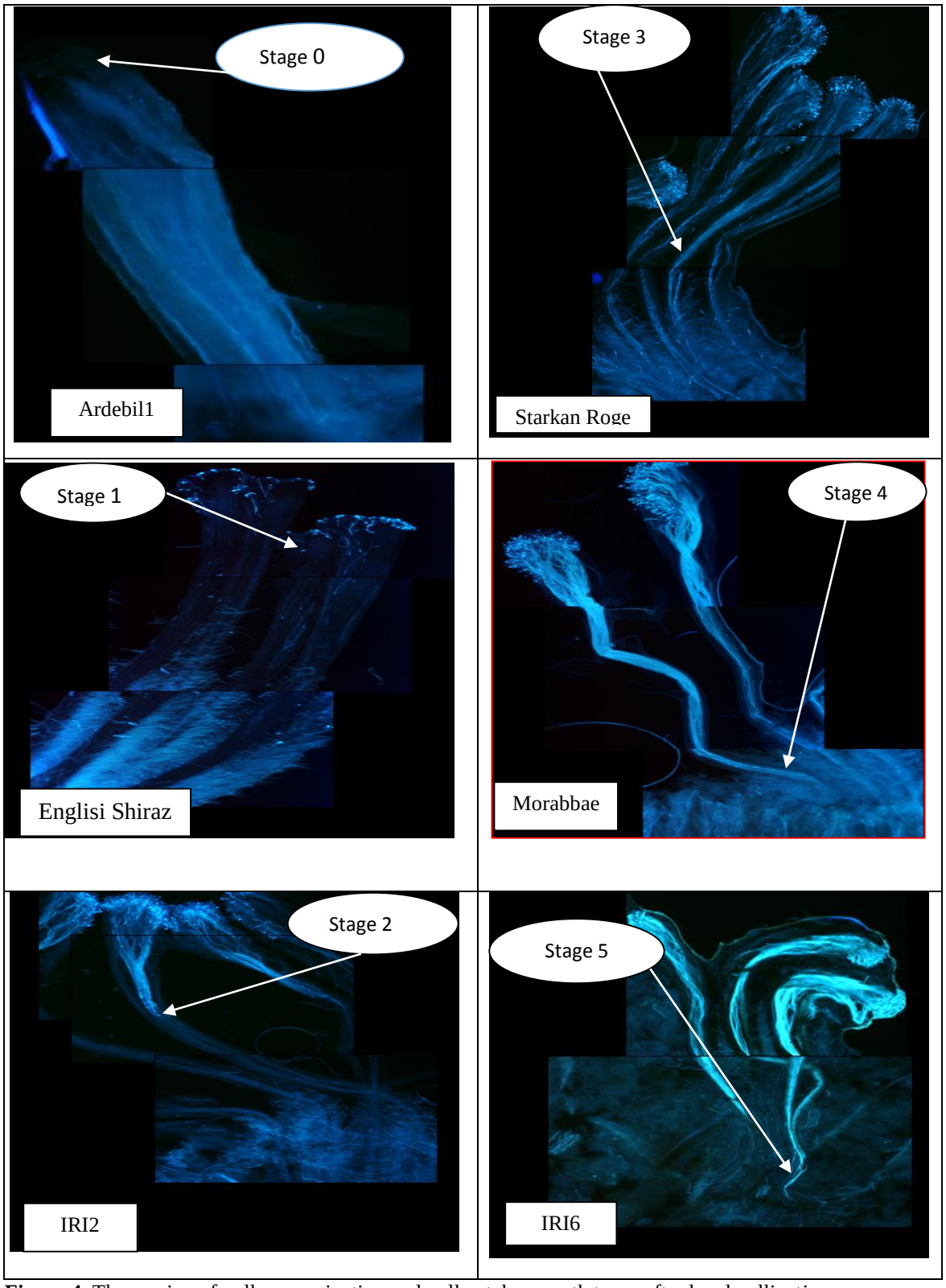

Figure 4. The scoring of pollen germination and pollen tube growth types after hand pollination.

Table 4. Spearman's rank correlation coefficient of the pollen tube growth into style and ovary with the fruit set percentage of 15 apple cultivars.

Trait	Pollen tube growth
Fruit set percentage	0.723**

**Significant at 1% of probability level

As shown in Table 3, The hand-pollinated treatment of CVs Morabbaei and IRI6 had higher FSP of 92.53% and 46.56% than the free-pollinated treatments as the control (Table 3, Figure 2), confirming them as quite self-compatible. In a 5-Year trial, among 108 apple CVs grown in commercial apple collection, considerable levels of self-(in) compatibility were individuated particularly in the native CVs Morabbaei, Zinati, Paeyze-Mashhad and Sheikh-Ahmed (Hajnajari 2008). In supplementary re-screening of pre-selected apple CVs at field conditions, Hajnajari and Moradi (2014), determined the level of self-compatibility of 47 apple CVs as well as a promising chance seedling IRI6 and reported them as fully self-compatible based on the FSP though with noted fruit disorders caused by inbreeding pressure.

Someway, the high FSP (%78.57) of the hand-pollinated Red Rome Beauty shoots were not conformed to the observed PTG as 0-1, while, the high PTG of Golden Delicious (4) conformed to its FSP (66.67) in inbred treatments. Cultivated in rainy and fuggy springs, Golden Delicious has a higher yield than Red Delicious for its relative self-compatibility. Golden Smoothee as a clone of Golden Delicious, with high genetic and morphological similarity, was almost completely self-incompatible based on FSP and PTG.

To widen the genetic basis of the commercial apples for resistance breeding, the *S*-genotyping of the collected apple germplasm and among them, 23 apple CVs, revealed the old CVs as self-incompatible carrying alleles *S9, S24, S26,* and *S2,* indicating prevalence of this group of alleles over others (Broothaerts *et al.* 2004a; Long *et al.* 2010, de Albuquerque *et al.* 2011). However, there were some perplexes. For example, although IRI6 carried the *S3* allele of self-incompatibility, it showed the highest self-compatibility level based on the fluorescent assay (4) and field experiment (%46.56). The same result was noted in the Morabbaei cultivar, carrying the *S3* allele, which had still a higher FSP (92.53%), than the control (%88.86) as well as the salient PTG (4) till ovary in the fluorescence microscopy test, therefore indicating the high level of self-compatibility. Analyzing a group of imported and native apple CVs by PCR, the self-(in) compatibility allele *S3* was identified in the fully self-compatible promising genotype IRI6 (Niazi Zeinivani *et al.* 2013).

Mirzaei *et al.* (2014) searching molecular aspects of Morabbaei, expressed the formation of a band with the approximate size of the *S10* allele by using a specific primer (MdS2SpF + R).

Therefore, the high degree of self-compatibility encountered in the studied genotypes can be attributed to other alleles in the locus *S* (Figure 5). Others have identified several self-(in) compatibility alleles including *S1* as the most frequent among their studied CVs (Kim *et al.* 2006).

Considering the field results, as well as PT tracing, it appears that in those CVs/genotypes with a high degree of self-compatibility, despite the detection of the self-(in) compatibility alleles, other factors outside the locus S may be involved. Thus far, it is deducible that the responsible alleles for self-compatibility have not still been specifically identified and/or never reported in apples. Probably, the self-compatibility declared for the Japanese apple Megumi (Matsumoto *et al.* 1999), is not related to the defect in the locus S.

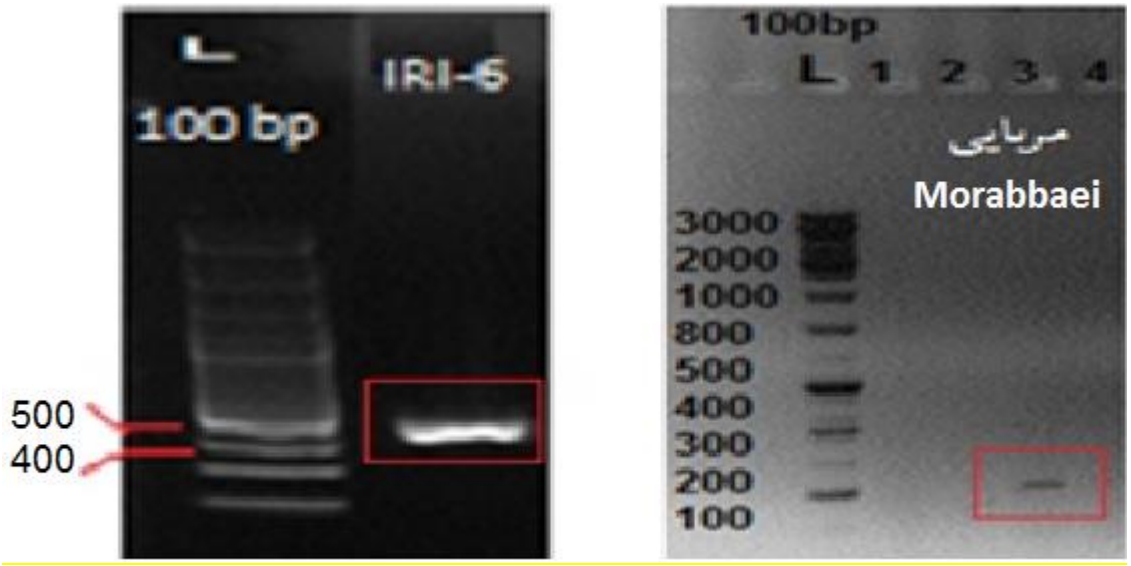

Figure 5. The *S10*-specific band in the Morabbaei cultivar (right) and the *S3*-specific band in the IRI6 cultivar (left).

Conclusion

We may conclude the general harmony, related to self-compatibility level, amongst the results for FSP at field conditions, PTG under fluorescent microscopy, and S-genotyping of the apple CVs under study. The noted differences in the self (in)-compatibility level among the CVs are attributed to the high genetic variability of the studied germplasm, a phenomenon that warrants food security against abiotic adversities. Surly, a smart selection of self-compatible apples for cold, windy, and rainy climates in the spring may extend the apple cultivation area with better performance.

Conflict of Interest

The authors declare that they do not have any relevant competing interests.

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