Spanish Journal of Agricultural Research
17 (3), e0804, 10 pages (2019)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2019173-14761
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Comparison of different classical and molecular methods for identifying self-incompatibility in two olive cultivars

Elham Aslmoshtaghi (Aslmoshtaghi, E)[1], Ali Reza Shahsavar (Shahsavar, AR)[1], Majid Talebi (Talebi, M)[2] and Aziz Dazeh (Dazeh, A)[2]

[1]*University of Shiraz, College of Agriculture, Dept. Horticultural Science, Shiraz-Esfahan Road, Shiraz, Iran.* [2]*Isfahan University of Technology, College of Agriculture, Dept. Biotechnology, Isfahan, 8415683111, Iran.*

Abstract

Aim of study: To determine compatibility relationships and select suitable pollinizers for two olive (*Olea europaea* L.) cultivars ('Amygdalolia' and 'Konservalia').

Area of study: The Kazeroun Olive Research Station, Fars, Iran.

Material and methods: Emasculated flowers from 'Amygdalolia' and 'Konservalia' cultivars were treated with self-pollination, open-pollination, and cross-pollination with pollen from cultivars such as 'Dacal', 'Amygdalolia','Konservalia', 'Koroniki', and 'Manzanilla'. Controlled pollination, pollen tube growth, and molecular analysis were employed.

Main results: Controlled pollination, pollen tube growth, and molecular analysis showed that cross-pollination was beneficial for 'Amygdalolia' compared to self-pollination. The results showed that this cultivar is self-incompatible, and its best pollinator is the 'Dacal' cultivar. Experiment results indicated that 'Konservalia' behaves as a self-compatible cultivar. The highest fruit percentage and higher pollen tube growth rates were found in self-pollination treatments. Molecular attempts to isolate candidates for sporophytic self-incompatibility (SSI) led the researchers to analyze the expression of SRK and SLG genes.

Research highlights: The results indicated an antagonist transcriptional expression pattern in the flowers of 'Amygdalolia', classified as a self-incompatible cultivar, and 'Konservalia', classified as a self-compatible cultivar, for the SRK and SLG genes.

Additional keywords: cross-pollination; fertilization; fruit setting; *Olea europaea*; pollen tube growth.

Abbreviations used: GSI (gametophytic self-incompatibility); ISI (self-incompatibility index); SI (self-incompatibility); SLG (S-locus glycoprotein); SRK (S-locus receptor kinase); SSI (sporophytic self-incompatibility).

Authors' contributions: All authors performed the field and laboratory experiments and/or analysis, read and approved the final manuscript.

Citation: Aslmoshtaghi, E; Shahsavar, AR; Talebi, M; Dazeh, A (2019). Comparison of different classical and molecular methods for identifying self-incompatibility in two olive cultivars. Spanish Journal of Agricultural Research, Volume 17, Issue 3, e0804. https://doi.org/10.5424/sjar/2019173-14761

Received: 22 Feb 2019. **Accepted:** 10 Oct 2019.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Elham Aslmoshtaghi: emoshtaghi11@gmail.com

Introduction

Olive (*Olea europaea* L.) is an allogamous species that is wind-pollinated. Olive trees usually produce pollen in great amounts, but only a small percentage of their flowers set normal fruit (Lavee *et al.*, 2002; Spinardi & Bassi, 2012). Olive yields depend on the pollen sources (pollinizers) planted in the orchard and on the level of self-incompatibility (Selak *et al.*, 2011). Most cultivars produce a small amount of fruit through self-pollination; however, a clear increase in fruit set under cross-pollination has been documented for many cultivars in different regions (Bartolini & Guerriero, 1995; Cuevas & Polito, 1997; Lavee *et al.*, 2002; Selak *et al.*, 2011; Taslimpour & Aslmoshtaghi, 2013). A self-incompatibility reaction causes an inhibition or delay in pollen tube growth, resulting in a lower percentage of fertilization (Taslimpour & Aslmoshtaghi, 2013). The degree of self-incompatibility varies among olive cultivars (Wu *et al.*, 2002; El-Hady *et al.*, 2007). Studying the self-incompatibility and cross-compatibility relationships of olives is important in pollination design for maximizing fruit set and yields (Bartolini *et al.*, 2002; Selak *et al.*, 2011). It is especially important when orchards are planted in isolated areas where the only available sources of pollen are within

the orchard itself (Selak *et al.*, 2011). While most studies agree on the benefits of cross-pollination, there is still uncertainty about the most effective pollen donors for even the most commonly grown cultivars. Genetic expression of self-incompatibility in the olive depends greatly on environmental and growing conditions (Selak *et al.*, 2013). High temperatures (30-35 °C) have inhibitory effects on pollen germination and pollen tube growth, and they increase the level of self-incompatibility (Lavee *et al.*, 2002). Quero *et al.* (2002) assumed that slower tube elongation under self-pollination makes ovule longevity critical, as the embryo sac begins to degenerate before the pollen tube reaches it. Different methods, including fruit setting after flower bagging or pollen-tube growth after hand pollination, have been used to determine self-incompatibility mechanisms in olive flowers, and each has demonstrated advantages and limitations (Serrano & Olmedilla, 2012). Contradictory results can be found in the literature about self-incompatibility in the same cultivar (Serrano & Olmedilla, 2012). Pollen-tube observation using florescence microscopy is an important method for studying self-incompatibility. During pollen tube growth, calluses are deposited within the tube plugs as well as on the pollen tube wall (Seifi *et al.*, 2012). Pollen-tube growth studies have been employed to examine many fruit crops (Selak *et al.*, 2014). Studies on pollen-tube growth have often been associated with fruit set percentage after artificial pollination (Seifi *et al.*, 2015). During those processes, the pollen has to be adequately transferred on the stigma, the pollen tubes have to accomplish growth in the pistil and then fertilize the ovules, and the absence of adequate pollen sources during the life span of olive flowers can cause variations in pollination (Bartolini & Guerriero 1995; Selak *et al.*, 2011). In paternity analysis, the genotype of the mother plant is compared to the genotype of the offspring to distinguish the father (Seifi *et al.*, 2012).

The effectiveness of microsatellite markers in identifying parent plants has been demonstrated by De la Rosa *et al.* (2004). Mookerjee *et al.* (2005) used microsatellite markers to identify 17 genotypes as potential pollen donors in a commercial olive orchard. However, applying a combination of methods can be accurate.

Self-incompatibility (SI) is one of the most important systems in preventing inbreeding. It is divided into three main categories: two systems of gametophytic self-incompatibility (GSI) and one of sporophytic self-incompatibility (SSI) (Breton & Bervillé, 2012; Selak *et al.*, 2014). The olive is currently classified as an S-RNase-based gametophytic self-incompatible plant based on morphological traits, such as wet stigma type and bi-nucleate pollen, features usually found in model taxa showing GSI, although no molecular evidence has been provided (Saumitou-Laprade *et al.*, 2010; Selak *et al.*, 2014). SSI is a sporophytic system, and the pollen SI phenotype is determined by the diploid genome of the parental plant (Nasrallah *et al.*, 1987). SSI was initially discovered in Brassicaceae, and the inhibition of self-fertilization is reached by avoiding pollen hydration or by inducing a rapid arrest of the pollen tube growth at the stigma surface (Nasrallah *et al.*, 1987). The incompatibility response is triggered by the interaction of two highly polymorphic genes encoding for the female determinant, S-locus receptor kinase (SRK), and a further S-locus protein encoding for secreted glycoprotein (S-locus glycoprotein, SLG), which is the first protein belonging to the S-locus isolated from *Brassica* (Breton & Bervillé, 2012).

Furthermore, the identification of the main genes known to play a crucial role in SSI (SRK and SLG) indicated that SSI may be present in the olive (Selak *et al.*, 2014). A new hypothesis seems to be in agreement with that of Saumitou-Laprade *et al.* (2010), who reported that *Phillyrea angustifolia* is a member of the Oleacea family. The SLG, present in the stigmatic papilla cell wall, and the SRK, localized in the stigmatic papilla plasma membrane, have been implicated in the recognition of self-pollen (Nasrallah *et al.*, 1987). SRK may be related to the plant proteins involved in defense against pathogens (Pastuglia *et al.*, 1997). SLG and SRK predominate expression in the stigma papillae into direct contact with pollen and expression that occurs just prior to flower opening and coincides with the timing of SI acquisition by the stigma (Nasrallah *et al.*, 1992).

The present study aimed to identify pollen donors for two olive cultivars, 'Konservalia' and 'Amygdalolia', using three methods (fruit setting after flower bagging; pollen tube growth after hand pollination; and paternity analysis using microsatellite markers) and to identify the gene expression involved in the SI of olive flowers.

Material and methods

Experimental orchard and plant material

Experiments were conducted during 2015 and 2016 using 15-year-old 'Konservalia' and 'Amygdalolia' olive trees growing at the Kazeroun Olive Research Station, Fars, Iran (49° 29' N, 37° 51' E). The soil is sandy, clay loam with the following properties: 55% sand, 30% clay, and 15% silt; pH=7.8 and EC=1.4 dS m^{-1}. The trees were spaced 5 m × 5 m apart under a drip irrigation system with hot and dry weather conditions

at an altitude of 960 m, with maximum and minimum annual temperatures of 48 °C and -5 °C, respectively. The mean annual temperature is 20.8 °C, and annual relative humidity is 52.33%. Temperature and relative humidity at flowering time were 19.7 °C and 49.9%, respectively. The orchard received regular irrigation, fertilization, and management. Shoots that were almost 1-cm in diameter, at similar heights from the ground, and with similar branch orders were selected and randomly assigned to three treatments: self-pollination, open pollination, and cross-pollination with pollen from five cultivars ('Dacal', 'Amygdalolia', 'Konservalia', 'Koroniki', and 'Manzanilla').

Pollination procedure

In both experimental years, four olive trees were chosen, being the same as each other in age, size, and fruiting state. Six branches, ~1 cm in diameter, at similar heights from the ground, and having around 20 inflorescences were selected. One from each of four replicate trees per treatment, at least 100 flowers aimed for pollination with each pollen donor cultivar, was emasculated at the white balloon stage and covered with paper bags to prevent uncontrolled pollination. Consistent flower age was assured by removing the open and immature flowers at the date of emasculation. One day after emasculation, all emasculated flowers were hand-pollinated with pollen grains from different pollen donor cultivars; the pollen was collected into Petri dishes on the day pollinations were performed, and applied to the recipient flowers using a paintbrush, and bagged again to prevent undesirable open-pollination. The open-pollination treatment consisted of the free exposition of shoots to wind-pollination. Self-pollination treatments were pollinated with their own pollen.

The initial and final fruit sets in response to the pollination treatments were assessed in the isolated shoots. The initial fruit set, expressed as the percentage of the number of developing fruits within the number of flowers counted in 20 inflorescences, was calculated 20 d after full bloom and before heavy fruit drop (Cuevas & Polito, 1997). The final fruit set, expressed as the percentage of the number of developed fruits within the number of flowers counted in 20 inflorescences, was calculated at 60 d after full bloom, when the abscission of young fruitlets was complete (Cuevas & Polito, 1997). The self-incompatibility index (ISI) was calculated by dividing the final fruit set under self-pollination by the fruit set under free pollination or cross pollination for each experimental year. As previously proposed, the self-incompatibility of olive cultivars was determined according to thresholds: ISI>1 = self-compatible, 0.29 to 1 = partially self-incompatible, <0.2 = severely self-incompatible, and 0 = completely self-incompatible (Zapata & Arroyo, 1978).

Analyses of pollen tube growth

Flowers of each pollination treatment (20) were collected 24 h after pollination and fixed in FAA (formalin, acetic acid, 70% ethanol at ratio 1:2:17) for at least 24h. Then they were washed with water and softened in 0.8 M NaOH. In each pistil, the stigma and style were cut from the ovary by transverse sectioning at the end of the style. The separated pistil parts were stained with 0.1% aniline blue in phosphate buffer in order to observe the pollen tube growth. The stigma and style were gently squashed using glass cover slips and were then examined with a microscope equipped with UV excitation filters (Jena, Germany). The pollen tube growth in the style was expressed as the percentage of the pistils with the most advanced pollen tube that reached the base of the style (Cuevas *et al.*, 1994).

Paternity analysis

For molecular paternity analysis, flowers within the bags were not emasculated in an effort to replicate field conditions. Flowers were hand-pollinated with pollen grains from the cultivars of different pollen donors. Leaf samples were collected from 'Konservalia' and 'Amygdalolia' as the mother trees and 'Dacal', 'Amygdalolia', 'Konservalia', 'Koroniki', and 'Manzanilla' as potential pollen donors. The samples were transferred on ice to the laboratory and kept at -80 °C for later DNA extraction. Forty mature fruits were collected, and these samples were transferred on ice to the laboratory and kept at 4 °C until use. The fruit flesh was removed, the stones were cracked open using a vise, and the embryos were separated from the endosperm using a pair of forceps. Genomic DNA was extracted using the CTAB method described by Murray & Thompson (1980). The quality and quantity of the DNA samples were estimated using agarose gel electrophoresis against the known concentration of the lambda DNA fragments and further verified by spectrophotometry (Beckmann, Germany). DNA samples were stored at -20 ºC. A set of four olive microsatellites with great informative potential (high polymorphism) were selected and the amplification efficiency, EMO2 and EMO3 (De la Rosa *et al.*, 2002), and DCA9 and DCA18 (Sefc *et al.*, 2000), were proven for paternity analyses. The PCR reaction was performed in a total volume of 25 µL of reaction mixture containing 50 ng of genomic DNA of the parents or 0.1 µL DNA of the embryos (measurements not performed due to

the small quantity of DNA extracted for each embryo), 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 2.5 µL of PCR buffer, 1 unit of Taq DNA polymerase, and sterile doubled-distilled water. All reactions were performed in a Mastercycler gradient 96 thermocycler (Eppendorf, Hamburg, Germany). The PCR program included an initial denaturation at 95 ºC for 7 min, 35 cycles of 45 s at 95 ºC, 45 s at 55 ºC, 45 s at 72 ºC, and a final extension at 72 ºC for 20 min. The reaction products were subjected to electrophoresis on a 12% denaturing polyacrylamide gel with 100 bp DNA ladder (Fermentase) and then stained with silver nitrate according to the method described by Bassam *et al.* (1993). The paternity assignment was made by eye and only when all paternal alleles for the 4 markers matched the putative father. For the four primer pairs used in the paternity analysis, the alleles were scored for each seedling. If a seedling presented only maternal alleles, it was considered the product of self-fertilization; if one maternal and one paternal allele were present for each primer, the seedling was considered true.

Gene expression

Trees of each cultivar were selected for their uniform size and high level of flowering. Three branches with 30 inflorescences were chosen for the self-pollination treatment on each replicate tree. Flowers were bagged using paper bags to prevent cross-pollination by foreign pollen. When the bagged flowers started to open, each branch was shaken by hand to ensure self-pollination. The paper bags were removed 10 d after flowering. Molecular analyses were performed using total RNA extracted from pistils of the cultivars 'Amygdalolia' and 'Konservalia' at different developmental stages after self-pollination (Stage 1: open anthers and completely open flowers), and after fertilization (Stage 2: following petal abscission). Pistil samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted using a total RNA isolation kit (Yekta Tajhiz Azma, Iran). RNA quantity was assessed using a Picodrop spectrophotometer (Pico200, UK) and stored at −80 °C until use. Total RNA was treated with DNase (Promega, USA) before being subjected to reverse transcription to cDNA synthase using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. RT-PCR was performed using the step one real-time PCR system (Applied Biosystem). Each reaction was performed in a total volume of 10 µL including 1 µL of diluted DNA (1:5) as template, 0.5 µL of each primer (1 Pmol/µL), and 5 µL of 2X SYBR Green Master Mix (Yekta Tajhiz Azma, Iran), using the following program: 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C, 1 min at 72 °C, and 5 min at 72 °C for final extension using different subdomain-specific primer combinations. The transcript levels were analyzed in two technical replicates using the $2^{-\Delta\Delta Ct}$ method and the *ef1* as the reference gene (Collani *et al.*, 2012).

Data analysis

All data was subjected to analysis of variance (ANOVA). Means were compared by the least significant difference (LSD) test at $p \leq 0.05$ using the SAS Version 9.0 software (SAS Institute, Cary, NC, USA).

Results

Artificial cross pollination

This study was an attempt to identify the optimal pollen donor cultivar for 'Amygdalolia' and 'Konservalia' in order to improve yields through increased rates of fertilization. Artificial cross-pollination as well as paternity were used to rank the efficiency of the various potential pollen donors of 'Amygdalolia' and 'Konservalia' in Iran. Because there was no difference between years, data from both years was combined. During both years, the initial and final fruit sets of 'Konservalia' were significantly higher following self-pollination and open-pollination than following cross-pollination (Table 1). Significant differences in initial and final fruit sets were found between self-pollinated and cross-pollinated flowers in the 'Amygdalolia' cultivar. The highest initial and final fruit sets were observed when 'Amygdalolia' was cross-pollinated with the 'Dacal' cultivar (Table 1). The final fruit set in 'Amygdalolia' was significantly increased by the application of pollen from 'Dacal' (26.81%) when compared with the fruit set after pollination with its own pollen (10.01%). A higher final fruit set in the open-pollination treatment when compared with the other treatments was recorded in two cultivars.

The self-incompatibility index for 'Konservalia' was calculated (1.05), and this cultivar was classified as being self-compatible. The quoted ISI values classified Amygdaloidal (0.45) as a partially self-incompatible cultivar. Under two years of experiments, low ISI was noticed for 'Amygdalolia' in which the self-incompatibility response was correlated with low self-pollination efficiency when compared with cross-pollination and free pollination treatments.

Table 1. Initial and final fruit set in 'Konservali' and 'Amygdalolia' olive cultivars following pollination treatments in 2015 and 2016. Values represent the mean of four replicates and different letters denote significant differences ($p < 0.05$) among treatments.

	Pollen source					
	'Konservalia'	**'Amygdalolia'**	**'Dacal'**	**'Koroniki'**	**'Manzanilla'**	**Open- pollination**
'Konservali'						
Initial fruit set	33.13^{a}	21.11^{c}	21.44^{c}	20.43^{c}	19.86^{c}	30.71^{b}
Final fruit set	21.13^{a}	13.98^{b}	16.07^{b}	17.11^{b}	13.8^{b}	21.04^{a}
'Amygdalolia'						
Initial fruit set	17.49^{c}	13.38^{d}	43.96^{a}	12.12^{d}	8.45^{e}	39.92^{b}
Final fruit set	10.01^{c}	9.61^{c}	26.81^{a}	8.16^{c}	3.82^{d}	20.88^{b}

Pollen tube growth

The highest pollen tube growth rate in 'Konservalia' was observed in the self-pollinated and open pollinated treatments (Fig. 1a). The highest percentage of 'Amygdalolia' flowers with pollen tubes at the end of the styles was found after cross-pollination with 'Dacal' (90%) in both years. However, there was no significant difference in the growth rate between the self- and cross-pollination treatments when 'Konservalia' and 'Koroniki' were used as pollen donors (Fig. 1b).

Paternity analysis

Paternity analysis suggested a strong self-incompatibility mechanism in the 'Amygdalolia' cultivar. In both years, 'Dacal' was the most frequent male parent, and 57% of the seeds were identified as progenies of a cross between 'Amygdalolia' and 'Dacal', 15% could be

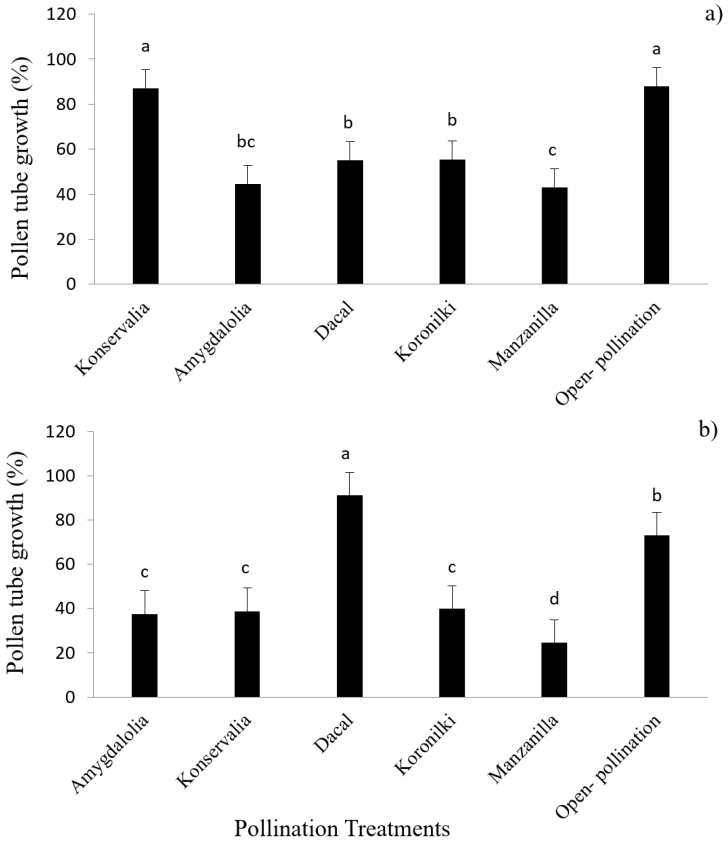

Figure 1. Percentage of flowers with pollen tubes at the end of style in 'Konservali' (a) and 'Amygdalolia' (b) cultivars following pollination treatments in 2015 and 2016. Vertical bars (where visible) indicate ± standard errors. Values represent means of four replicates. Different letters denote significant differences ($p < 0.05$) among treatments.

ascribed to 'Koroniki', 12.5% to 'Amygdalolia', 10% to 'Konservalia', and 5% to 'Manzanilla'. 'Koroniki', 'Amygdalolia', 'Konservalia', and 'Manzanilla' were identified as poor pollen donors to 'Amygdalolia' in both years (Fig. 2a). 'Konservalia' showed a very low compatibility with cross-pollination treatments, and 60% of the seeds were identified as progenies of the self-pollination treatment. Artificial cross-pollination of 'Konservalia' flowers with pollen from 'Amygdalolia', 'Koroniki', 'Dacal', and 'Manzanilla' resulted in a low percentage of seeds identified with paternity analysis. 'Amygdalolia', 'Koroniki', 'Dacal', and 'Manzanilla' were identified as almost completely incompatible with the 'Konservalia' cultivar (Fig. 2b).

Gene expression

Expression analysis of SLG and SRK genes showed similar patterns within the same cultivar, but opposite patterns between different cultivars. In fact, in 'Amygdalolia', SLG and SRK were expressed more after self-pollination (Stage 1), whereas in 'Konservalia' the expression was high after fertilization (Stage 2). SLG and SRK expression levels decreased from after self-pollination (Stage 1) to after fertilization (Stage 2) in 'Amygdalolia', whereas in 'Konservalia', the expression levels increased from after self-pollination (Stage 1) to after fertilization (Stage 2) (Fig. 3a,b).

Discussion

Differences between treatments (self-pollination, cross-pollination, and open pollination) were investigated on the level of initial and final fruit set to determine if the relations that exist at early response (initial fruit set) correspond to those that follow (final fruit set). Results showed that 'Konservalia' behaved as a self-compatible cultivar, while 'Amygdalolia' behaved as a relatively self-incompatible cultivar.

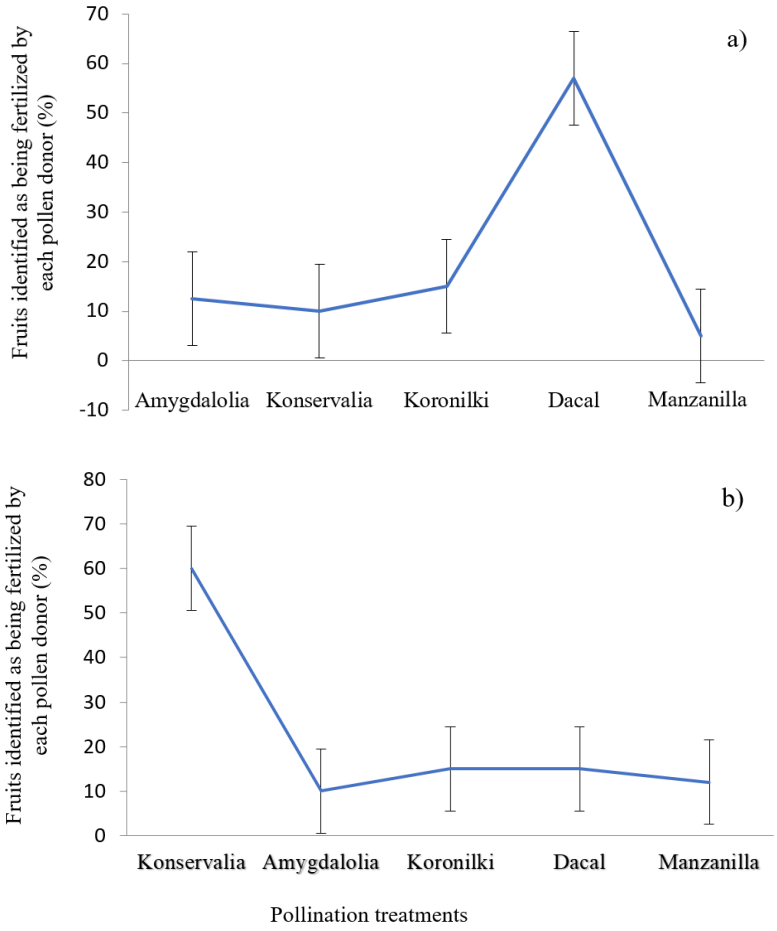

Figure 2. Percentage of 'Amygdalolia' (a) and 'Konservalia' (b) fruits identified as being fertilized by each pollen donor in 2015 and 2016. Vertical bars indicate ± standard errors. Values represent means of four replicates.

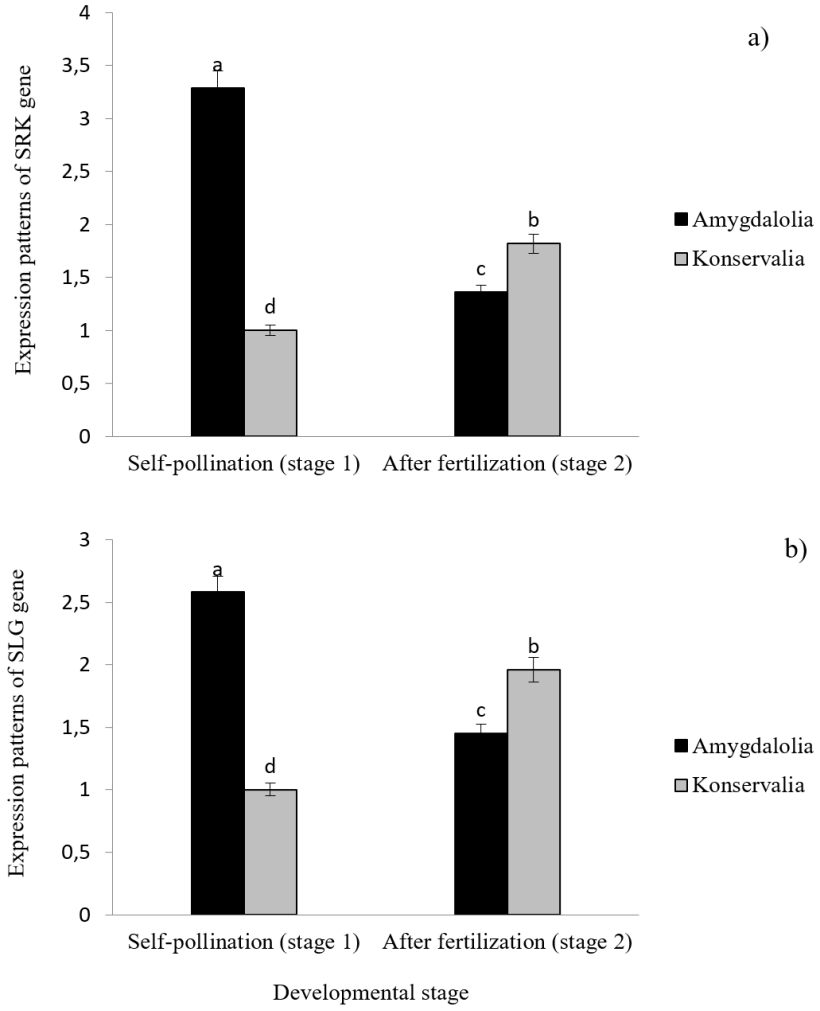

Figure 3. Expression pattern of S-locus receptor kinase (SRK) (a) and S-locus glycoprotein (SLG) (b) genes as assessed by qRT- PCR analysis in pistils. Vertical bars indicate ± standard errors. Values represent means of four replicates.

Pollination with 'Dacal' increased in the final fruit set over that of self-pollination. Fruit set following self-pollination was consistently lower than that following cross-pollination with 'Dacal' and that following open pollination. Because the final fruit set is the main factor in determining potential yield, pollinizers were compared based on the final fruit set. 'Dacal' was identified as the major pollen donor to the 'Amygdalolia' cultivar. It is not enough that the donor and acceptor flowers are open at the same time; the effective pollination periods, depending on stigma receptiveness and pollen viability, should overlap. 'Dacal' was cross-compatible with the 'Amygdalolia' cultivar. Lavee *et al.* (2002) divided the pollinizer into four efficiency groups based on the level of fruit set in relation to self-pollination. According to the proposed methodology, 'Dacal' was an efficient pollinator for the 'Amygdalolia' cultivar.

These results confirm the presence of self-incompatibility in most olive cultivars as reported by other researchers (Selak *et al.*, 2011; Serrano & Olmedilla, 2012; Taslimpour & Aslmoshtaghi, 2013). Although the similar levels of fruit set suggest that open-pollination provided enough cross-pollen, the source of this pollen is a matter of discussion. This result was similar to that of Orlandi *et al.* (2005) who showed that free-pollination improved fruit set. Open-pollination in each cultivar improved final fruit set when compared with the self-pollination treatment. The increased fruit set under open-pollination was probably the results of more favorable environmental conditions in opened branches than those in enclosed bags (Selak *et al.*, 2011; Bartolini & Viti, 2018). The index of self-incompatibility (ISI), a concept in analysis of tree breeding systems, is useful for assessing the degree of self-incompatibility in plant species. The quoted ISI values classified

'Amygdalolia' as a partially self-incompatible cultivar. Low ISI was noticed for 'Amygdalolia', in which the self-incompatibility response was correlated with low self-pollination efficiency when compared with the cross-pollination and open-pollination treatments. The degree of ISI in olive is widely influenced by climatic conditions and, therefore, varies from environment to environment and from year to year (Bartolini & Guerriero, 1995; Selak *et al.*, 2011). In fruit set trials, this may result in misleading conclusions regarding the self-compatibility and pollination relationships between cvs. Pollen adhesion on the host flower stigma is a key prerequisite for successful fertilization. Where artificial pollination takes place, the pollen supply could be neither sufficient nor uniform. Microcopy techniques could be valuable tools in pollination studies to avoid misleading artifacts produced because of technical mistakes (Koubouris *et al.*, 2014).

Incompatibility occurs when a perfect pollen grain fails to germinate on the stigma or germinates but its tube growth is somehow impeded. Incompatibility may occur between two cultivars or when a cultivar is genetically programmed not to be fertilized by its own pollen (Serrano & Olmedilla, 2012; Koubouris *et al.*, 2014). Self- and cross-incompatibility mechanisms are both common in olive and have been the main reason for the large genetic variability typical of the species (Wu *et al.*, 2002). The results obtained from traditional methods for assessing self-incompatibility, bagging flowers on the tree and scoring fruit set through self-pollination, and also observing pollen tube growth by fluorescence microscopy are close to real conditions, because pre-harvest drop is revealed by flower bagging and counting fruit. However, the results of compatibility tests in olive cultivars are quite contradictory, particularly between seasons and locations. Thus, it is necessary to research this mechanism in different olive cultivars in different regions (Cuevas *et al.*, 1994; Orlandi *et al.*, 2005; Seifi *et al.*, 2015). Cuevas and Polito (1997) observed that during self-incompatibility, most pollen tubes were unable to grow through the style and reach the ovule for fertilization, while pollen tubes arising from cross-pollination grew faster and reached the ovule. Pollen grains change their metabolism from the autotrophic phase on the stigma to the completely heterotrophic phase at the expense of the style's carbohydrate reserves (Herrero & Arbeloa, 1989). The pollen-pistil reaction is an important component in the fertilization success of flowering plants (Aslmoshtaghi & Shahsavar, 2016). Pollen tube behavior has been efficiently used to study incompatibility relationships in many fruit species (Ortega & Dicente, 2008; Distefano *et al.*, 2009). Incompatibility reactions can become evident at different levels of the style in flowers of different fruit species (Selak *et al.*, 2014). In the current study, pollen tube growth was considered as a fast procedure to assess compatibility between olive cultivars. The percentage of pollen tube growth in 'Amygdalolia' was significantly higher in cross-pollinated flowers in comparison with self-pollinated flowers, although the percentage for pollen tube growth in 'Konservalia' was significantly higher in self-pollinated flowers. The results are in line with those of Selak *et al.* (2013, 2014). Florescence microscopy can be efficient, provided that climatic conditions are suitable during the flowering period (Selak *et al.*, 2013). The results presented in this study demonstrate that the identification of the paternal parent using molecular techniques is a reliable method, because the genetic contribution of alleles is traced from the parents to the offspring (De la Rosa *et al.*, 2004). Microsatellite markers are suitable for this purpose, because of their codominant segregation and high level of polymorphism in olive (De la Rosa *et al.*, 2004). The short DNA extraction protocol and highly polymorphic nature of the microsatellites developed in olive make microsatellite testing a convenient technique for routinely assessing the crosses made in breeding programs and for confirming cross- and self-compatibility (Mookerjee *et al.*, 2005; Seifi *et al.*, 2012). The most likely methods of paternity analyses identify the maternal contribution of alleles in the genotype of the embryo and then compare the remaining alleles with the genotypes of potential fathers to identify the most likely father (Mookerjee *et al.*, 2005; Seifi *et al.*, 2012). Considering that 'Amygdalolia' is a self-incompatible cultivar and 'Konservalia' is a self-compatible cultivar, the results were in agreement with those expected by putative genes involved in self-incompatibility. In fact, after self-pollination (Stage 1), the whole set of transcripts codifying for the proteins involved in self-incompatibility mechanism should be present in flower tissue, whereas, after fertilization (Stage 2), the number of transcripts should decrease, since the self-incompatibility reaction has already taken place. SRK and SLG should be linked in the same locus, and the similar expression pattern could be proof of their association (Stone *et al.*, 1999). One is the arm repeat-containing protein, ARC1 (Armadillo-repeat containing 1), a stigma protein first identified in a yeast two-hybrid screen as a protein interacting with the cytoplasmic domain of SRK (Stone *et al.*, 1999). Therefore, it was proposed that ARC1 is activated by SRK to promote the ubiquitination and proteasomal degradation of stigmatic proteins that support pollen germination and/or pollen tube growth (Tantikanjana *et al.*, 2010). The expression reduction of Exo70A1, which is essential for the proper germination of pollen grains, disrupts compatible pollen tube growth, whereas its over-expression leads to a partial breakdown of SI

(Murase *et al.*, 2004). ARC1-mediated degradation of Exo70A1 leads to self-pollen rejection by inhibiting polarized secretion of compatibility factors (Tantikanjana *et al.*, 2010). The specific expression of SRK in the self-incompatible cv. 'Amygdalolia' along with the low expression in the self-compatible cv. 'Konservalia' strongly support the hypothesis that an SSI similar to that of *Brassica* may also occur in *Olea*, taking into account the crucial role played by SRK in the SSI of *Brassica*. The identification of the paternal parent using molecular techniques is a reliable method, because the genetic contribution of alleles is traced from the parents to the offspring (Mookerjee *et al.*, 2005). Consequently, paternity testing with SSRs is considered a very useful tool in olive breeding programs to demonstrate compatibility relationships (De la Rosa *et al.*, 2004). It is more precise, less expensive, simpler and faster than classic methods (Seifi *et al.*, 2012). Additionally, it allows the paternity of the selections to be checked at any step of the vegetative or seed propagation procedure, which could be an important piece of information for successive generations of the breeding program (Mookerjee *et al.*, 2005; Seifi *et al.*, 2012).

The results obtained in this study indicated that 'Amygdalolia' is partially self-incompatible and cross-compatible with 'Dacal'. These observations showed that replanting or grafting a number of trees with 'Dacal' may enhance the tree productivity in 'Amygdalolia' in mono-cultivar orchards. It was noticed that 'Konservalia' is a self-compatible cultivar. The results obtained from classical methods were in agreement with the results of the paternity analysis. In general, the results showed that a combination of all three methods (flower bagging, florescence microscopy, and molecular analysis) was most efficient for identifying self-incompatibility cultivars and were complementary. These results will be valuable guidelines to anyone planning new planting. In addition, they can be used to increase productivity in established groves through the addition of highly compatible pollen donors.

Acknowledgements

We thank the cooperative of Kazeroun Olive Research Station, Fars, Iran for providing the experimental farm and department of horticultural science, college of agriculture, Shiraz University, Iran for their support.

References

Aslmoshtaghi E, Shahsavar AR, 2016. Biochemical changes involved in self-incompatibility in two cultivars of olive (*Olea europaea* L.) during flower development. J Hort Sci Biotechnol 91 (2): 189-195. https://doi.org/10.1080/14620316.2015.1133606

Bartolini S, Guerriero R, 1995. Self-compatibility in several clones of oil olive cv. Leccino. Adv Hort Sci 9: 71-74.

Bartolini S, Viti R, 2018. Olive floral biology and climatic elements: twenty-eight years of observations. Acta Hort 1229: 299-304. https://doi.org/10.17660/ActaHortic.2018.1229.45

Bartolini S, Viti R, Guerriero R, 2002. Observations on fertilization process in self-pollinated flowers of cultivar 'Leccino'. Acta Hort 586: 521-524. https://doi.org/10.17660/ActaHortic.2002.586.108

Breton CM, Bervillé A, 2012. A new hypothesis elucidates self-incompatibility in the olive tree regarding S-alleles dominance relationships as in the sporophytic model. C R Biol 335: 563-572. https://doi.org/10.1016/j.crvi.2012.07.006

Bassam BJ, Caetano-Anolles G, 1993. Silver staining of DNA in polyacrylamide gels. Appl Biochem Biotechnol 42: 181-188. https://doi.org/10.1007/BF02788051

Collani S, Galla G, Ramina A, Barcaccia G, Alagna F, Caceres EM, Baldoni L, Muleo R, Perrotta G, 2012. Self-incompatibility in olive: a new hypothesis on the S-locus genes controlling pollen- pistil interaction. Acta Hort 967: 133-140. https://doi.org/10.17660/ActaHortic.2012.967.15

Cuevas J, Polito VS, 1997. Compatibility relationships in 'Manzanillo' olive. Hort Sci 32 (6): 1056-1058. https://doi.org/10.21273/HORTSCI.32.6.1056

Cuevas J, Rallo L, Rapoport H, 1994. Initial fruit set at high temperature in olive, *Olea europaea* L. J Hort Sci 69 (4): 665-672. https://doi.org/10.1080/14620316.1994.11516498

De la Rosa R, James CM, Tobutt KR, 2002. Isolation and characterization of polymorphic microsatellites in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. Mol Ecol Notes 2 (3): 265-267. https://doi.org/10.1046/j.1471-8286.2002.00217.x

De la Rosa R, James CM, Tobutt KR, 2004. Using microsatellites for paternity testing in olive progenies. Hort Sci 39 (2): 351-354. https://doi.org/10.21273/HORTSCI.39.2.351

Distefano G, Las Casas G, La Malfa S, Gentile A, Tribulato E, Herrero M, 2009. Pollen tube behavior in different mandarin hybrids. J Amer Soc Hort Sci 134 (6): 583-588. https://doi.org/10.21273/JASHS.134.6.583

El-Hady S, Eman LF, Haggag MM, Abdel-Migeed M, Desouky IM, 2007. Studies on sex compatibility of some olive cultivars. Res J Agri Biol Sci 3: 504-509.

Herrero M, Arbeloa A, 1989. Influence of the pistil on pollen tube kinetics in peach (Prunus persica). Am J Bot 76: 1441-1447. https://doi.org/10.1002/j.1537-2197.1989.tb15124.x

Koubouris GC, Breton CM, Metzidakis IT, Vasilakakis MD, 2014. Self-incompatibility and pollination relationships for four Greek olive cultivars. Scientia Hort 176: 91-96. https://doi.org/10.1016/j.scienta.2014.06.043

Lavee S, Taryan J, Levin J, Haskal A, 2002. The significance of cross-pollination for various olive cultivars under irrigated intensive growing conditions. Olivae 91: 25-36.

Mookerjee S, Guerin J, Collins G, Ford C, Sedgley M, 2005. Paternity analysis using microsatellite markers to identify pollen donors in an olive grove. Theor Appl Genet 111 (6): 1174-1182. https://doi.org/10.1007/s00122-005-0049-5

Murase K, Shiba H, Iwano M, Che FS, Watanabe M, Isogai A, Takayama S, 2004. A membrane-anchored protein kinase involved in Brassica self-incompatibility signaling. Sci 303 (5663): 1516-1519. https://doi.org/10.1126/science.1093586

Murray HC, Thompson WF, 1980. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8: 4321-4325. https://doi.org/10.1093/nar/8.19.4321

Nasrallah JB, Kao TH, Chen CH, Goldberg ML, Nasrallah ME, 1987. Amino-acid sequence of glycoproteins encoded by three alleles of the S locus of *Brassica oleracea*. Nature 326: 617-619. https://doi.org/10.1038/326617a0

Nasrallah ME, Kandasamy MK, Nasrallah JB, 1992. A genetically defined trans-acting locus regulates S-locus function in Brassica. The Plant J 2 (4): 497-506. https://doi.org/10.1111/j.1365-313X.1992.00497.x

Orlandi F, Romano B, Fornaciari M, 2005. Effective pollination period estimation in olive (*Olea europaea* L.): a pollen monitoring application. Sci Hortic 105 (3): 313-318. https://doi.org/10.1016/j.scienta.2005.01.012

Ortega E, Dicente F, 2008. Inheritance of self-incompatibility in almond. Theor Appl Genet 106: 904-911. https://doi.org/10.1007/s00122-002-1159-y

Pastuglia M, Roby D, Dumas C, Cock JM, 1997. Rapid induction by wounding and bacterial infection of an S- gene family receptor-like kinase gene in *Brassica oleracea*. Plant Cell 9: 49-60. https://doi.org/10.1105/tpc.9.1.49

Quero A, Pinillos V, Cuevas J, 2002. Reduced ovule longevity increase cross pollination response in olive. Acta Hort 586: 469-472. https://doi.org/10.17660/ActaHortic.2002.586.97

Saumitou-Laprade P, Vernet P, Vassiliadis C, Hoareau Y, Magny G, Dommée B, Lepart J, 2010. A self-incompatibility system explains high male frequencies in an androdioecious. Plant Sci 327 (5973): 1648-1650. https://doi.org/10.1126/science.1186687

Sefc KM, Lopes MS, Lefort F, Botta R, Roubelakis-Angelakis KA, Ibanez J, Steinkellner H, 2000. Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. Theor Appl Genet 100 (3-4): 498-505. https://doi.org/10.1007/s001220050065

Seifi E, Guerin J, Kaiser B, Sedgley M, 2012. Sexual compatibility of the olive cultivar 'Kalamata' assessed by paternity analysis. Span J Agr Res 10 (3): 731-740. https://doi.org/10.5424/sjar/2012103-501-11

Seifi E, Guerin J, Kaiser B, Sedgley M, 2015. Flowering and fruit set in olive: a review. J Plant Physiol 5 (2): 1263-1272.

Selak GV, Perica S, Ban SG, Radunic M, Poljak M, 2011. Reproductive success after self-pollination and cross-pollination of olive cultivars in Croatia. Hort Sci 46 (2): 186-191. https://doi.org/10.21273/HORTSCI.46.2.186

Selak GV, Perica S, Ban SG, Poljak M, 2013. The effect of temperature and genotype on pollen performance in olive (*Olea europaea* L.). Sci Hortic 156: 38-46. https://doi.org/10.1016/j.scienta.2013.03.029

Selak GV, Cuevas J, Ban SG, Perica S, 2014. Pollen tube performance in assessment of compatibility in olive (*Olea europaea* L.) cultivars. Sci Hortic 165: 36-43. https://doi.org/10.1016/j.scienta.2013.10.041

Serrano I, Olmedilla A, 2012. Histochemical location of key enzyme activities involved in receptivity and self-incompatibility in the olive tree (*Olea europaea* L.). Plant Sci 197: 40-49. https://doi.org/10.1016/j.plantsci.2012.07.007

Spinardi A, Bassi D, 2012. Olive fertility as affected by cross-pollination and boron. The Sci World J 2012: art ID 375631. https://doi.org/10.1100/2012/375631

Stone SL, Arnoldo M, Goring DR, 1999. A breakdown of Brassica self-incompatibility in ARC1 antisense transgenic plants. Science 286 (5445): 1729-1731. https://doi.org/10.1126/science.286.5445.1729

Tantikanjana T, Nasrallah ME, Nasrallah JB, 2010. Complex networks of self-incompatibility signaling in the Brassicaceae. Curr Opin Plant Biol 13 (5): 520-526. https://doi.org/10.1016/j.pbi.2010.06.004

Taslimpour MR, Aslmoshtaghi E, 2013. Study of self-incompatibility in some Iranian olive cultivars. Crop Breeding J 3 (2): 123-127.

Wu SB, Collins G, Sedgley M, 2002. Sexual compatibility within and between olive cultivars. The J Hort Sci Biotechnol 77 (6): 665-673. https://doi.org/10.1080/14620316.2002.11511554

Zapata TR, Arroyo MTK, 1978. Plant reproductive ecology of a secondary deciduous tropical forest in Venezuela. Biotropica 10 (3): 221-230. https://doi.org/10.2307/2387907