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TITLE:

Establishing Pollination Requirements in Japanese Plum by Phenological Monitoring, Hand Pollinations, Fluorescence Microscopy and Molecular Genotyping

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KEYWORDS:

Japanese plum, flowering, fluorescence microscopy, gametophytic self-incompatibility, hybrids, pollen tube, pollen grain, pollination, pollinizers, *Prunus salicina, S*-alleles

SUMMARY:

A methodology for the determination of pollination requirements in Japanese plum-type hybrids is described, which combines field- and laboratory-pollinations and observations of pollen tubes under the fluorescence microscopy with the identification of *S*-genotypes by PCR and the monitoring of flowering for the selection of pollinizers.

ABSTRACT:

The Japanese plum cultivars commonly grown are interspecific hybrids derived from crosses between the original *Prunus salicina* with other *Prunus* species. Most hybrids exhibit gametophytic self-incompatibility, which is controlled by a single and highly polymorphic *S*-locus that contains multiple alleles. Most cultivated hybrids are self-incompatible and need pollen from a compatible donor to fertilize their flowers. Establishing pollination requirements in Japanese plum is becoming increasingly important due to the high number of new cultivars with unknown pollination requirements. In this work, a methodology for the determination of pollination requirements in Japanese plum-type hybrids is described. Self-(in)compatibility is determined by hand-pollinations in both the field and in the laboratory, followed by monitoring pollen tube elongation with fluorescence microscopy, and also monitoring fruit maturation in the field. Selection of pollinizer cultivars is assessed by combining the identification of *S*-genotypes by PCR analysis with the monitoring of flowering time in the field. Knowing the pollination requirements

of cultivars facilitates the selection of cultivars for the design of new orchards and allows the early detection of productivity problems related with pollination deficiency in established orchards.

INTRODUCTION:

Japanese plum (*Prunus salicina* Lindl.) is native to China[1]. In the 19th century, this crop was introduced from Japan to the United States, where it was intercrossed with other North American diploid plums[2]. In the 20th century, some of these hybrids were spread to temperate regions around the world. Nowadays, the term "Japanese plum" refers to a wide range of interspecific hybrids derived from crosses between the original *P. salicina* with up to 15 other diploid *Prunus* spp.[3–5].

Japanese plum, like other species of the *Rosaceae* family, exhibits Gametophytic Self-Incompatibility (GSI), which is controlled by a single and highly polymorphic *S*-locus containing multiple alleles[6]. The *S*-locus contains two genes that encode a ribonuclease (*S-RNase*) expressed in the pistil, and an F-box protein (SFB) expressed in the pollen grain[7]. In the self-Incompatibility reaction, when the *S*-allele expressed in the pollen grain (haploid) is the same as one of the two expressed in the pistil (diploid), the growth of the pollen tube across the style is arrested due to the degradation of the pollen tube RNA by the action of the *S-RNase*[8]. Since this process prevents fertilization of the female gametophyte in the ovule, GSI promotes the outcrossing between cultivars.

Although some Japanese plum cultivars are self-compatible, most cultivars currently grown are self-incompatible, and need pollen from inter-compatible donors to fertilize their flowers[3]. In stone fruit species of genus *Prunus* such as almond[9], apricot[10–12] and sweet cherry[13], pollination requirements of cultivars can be established by different approaches. Self-(in)compatibility can be determined by self-pollination of flowers in the field and subsequent monitoring of fruit set, or by semi-in vivo self-pollinations at controlled conditions in a laboratory and the observation of pollen tubes under the microscope[14–18]. Incompatibility relationships among cultivars can be determined by cross pollinations in the field or the laboratory using pollen of the potential pollinizer cultivar, and by the identification of *S*-alleles of each cultivar by PCR analysis[14–16,19–22]. In species such as sweet cherry or almond, self-(in)compatibility can be also assessed by the identification of particular *S* alleles associated to self-compatibility, as S_4' in sweet cherry[13] or S_f in almond[23].

Several plum breeding programs from the main producing countries are releasing a number of new cultivars[2,14], many of them with unknown pollination requirements. In this work, a methodology for the determination of pollination requirements in Japanese plum-type hybrids is described. Self-(in)compatibility is determined by self-pollinations in both the field and the laboratory, followed by observations of pollen tubes under the fluorescence microscopy. Selection of pollinizer cultivars combines the identification of *S*-genotypes by PCR analysis with the monitoring of flowering time in the field.

PROTOCOL:

1. Hand-pollination in the field

1.1. Pollen extraction

1.1.1. To obtain pollen, collect flower buds at stage D[24], according to stage 57 on the BBCH scale[25,26].

NOTE: More flower buds are necessary in Japanese plum than in other *Prunus* species because their anthers produce less pollen.

1.1.2. Remove the anthers using a plastic mesh (2 mm x 2 mm pore size) and place them on paper at room temperature for 24 h until anther dehiscence.

1.1.3. Sieve the pollen grains through a fine mesh (0.26 mm x 0.26 mm pore size), and conserve them in a 10 mL glass tube with a cap at 4 °C until use.

1.2. Pollination of emasculated flowers

1.2.1. When between 10%–20% of flowers are open, select and label several branches. Remove open flowers and young buds, leaving only flower buds at stage D[24], according to stage 57 on the BBCH scale[25,26].

1.2.2. Remove the petals, sepals, and stamens of between 800 and 1,000 flower buds per treatment with either fingernails or tweezers.

1.2.3. Hand pollinate the pistils with the help of a fine paintbrush 24 h after emasculation. Some branches containing half of the pistils with pollen of the same cultivar, and the other half with compatible pollen from other cultivar as a control. Be careful not to contaminate the fingertip or paintbrush with pollen grains from other cultivars.

1.2.4. Record weekly counts of flowers and developing fruits to characterize fruit drop pattern and quantify the final fruit set in each pollination treatment.

1.3. Supplementary pollination in the field

1.3.1. A few days before the first flowers open, enclose selected trees in a 0.8 mm mesh cage to avoid the arrival of pollinating insects.

1.3.2. When 10%–20% flowers are open, select and label several branches per pollination treatment, leaving 1,000–1,500 flowers per treatment.

1.3.3. On the next day, when flowers are open, pollinate each flower with the help of a paintbrush with the corresponding pollen (pollen from the same cultivar for self-pollination, and from other compatible cultivars as crosspollination control).

1.3.4. Pollinate every other day until all flowers open.

1.3.5. Record weekly counts of flowers and developing fruits from anthesis to harvest to characterize fruit drop pattern and quantify final fruit set in each pollination treatment.

2. Hand-pollinations in the laboratory

2.1. Collect 50–100 flowers at stage D[24], according to stage 57 on the BBCH scale[25,26].

2.2. In the laboratory, emasculate 30 flowers per treatment (self- and cross-pollination).

NOTE: Emasculation should be proceeded carefully to avoid any damage on the pistils.

2.3. Make a fresh cut on the base of each flower pedicel underwater before placing it on a piece of wet florist foam (one piece of foam for each pollination treatment).

2.4. Hand pollinate each pistil 24 h later using a fine paintbrush with pollen collected previously (see section 1.1). Pollinate one set of pistils with pollen from the same cultivar, and the other set with pollen from a compatible cultivar as control.

2.5. Leave the pollinated pistils 72 h after pollination at room temperature. The floral foam should be continuously wet with water.

2.6. Fix the pistils in a fixative solution of ethanol/acetic acid (3:1) for at least 24 h at 4 °C. Replace the fixative with 75% ethanol. Samples can be conserved in this solution at 4 °C until use[27].

NOTE: Ensure that the samples are completely submerged in the solution.

3. Microscopic observations

3.1. Evaluation of in vitro pollen germination

3.1.1. To elaborate pollen germination medium, dissolve 25 g of sucrose on 250 mL of distilled water, then add 0.075 g of calcium nitrate [$Ca(NO_3)_2$] and 0.075 g of boric acid (H_3BO_3).

3.1.2. Add 2 g of agar to the solution and mix until completely dissolved[28].

3.1.3. To sterilize the medium, autoclave it at 120 °C for 20 min. Cool the medium and, before it solidifies, distribute 3 mL per sterile Petri dish (55 mm x 12 mm) in a sterile laminar flow hood. After medium solidification, conserve the Petri dishes wrapped in aluminum foil at 4 °C until use.

3.1.4. Spread the pollen of each cultivar previously used as pollen donor in the controlled

pollinations in two Petri dishes and incubate them at 25 °C for 24 h.

NOTE. The inoculated culture media can be observed with microscopy immediately after or stored at -20 °C until use. For this purpose, the Petri dishes should be changed from the freezer to the fridge 24 h before microscopy observations.

3.1.5. To observe the pollen grains, prepare 1% (v/v) aniline blue solution that stains callose. First, prepare a 0.1 N potassium phosphate tribasic (K_3PO_4) solution by dissolving 7.97 g of K_3PO_4 in 1,000 mL of distilled water. To elaborate the 1% (v/v) aniline blue solution, dissolve 1 mL of aniline blue in 100 mL of 0.1 N K_3PO_4.

3.1.6. Add 2–3 drops of aniline blue solution to each Petri dish plate and observe after 5 min under a UV epifluorescence microscope using exciter filter BP340-390 and barrier filter LP425. Count viable and non-viable pollen grains in three fields per plate, each field containing 100-200 pollen grains, in two Petri dishes for each cultivar.

3.2. **Pollen tube growth**

3.2.1. Rinse the fixed pistils with distilled water three times (1 h each, 3 h in total) and transfer to 5% (w/v) sodium sulphite (Na_2SO_3) at 4 °C for 24 h. To prepare this solution, dissolve 5 g of sodium sulphite in 100 mL of distilled water.

3.2.2. Autoclave the pistils at 120 °C for 8 min in 5% (w/v) sodium sulphite to soften the tissues.

3.2.3. Squash softened pistils in a drop of 1% (v/v) aniline blue solution under a cover glass on a slide to stain callose.

3.2.4. Observe pollen tube growth along the style under a microscope with UV epifluorescence using exciter filter BP340-390 and barrier filter LP425.

4. **Determining incompatibility relationships**

4.1. DNA extraction from leaves

4.1.1. To extract DNA, collect 3–4 young leaves of each cultivar in the field, preferably in spring.

NOTE: DNA can also be extracted from mature leaves, but DNA from young leaves has less phenolic compounds.

4.1.2. Isolate DNA using a commercial kit and follow the provided protocol kit (see **Table of Materials**).

4.1.3. Quantify the DNA concentration and evaluate the quality of the DNA of each sample at 260 nm in an UV-Vis microvolume spectrophotometer. Adjust the DNA concentration to 10 ng/μL.

4.2. PCR conditions for fragment amplification

4.2.1. Label 0.2 mL PCR tubes and caps.

4.2.2. Prepare the PCR reagents according to **Table 1** and let them thaw on ice.

4.2.3. Set up a volume of master mix of each pair of primers in a 1.5 mL microtube according to the number of reactions plus 10% of excess, considering a volume of 16 μL per reaction. Add the reagents following the order in **Table 1** and mix thoroughly.

4.2.4. Aliquot 16 μL of master mix into each 0.2 mL PCR tube containing 4 μL of DNA template or 4 μL of sterilized distilled water as negative control (C-). Use DNA of cultivars with known genotype as positive controls. Mix gently, close the reaction tubes with the caps, and centrifugate at 2,000 x *g* for 30 s to collect the entire volume at the bottom of the reaction tube.

4.2.5. Place the reaction tubes in the thermocycler and set up the PCR program using the following temperature profile: an initial step of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 3 min at 72 °C, and a final step of 7 min at 72 °C[20].

4.3. Electrophoresis and estimation of fragment size

4.3.1. To prepare a 1.7% (w/v) agarose mini gel, dissolve 0.68 g of agarose and 40 mL of 1x TBE buffer into a 100 mL Erlenmeyer flask. Melt the solution by heating in a microwave at 600 W at 30 s intervals to avoid boiling.

4.3.2. Let the solution stay on the bench to cool down, and then add 3.5 μL of nucleic acid staining solution.

4.3.3. Place the gel tray into the casting stand and put the selected comb into the gel mold. Be sure to have enough wells for each PCR product and DNA ladder.

4.3.4. Pour the agarose solution into the gel mold and let it cool down until polymerization. Remove the comb of the polymerized gel. Place the gel in the horizontal electrophoresis system containing enough 1x TBE buffer to cover the surface of the gel.

4.3.5. Load the first and last wells with 2 μL of the DNA molecular weight ladder (1 kb DNA Ladder). Load 3 μL of each product of the PCR in the other wells. Close the chamber, turn on the power, and run the gel at 100 V for 30 min.

4.3.6. Observe the gel under UV light using a gel documentation system. Use the DNA molecular weight ladder to determine the size of the amplified fragment and compare it with the positive controls in order to identify the corresponding alleles.

5. Monitoring flower dates

5.1. Monitor the phenology of different trees of each cultivar at flowering over different years. Establish the length of the flowering period from the first (about 5%) to the last open flowers (about 95%). Full bloom is considered when at least 50% of flowers are at stage F[24], according to stage 65 on the BBCH scale[25,26].

5.2. To compare the flowering dates of inter-compatible cultivars and determine those that coincide at flowering time every year, elaborate a calendar of flowering times with data from several years.

REPRESENTATIVE RESULTS:

Each Japanese plum flower bud contains an inflorescence with 1–3 flowers. As in other stone fruit species, each flower is made up of four whorls: carpel, stamens, petals, and sepals, which are fused forming a cup at the base of the flower. Flower structures are smaller than other stone fruits, with a short and fragile pistil surrounded by the stamens that contain a small amount of pollen grains. At full bloom, the flowers of each inflorescence appear separated on short stalks, showing the white petals forming a balloon surrounded by the green sepals (stage D, 57 BBCH) (**Figure 1A**) in the days before anthesis. The flower is fully open at anthesis, showing the anthers and the pistil (stage F, 65 BBCH) (**Figure 2A**). Like other temperate *Prunus* spp., the flower buds open first, and the leaf buds sprout several days later. This makes blooming trees look spectacular showing a great number of flowers but no leaves.

Hand-pollinations in Japanese plum required the collection of flowers at the balloon stage previous to anthesis (**Figure 1A**), in which the pistil and stamens are nearly mature, but the anthers are still undehisced. This stage prevents the arrival of insects carrying external pollen because the petals are still closed. Although the number of flowers is much higher than in other *Prunus* species, most of them (85%–95%) are not able to set fruit. As a result, using a high number of flowers for pollination experiments is mandatory. The undehisced anthers could be easily separated from the flower in the laboratory and extended on a piece of paper (**Figure 1B**), where the anthers are dehisced after 24 h at room temperature, showing the pollen grains (**Figure 1C**). Then, the pollen grains were easily sieved through a fine mesh (**Figure 1D**), and could be used immediately or stored until use, both for field (**Figure 2A–D**) and/or laboratory pollinations (**Figure 2E,F**).

The monitoring of fruit drop of hand-pollinated flowers after emasculation (**Figure 2A,B**) or supplementary pollination of non-emasculated flowers (**Figure 2C,D**) showed clear differences between treatments. Most flowers dropped 2–3 weeks after pollination in both treatments. All self-pollinated flowers dropped. However, 4% of self-pollinated flowers from the supplementary pollination treatment remained in the tree until harvest, indicating that this cultivar (Rubirosa)

behaves as self-compatible (**Figure 3**). The behavior of cross-pollinated flowers used as control also varied between treatments. Cross-pollinated flowers with supplementary pollination dropped until 3 weeks after pollination, resulting in 7% of fruit set. However, all emasculated cross-pollinated flowers dropped coinciding with the drop of all self-pollinated flowers.

For hand pollinations in the laboratory, the pistils were placed in wet florist foam after a fresh cut on the base of each flower pedicel underwater (**Figure 2E**) and pollinated 24 h later with the help of a fine brush (**Figure 2F**). In self-pollinated flowers, self-compatible cultivars showed at least one pollen tube reaching the base of the style in most of the pistils examined, while in self-incompatible cultivars, pollen tube growth was arrested in the upper style (**Table 2**).

In vitro pollen germination differed significantly between cultivars (**Table 3**). Good pollen viability was considered when more than 20% of pollen grains showed a pollen tube longer than its length after 24 h in the culture medium (**Figure 4A**). However, when most of pollen grains did not germinate (**Figure 4B**), the cultivar was considered male sterile and not suitable as a pollinator.

Like in other *Prunus*, the pistil is made up of three structures: stigma, style, and ovary. The ovary has two ovules, and at least one of them should be fertilized for fruiting. During pollination, pollen grains are transferred to the stigma, where germination occurred within 24 h (**Figure 4C**). Each germinating pollen grain produced a pollen tube, which grew through the pistil structures. In self-incompatible cultivars in which the pollen grains have an *S* allele that coincides with one of the two *S* alleles of the pistil, the pollen tube stopped growing in the upper third of the style (**Figure 4D**), preventing the arrival to the ovary and the subsequent fertilization. However, when *S* allele of the pollen grain is different with that of the pistil, the pollen tube could grow through the style (**Figure 4E**), reach the ovary (**Figure 4F**) and fertilize an ovule.

PCR analysis (**Table 1**) was carried out using primers from conserved regions of *S-RNase* of sweet cherry and Japanese plum (**Figure 5**). The primer set used, PruC2-PCER and PruT2-PCER (See **Table of Materials**), allowed to determine the size of both *S*-alleles in each cultivar. The amplified fragments were run in agarose gel by electrophoresis, and seven different *S*-alleles (*Sa, Sb, Sc, Se, Sf, Sh, Sk*) were identified in the cultivars analyzed. The fragment sizes ranged between 393 and 1,580 bp using PruC2-PCER (**Figure 5**) and between 820 and 1,993 bp using PruT2-PCER. Six *S*-genotypes were identified (*SaSb, SbSc, SbSf, ScSh, SeSh, SfSk*).

The phenology of each cultivar allowed to calculate the length of the flowering period for a total period of four years, considering full bloom when most flowers were at stage F, stage 65 BBCH. Flowering in orchard conditions (**Figure 6**) allowed the comparison of flowering times between cultivars and years, and the determination of which cultivars are coincident at flowering time in each year (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1: Pollen extraction in Japanese plum. (**A**) Flower buds at balloon stage D, according to Baggiolini[24], stage 57 of the BBCH scale. (**B**) Removal of undehisced anthers from the flower. (**C**)

Dehisced anthers showing the pollen grains. (**D**) Sieve of pollen grains from anthers using a fine mesh.

Figure 2: Hand-pollination experiments to determine self-(in)compatibility in Japanese plum. Field pollinations: (**A**) Flowering in Japanese plum, with flowers at anthesis and at balloon stage. (**B**) Flowers at balloon stage D, according to Baggiolini[24], stage 57 of the BBCH scale after emasculation. (**C**) Caged tree to avoid the arrival of insects. (**D**) Supplementary hand-pollination of non-emasculated flowers. Laboratory pollinations: (**E**) Cut on the base of pedicel underwater and emasculated flowers placed on soaked foam. (**F**) Hand-pollination of the pistils with a fine paintbrush.

Figure 3: Fruit drop in Japanese plum as affected by different pollination treatments. Self- and cross-pollination in emasculated and non-emasculated flowers. Percentage of flowers and developing fruits from the original number of remaining flowers in the tree during the 5 weeks after pollination in Rubirosa.

Figure 4: Pollen germination and pollen tube growth in self-pollinated flowers in Japanese plum. (**A**) In vitro pollen germination. (**B**) Non-germinated pollen grains in vitro. (**C**) Pollen grain germination on the stigma surface. (**D**) Pollen tube arrested in the upper third of the style. (**E**) Pollen tube growing along the style. (**F**) Pollen tubes at the base of the style. Scale bars, 200 μm.

Figure 5: PCR amplification using primer set PruC2-PCER of nine Japanese plum cultivars. Identification of seven *S*-alleles (*Sa, Sb, Sc, Se, Sf, Sh, Sk*) and six *S*-genotypes (*SaSb, SbSc, SbSf, ScSh, SeSh, SfSk*). Incompatibility Group (I.G.[3]), 'Fortune' (F), 'TC Sun' (TS), 'Laroda' (LR), 'Queen Rosa (QR), 'Red Beaut' (RB), 'Golden Globe' (GG), 'Kelsey' (K), Negative control (distilled water) (C-), 'Zanzi Sun' (ZS), 'Laetitia' (LT). 1 kb: Size standard.

Figure 6: Monitoring of phenology of Japanese plum. Two Japanese plum cultivars with flowers in different phenological stage. Stage C[24], stage 55 of BBCH scale (left) and Stage F[24], stage 65 of BBCH scale (right).

Figure 7: Flowering time in four Japanese plum cultivars over 4 years. Period from the first to the last open flowers. Yellow cells indicate the days of full bloom in which most flowers were open.

Table 1: Reaction conditions used in this protocol.

Table 2: Pollen germination and pollen tube growth through the style for two Japanese plum cultivars after self- and cross-pollinations. Number of hand-pollinated pistils examined, percentage of germinated pollen grains on stigma, percentage of pistils with pollen tubes at the base of the style, mean number of pollen tubes at the base of the style, incompatible (-) or compatible (+) cross, and self-compatible (SC) or self-incompatible (SI).

Table 3: Percentage of in vitro pollen germination of 15 Japanese plum cultivars.

Mean ± SD of six replicates.

DISCUSSION:

The methodology described herein for pollination requirements of Japanese plum cultivars requires determining the self-(in)compatibility of each cultivar by controlled pollinations in the field or the laboratory, and the subsequent observation of pollen tube growth with fluorescence microscopy. The incompatibility relationships are established by the characterization of the *S*-alleles by molecular genotyping. Finally, the selection of pollinizers is performed by the monitoring phenology to detect those cultivars that coincide at flowering every year.

Establishing pollination requirements in new Japanese plum-type cultivars is becoming increasingly important due to the high number of new cultivars with unknown pollination requirements and that most of them are self-incompatible[3]. The approach described herein, combining phenological monitoring, controlled pollinations, fluorescence microscopy, and molecular genotyping, has proven to be useful in determining the pollination requirements of cultivars[14].

Japanese plum hybrids show a number of peculiarities that hinder the use of a single approach for determining pollination requirements as used in other *Prunus* species. First, the flowers are smaller and more fragile[3]. Pollen extraction and handling is similar to that of other *Prunus* spp.[12], although it is necessary to collect a higher number of flowers because the anthers have less amount of pollen grains[3]. In some cultivars such as Rubirosa, the emasculation of the flowers cannot be used because this technique causes ovule degeneration[22] and the subsequent drop of the flower[21], which can result in false diagnosis of self-incompatibility. For breeding purposes, flower emasculation in sensitive cultivars used as female parental can lead to a lack of offsprings[3].

The number of flowers is much higher than in other species of fruit trees, but the percentage of fruit set is very low[3]. This makes it necessary to use a higher number of flowers for both field and laboratory pollinations[21,22]. Laboratory pollinations and subsequent microscopic observations of pollen tube growth allow the assessment of self-(in)compatibility more accurately than by monitoring hand-pollinated flowers in the field until harvest, since this technique avoids environmental influence and allows the analysis of a higher number of cultivars than in field experiments. However, flower handling is more toilsome than in other *Prunus* species such as apricot[12], since it is necessary to cut the pedicel underwater before placing the pistils in the foam. Furthermore, a higher number of pistils must be analyzed under the microscope than in other species, because in the compatible pollen-pistil relationships between Japanese plum hybrids, the pollen tubes reach the ovary in a reduced percentage of flowers[3]. In addition, pollen tubes are more difficult to detect under the microscope, and the number of tubes reaching the base of the style is lower[15,18,20,28–34]. In those cultivars whose flowers are especially fragile and degenerate under laboratory conditions before the pollen tubes reach the base of the style, the self-(in)compatibility should be evaluated by field pollinations.

The evaluation of pollen viability allows to know whether the pollen used in the hand-pollinations is adequate and thus to discard possible false diagnoses of self-incompatibility in those cases of

low or null germination percentage. The use of this technique has reported considerable differences in pollen germination between Japanese plum cultivars[21,22,35]. Furthermore, this approach is also useful in detecting male sterility[22,36,37], which is of great importance for discarding male-sterile cultivars as pollen donors in commercial orchards and in crosses for breeding purposes.

Although the incompatibility relationships among cultivars can be determined using the same approach used for self-(in)compatibility assessment by laboratory pollinations, that technique has some disadvantages for this purpose. Pollinations can only be performed during the flowering season, and collections or orchards with adult trees placed near the laboratory are needed for the collection of flowers, whose lifespan is very short[3,38]. Furthermore, the number of relationships analyzed each year is low, because each pair of cultivars require a particular cross-pollination. As an alternative, the identification of the *S*-alleles by PCR does not require flowers, since DNA can be extracted from any plant tissue; therefore, the period during which the samples can be collected is longer. Furthermore, unlike flowers that need to be used immediately, the leaves or other plant tissues can be stored, so the analysis is not limited to a few days in spring, but can be done throughout the year[39]. The identification of the two *S*-alleles for each cultivar by the amplification of the second *S-RNase* intron using the primer set PruC2-PCER and PruT2-PCER[40,41] and the subsequent analysis of the size of the amplified fragments agarose gel electrophoresis[20,21] allow assigning the cultivars to their corresponding incompatibility group (I.G.[3]). Each I.G. includes those self-incompatible cultivars with the same two *S*-alleles, which are therefore inter-incompatible. Cultivars from different groups, carrying at least one different *S*-allele, are inter-compatible.

This technique has the limitation that it does not allow the determination of the self-(in)compatibility for Japanese plum cultivars as it occurs in other *Prunus* species, in which self-compatibility has been associated with a particular *S*-allele, such as S_f in almond[42] and S_4' in sweet cherry[43]. Some *S*-alleles were initially associated with self-compatibility in Japanese plum, such as S_b[20,44], S_e[19,20,44], S_g[45], and S_t[46]. However, subsequent works have reported self-incompatible cultivars that carry these *S*-alleles[14,20–22,47]. Therefore, further work of *S*-allele sequencing is required in Japanese plum to clarify whether different alleles of the same size or mutations have been erroneously identified as alleles S_b, S_e, S_g, or S_t[3]. Meanwhile, the assessment of self-(in)compatibility in Japanese plum should be analyzed by field- or laboratory-pollinations and the subsequent monitoring of fruit drop in the field or the behavior of pollen tubes under the microscope.

The identification of *S*-alleles by PCR analysis has been shown to be adequate to establish incompatibility relationships between cultivars[3]. However, to choose adequate pollinizers, it is necessary to combine this information with the data on the flowering times of each cultivar in each area for several years, since the mismatch in the flowering period, even if it only occurs in some years, may cause lack of fruit set with significant reduction in harvest[14].

Many of the differences observed in floral biology and agronomic behavior between cultivars may be related to their origin, since all the cultivars currently grown are hybrids derived from

crosses between the original species *P. salicina* with other species of the same genus but with different characteristics[5,48]. This may be the main reason why it is necessary to combine different techniques to determine pollination requirements, unlike other fruit species. Knowing the pollination requirements of each cultivar facilitates the adequate selection of cultivars for the design of new orchards and allows the detection and resolution of production problems related to the lack of pollination in established orchards.

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DISCLOSURES:

The authors have nothing to disclose.

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615

Figure 1

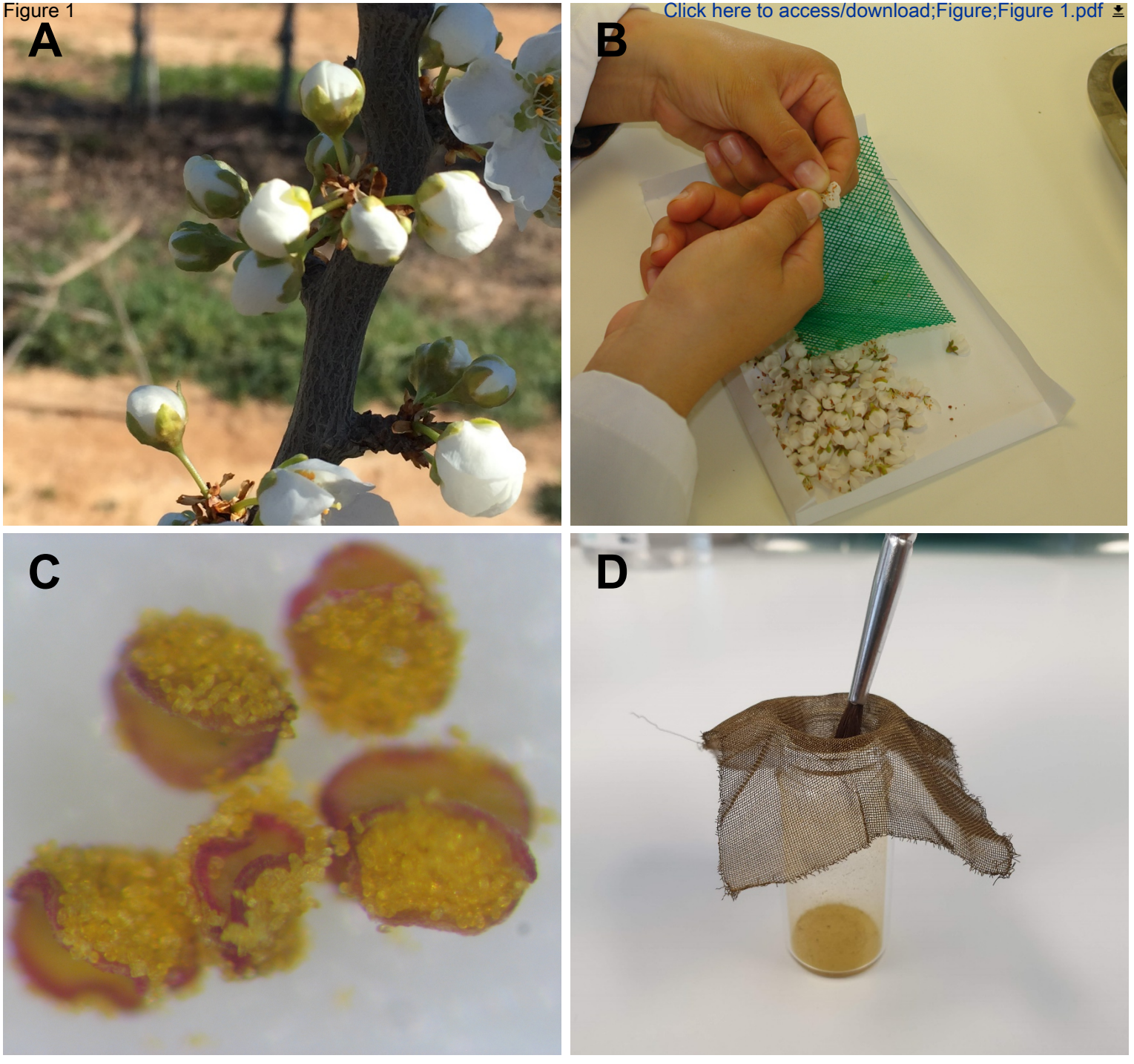


Figure 2

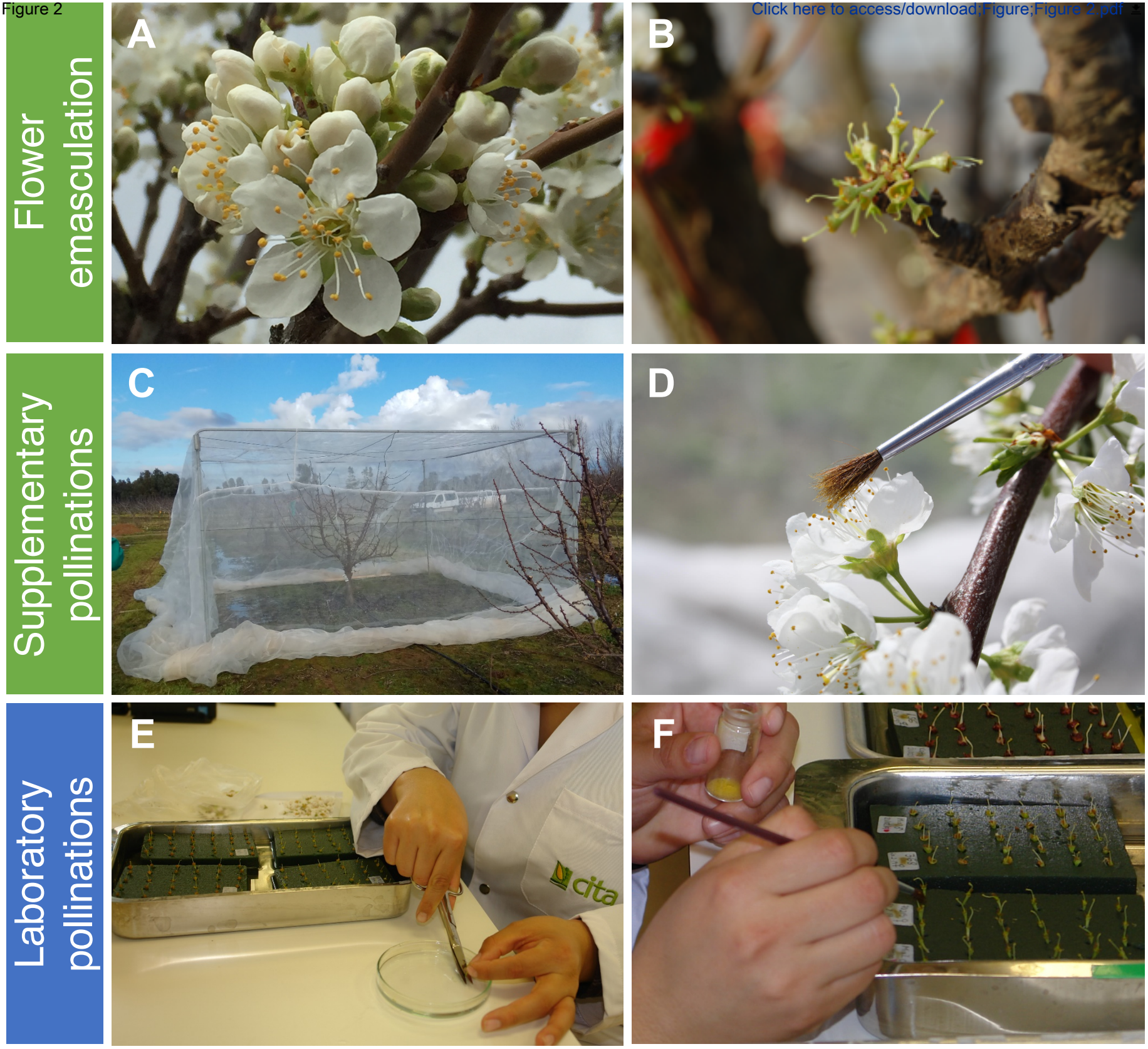


Figure 3

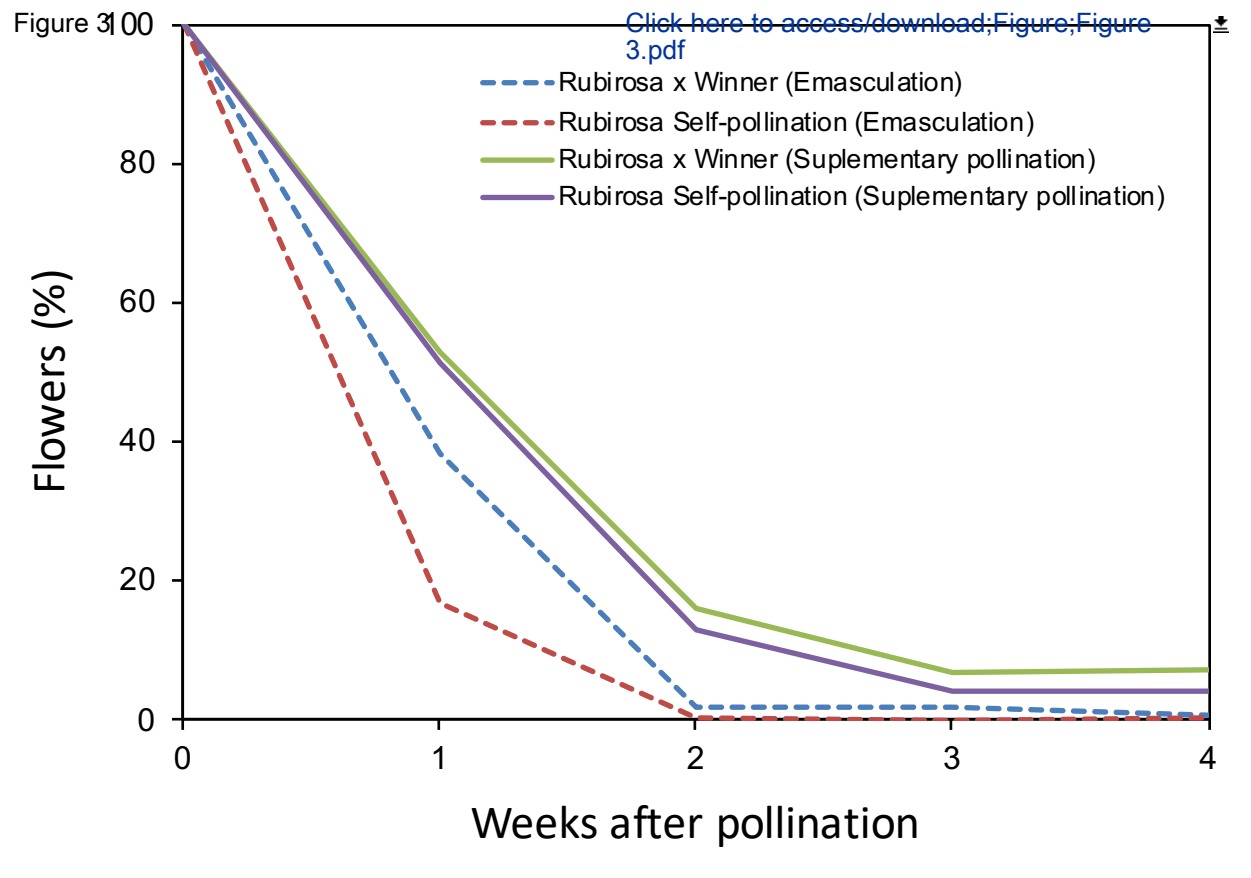


Figure 4

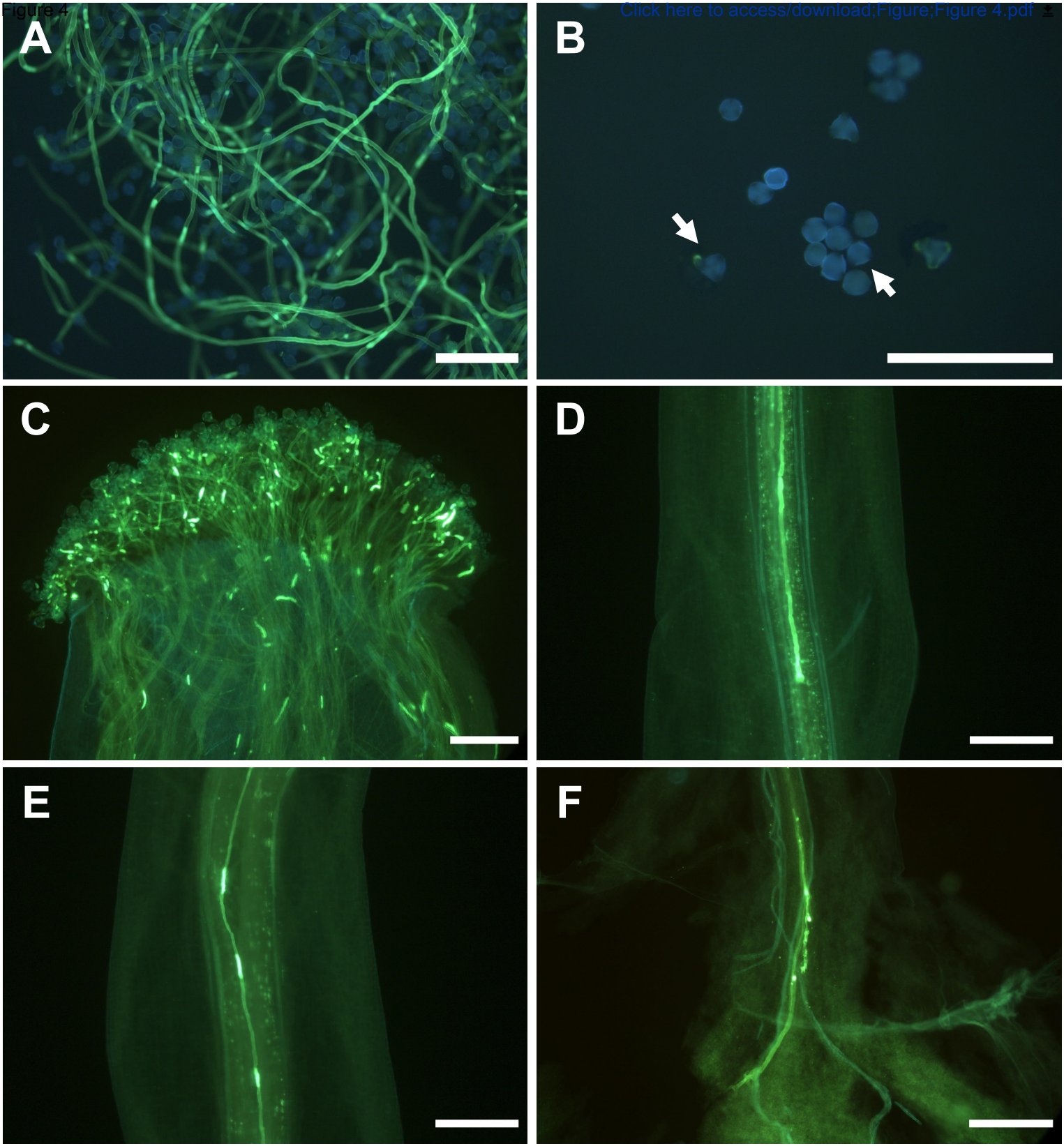


Figure5_R1

Click here to access/download;Figure;Figure 5_R1.pdf

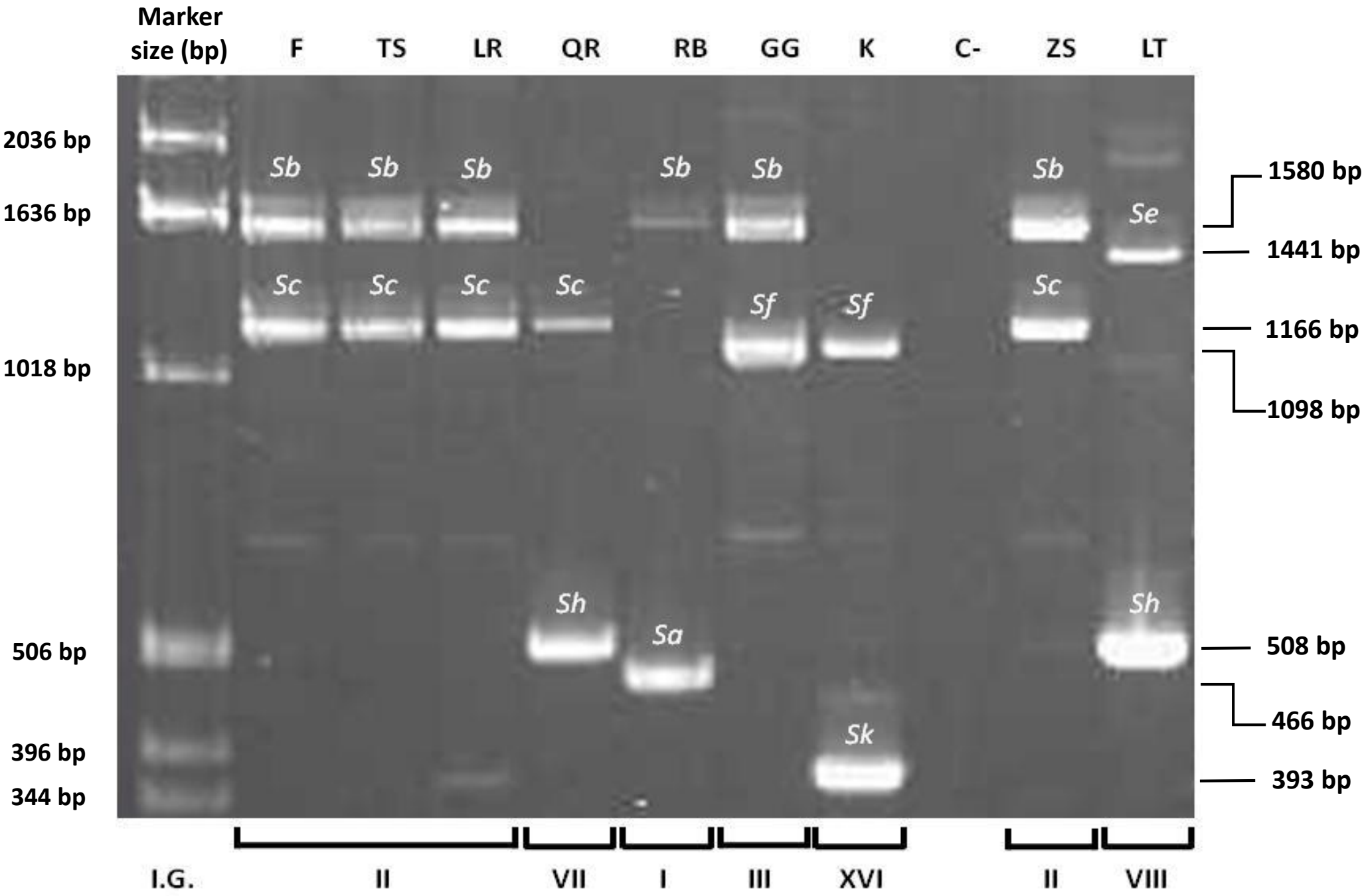
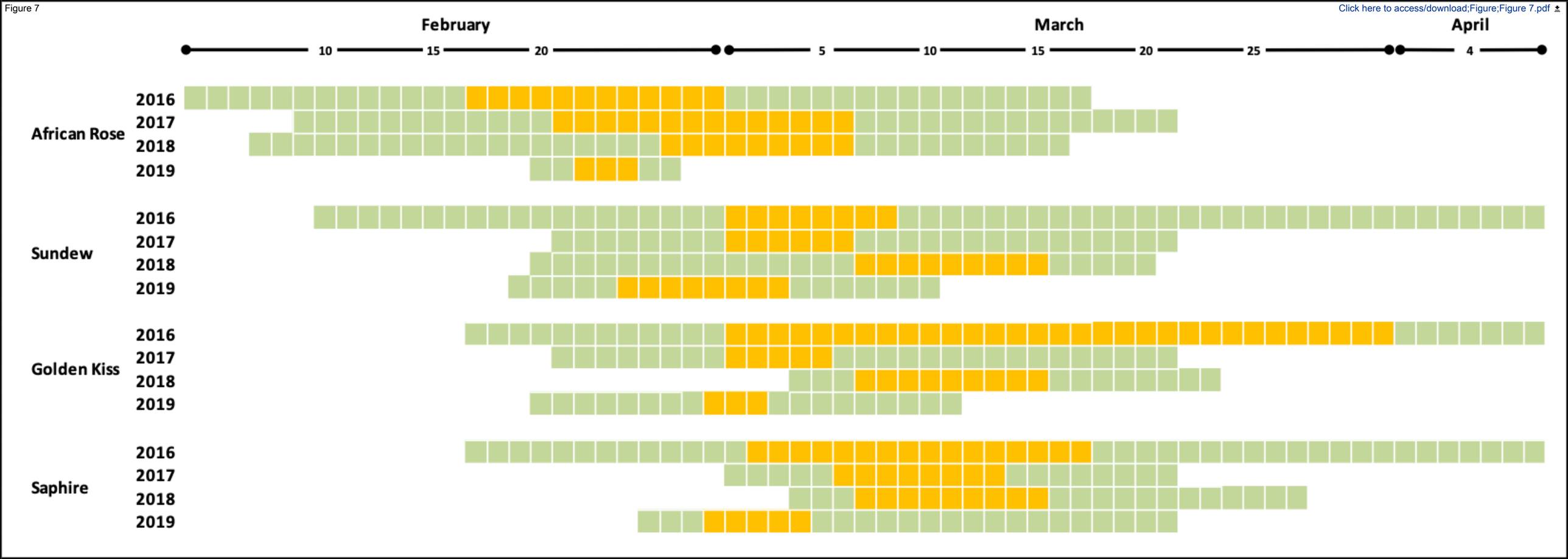


Figure 6

Figure 7

Click here to access/download;Figure;Figure 7.pdf



Reagents	**Volume per one reaction (L)**
H_2O	11.50
10X Buffer with 20 mM $MgCl_2$	2.80
dNTP mix , 10 mM each	0.80
Primer forward	0.40
PruC2 (5'-CTATGGCCAAGTAATTATTCAAACC-3')[40] or	
PruT2 (5'- TSTTSTTGSTTTTGCTTTCTT-3')[40]	
Primer reverse	0.40
PCER (5'-TGTTTGTTCCATTCGCCTTCCC-3')[41]	
DNA template	4.00
Taq DNA polymerase, 500 U	0.09
Final volume	20.0

Cultivars (S- genotype)	Number of pistils examined	Germinated pollen grains on stigma (%)	Pistils with pollen tubes at the base of style (%)	Pollen tubes at the base of style (mean number)	Self/cross - compatibility reaction
TC Sun (*SbSc*) × Larry Ann (*SbSh*)	22	92.3	27	1.2	+
TC Sun (*SbSc*) × Blackamber (*SbSc*)	10	74.8	0	0.0	-
TC Sun (*SbSc*) self-pollination	44	78.3	7	1.0	+
Golden Plum (*SbSc*) × Black Star (*SeSf*)	11	64.7	36	1.5	+
Golden Plum (*SbSc*) × TC sun (*SbSc*)	11	98.4	0	0.0	-
Golden Plum (*ShSk*) self-pollination	38	85.2	0	0.0	-

Cultivar	Germination (%)	SD*
Earlemoon	50	3.3
Earliqueen	30	4.2
Eldorado	10	1.3
Friar	52	2.0
Golden Japan	17	3.8
Golden Plumza	18	3.3
Laroda	46	3.6
Larry Ann	20	5.8
Methley	2	0.8
Owen T	16	0.3
Primetime	20	3.3
Queen Rosa	42	3.9
Royal Diamond	19	2.2
Santa Rosa	36	2.0
TC Sun	49	3.1

*SD = Standard deviation

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Acetic Acid Glacial	Panreac	131008.1611	
Agar	iNtRON Biotechnology	25999	
Aniline blue	Difco	8504-88	
Boric Acid (H_3BO_4)	Panreac	131015.1210	
Calcium Nitrate 4-hydrate ($Ca(NO_3)_2{\cdot}4H_2O$)	Panreac	131231.1211	
Coverglass	Deltalab	D102460	24 mm x 60 mm
Digital Camera	Imaging Developmet Systems	UI-1490SE	
Digital Camera Software Suite	Imaging Developmet Systems	4.93.0.	
DNA Oligos	ThermoFisher Scientific		
dNTP Mix, 10 mM each	ThermoSischer Scientific	R0193	
DreamTaq Green DNA polymerase	ThermoFisher Scientific	EP0713	
Ethanol 96°	VWR-Chemicals	83804.360	
1Kb DNA Ladder (U.S. Patent No. 4.403.036) (500pb-12Kb)	Invitrogen	15615-016	Size: 250µg; Conc: 1.0 µg/µl
Gel Documentation System	Bio-Rad	1708195	
Hand Counter	Tamaco	TM-4	
Image Lab Software	Bio-Rad		Image Analyse System for Gel Documentation System
MetaPhor Agarose	Lonza	50180	
Microcentrifuge 5415 R	Eppendorf	Z605212	
Microscope with UV epiflurescence	Leica	DM2500	Barrier filter LP425
Microslides	Deltalab	D100004	26 mm x 76 mm
Mini Electrophoresis System	Fisherbrand	14955170	
Minicentrifuge	ThermoFisher Scientific	15334204	
NanoDrop 1000 Spectrophotometer	ThermoFisher Scientific	ND1000	
Petri Dishes	Deltalab	200201	55 mm x 14 mm
Potassium Phosphate Tribasic ($K_3PO_4{\cdot}1.5H_2O$)	Panreac	141513	
Primer forward 'Pru C2'	ThermoFisher Scientific		
Primer forward Pru T2'	ThermoFisher Scientific		
Primer reverse 'PCER'	ThermoFisher Scientific		
RedSafe Nucleic Acid Staining Solution	iNtRON Biotechnology	21141	
Saccharose	Panreac	131621.1211	
Sodium sulphite anhydrous (Na_2SO_3)	Panreac	131717.1211	
Speedtools plant DNA extraction Kit	Biotools	21272	
TBE Buffer (10X)	Panreac	A0972,5000PE	
Thermal Cycler T100	Bio-Rad	1861096	
Thermomixer comfort	Eppendorf	T1317	
Vertical Autoclave Presoclave II	JP Selecta	4001725	
Vortex	Fisherbrand	11746744	

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Zaragoza, September 23th, 2020

Dear Editor,

We are pleased to enclose a revised version of our manuscript JoVE61897 ("*Establishing pollination requirements in Japanese plum by phenological monitoring, hand pollinations, fluorescence microscopy and molecular genotyping*" by B.I. Guerrero, M.E. Guerra and J. Rodrigo.

The paper has been revised giving careful consideration to the points raised by the editor and reviewers. We describe in detail below the changes made in this new version.

We do appreciate the comprehensive reviews that have clearly contributed to improve our paper over our original submission. We look forward to hearing from you on your final decision.

Yours sincerely,

Brenda I. Guerrero

Changes made addressing the comments of the handling editor and reviewers:

Editorial comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

* The document has been carefully revised.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this work, the authors describe in detail the sequence of research methodologies that lead to the establishment of the best pollen donors in Japanese plum, an important fruit crop in temperate environments. The description of the procedure combines fieldwork (hand pollinations and monitoring of flower phenology), and two ways of laboratory work, one including controlled pollinations with cut flowers, then observing pollen tubes with fluorescence microscopy, and another one extracting DNA and amplifying the alleles that associate with compatibility reactions. This link between agronomical measurements, microscopy evaluations, and molecular characterization, has proven to be successful knowing the excellent trajectory of the research group, and this is exemplified in the current work. The results show a nice set of techniques that can be applied not only to Japanese plum, but also to a number of other fruit tree species. However, the particularities of this species is well described, such as the fragile character of the flowers, limiting the data potentially obtained. The images are fantastic!

Major Concerns:

I have no major concerns.

Minor Concerns:

There are a number of language suggestions that I edited in the pdf enclosed, which may improve the manuscript, although I know that the main purpose is to record a video with the techniques, so the authors are free to take my suggestions or not.

* The paper has been revised giving careful consideration to the points raised by the reviewer in the pdf file.

Thank you very much for the very constructive review of our manuscript.

Reviewer #2:

Manuscript Summary:

Very helpful manuscript for evaluation of the pollination requirements in Asian plum. The procedures are well narrated in orderly way. If possible, the instructions can be used for other Rosaceae species including stone fruits. However, English expressions should be proofread through the whole manuscript. I could find several awkward expressions, and editing by English expert is needed for better manuscript.

* The paper has been revised giving careful consideration to the points raised by the reviewer. The English has been revised through the whole manuscript.

Major Concerns:

None.

Minor Concerns:

Line69. Self-(in)compatibility
* "Self(in)compatibility has been corrected to "Self-(in)compatibility" (line 72)

Line91. Hand-pollinations → Hand-pollination
* "Hand-pollinations" has been changed to "Hand-pollination" (line 94)

Line113. flower buds → flower buds per treatment. (If you mean 800 and 1000 flower buds per branch, change treatment into branch.)
* The sentence has been modified as suggested (lines 115-116)

Line117. (Add to the end of paragraph.) Be careful not to contaminate the fingertip or paintbrush with pollen grains from other cultivars.
* The sentence has been added to the end of paragraph (lines 120-121)

Line122. in field → in the field
* It has been corrected (line 126)

Line127. 10-25% flowers → Why different range of open flowers with pollination of emasculated flowers in Line108 (10-20%)?
* Thanks for your comment, we appreciate that you have notice this issue. The percentage have been revised (10-20%) in both sections (Line 111, line 131).

Line128. remove old flowers → Do you have reason for removing? Although the old flowers are open, they are not contaminated due to the mesh cage.
* Thank you for reporting the error. Old flowers should be removed before the emasculation of flowers (see section 1.2.1), but not in this treatment. The sentence has been corrected (line 134)

Line134. have opened → open
* It has been corrected (line 140)

Line143. In the laboratory, emasculate 30 flowers per treatment (self- and cross-pollination).
* The sentence has been rewritten as suggested (lines 149-150)

Line145. Emasculation should be proceeded carefully to avoid-
* The sentence has been rewritten as suggested (line 152)

Line158. ensure → Ensure
* "ensure" has been changed to"Ensure" (line 165)

Line170. 3 mL → I think the thickness of medium matters. Thickness of the medium will be different depend of the size of Petri dish. Please specify the size of Petri dish with the volume of medium (e.g. 3 mL), or mention the thickness of the medium.
* We agree that the thickness of medium matters. We have included both the volume of medium and the dimensions of Petri dish (line 177)

Line173. Spread pollen → Spread the pollen
* "the" has been included (line 181)

Line174. two Petri dishes → Why two Petri dishes? For replication?
* Indeed, two Petri dishes are used for replication, as mentioned in section 3.1.6 (line 197)

Line192. three times for 1 h → I'm confused this part. Is it 3 h in total or 3 times within 1 h?
* The sentence has been rewritten. The fixed pistils are rinsed with distilled water three times (1h each, 3 h in total) (lines 201-202)

Line258. Used de DNA → Use the DNA
* "de" has been corrected to "the" (line 269)

Line262. Monitoring of flowering dates. → Monitoring flower dates
* The sentence has been rewritten as suggested (line 273)

Line264. From the first to the last open flowers. → If the percentage of open flower is suggested, it will be better.
* The percentage of open flowers has been included: "…from the first (about 5%) to the last open flowers (about 95%) (lines 275-276)

Line274. reduce amount → small amount
* Reduce has been changed to small (line 285)

Line306-309. For hand pollination in the laboratory, the pistils ~ with the pollen previously extracted. → Remove this sentence which was already mentioned in section 2.4.
* The paragraph has been rewritten. Although some information is also mentioned in "Protocol", we have kept some information to describe Figure 2, since it is mandatory to cite all figures in this section according to the JoVE guidelines for authors (lines 318-321)

Line309. In hand pollinations in the laboratory, self-compatible
* The sentence has been revised, taking in account this suggestion and that of Reviewer 1 (line 321)

Line316. resulting in a low percentage of germination, the cultivar
* The sentence has been revised, taking in account this suggestion and that of Reviewer 1 (lines 329-330)

Line322. after pollination (Figure 4C).
* The sentence has been revised, taking in account this suggestion and that of Reviewer 1 (line 336)

Line327. However, when S allele of the pollen grain is different with that of pistil, the tube
* The sentence has been corrected as suggested (lines 341-343)

Line335. 1580bp → 1580 bp
* It has been corrected (line 351)

Line335. "Figure 5" should be in bold.
* It has been corrected (line 352)

Line356. Figure1A is mentioned as 57 BBCH in Line 277. Please unify the level of BBCH. I think 59 BBCH is more suitable for Figure 1A rather than 57 BBCH.

* In other to avoid confusion, we consider BBCH code 57 more appropriate than 59, because in some references code 59 is described as "The stamens are apparent (Stage E of the Baggiolini code)" (Pérez-Pastor et al., 2004; Fadón et al., 2015). Therefore, we have kept the code 57 BBCH for this phenological stage throughout the manuscript (line 375)

Fadón, E., Herrero, M., Rodrigo, J. Flower development in sweet cherry framed in the BBCH scale. *Scientia Horticulturae*. **192**, 141–147 (2015).

Pérez-Pastor, A., Ruíz-Sánchez, M.C., Domingo, R., Torrecillas, A. Growth and phenological stages of Búlida apricot trees in south-east Spain. *Agronomie*, **24** (2), 93-100 (2004).

Line362. Figure2A is mentioned as 65 BBCH in Line 278. The different levels for one picture is confusing. Please unify the BBCH level or remove "D, according to Baggiolini24, stage 57 of the BBCH scale".

* The sentence has been revised as suggested (line 381)

Line380. Please add reference to I.G. such as Guerra and Rodrigo, 2015 (a review paper).

* The reference "Guerra and Rodrigo, 2015" has been included (line 399)

Line396. percentage of germinated pollen grains on stigma

* It has been corrected as suggested (line 415)

Line421. small → low

* "small" has been changed to "low" (line 448)

Line432. w hose → whose

* It has been corrected (line 459)

Line433. Please remove "have time to".

* "have time to" has been removed (line 460)

Line463. S-allele → S should be in italic.

* "*S*" has been formatted in italic (line 490)

Line474. Please put a period (.) behind "cultivars".

* A period (.) has been put behind "cultivars" (line 501)

Line501. Please put the publisher and city between 357 and (2008).

* The publisher and city have been included (line 529)

Line506. In Fruit Breeding → Fruit Breeding
* “In” has been removed (line 534)

Line507. Jhon → John
* “Jhon” has been changed to “John” (line 535)

Line511. Please put the pages behind (2).
* The pages “7-15” have been put behind “(2)” (line 539)

Line527. sweetcherry → sweet cherry
* It has been corrected (line 555)

Line532. 261 → 261, 109022
* It has been corrected (line 560)

Line544. 'Moniqui.' → 'Moniqui'.
* It has been corrected (line 572)

Line553. Please put a period (.) behind "Science"
* A period (.) has been put behind “Science” (line 581)

Line575. Self-pollination
* “Self- pollination” has been corrected to “Self-pollination” (line 603)

Line581. Peach →peach
* It has been corrected (line 609)

Line593. 30 → 30 (6)
* It has been corrected (line 621)

Line599. S-allele → S should be in italic.
* “*S*” has been formatted in italic (line 627)

Line599. identification,
* “identification ,” has been corrected to “identification,” (line 627)

Line605. Self-(in)compatibility
* “Self-(in) compability” has been corrected to “Self-(in)compability” (line 633)

Line615. self-compatible
* “self- compatible” has been corrected to “self-compatible” (line 643)

Line624. plums
* “Plums” has been corrected to “plums” (line 652)

Figure 5. Please inform the size of each bands in marker (in bp). "1kb" is not enough to estimate the size of bands in the figure.
* Figure 5 has been modified, including the size of each band

Table 2.
- *Mean percentage of pollen grains germinated on stigma → Germinated pollen grains on stigma (%)*
* It has been changed as suggested

- *Mean of pollen tubes at the base of style → Confusing. What is difference with left column, "Pistils (%) with pollen tubes at the base of style". And please mention the unit. Table3.*
* It has been changed as suggested. This column refers to the number of pollen tubes observed at the base of the style

- *Add a footnote to SE.*
* A footnote has been added

* Thank you very much for the very constructive review of our manuscript.

Reviewer #3:

I don't think there is any problem with the contents of the method, the results obtained and the interpretation. However, the description manner of the results and discussion sections should be modified. In the results section, authors should only describe the data obtained by conducting experiments, and the text should usually be written in the past form.
* The text in this section has been revised using the past form. However, we have used the present form in the paragraphs corresponding to the botanical descriptions, since it is mandatory to reference all figures in this section according to the JoVE guidelines for authors. This affects to paragraph 1 (lines 282-292) and 6 (lines 333-343)

For example, what is written in L322-324 is not revealed through the experiments.
* The sentence has been removed (lines 337-338)

Is the part of L345-352 a consideration rather than a result?
* This paragraph has been moved from Results to Discussion (lines 424-429)

The discussion section should consciously write what is suggested based on the results.

For example, I think that the part of L415-417 is suggested from the result of Fig.3. There is no point in conducting an experiment if what has already been clarified is only discussed. Thus, I recommend the authors revise the manuscript as needed.
* This section has been revised as suggested, but also taking into account JoVE's guidelines for authors ("The discussion section of the article should be focused on the protocol and not on the representative results"). In addition to the new first paragraph, the name of the cultivar whose data from Figure 3 are discussed is included in the new version (line 442)

Other minor corrections are as follows.

L63. SRNAse -> S-RNase?
* "*SRNAse*" has been corrected to "*S-RNase*" (line 65)

L153. How did you keep the floral foam wet? Which did you use, water or life-prolonging agent such as cut flowers?
* We use water, which has been included in the protocol (lines 159-160)

L226. Where can I find a description of information on primer sequence? I'm sorry if I just overlooked the description about it.
* The information on primer sequence has been included in Table 1

* Thank you very much for the very constructive review of our manuscript.