Submission ID #: 61897
Scriptwriter Name: Domnic Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18878633

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

Authors and Affiliations:

Brenda I. Guerrero[1,2], Mª Engracia Guerra[3], Javier Rodrigo[1,2]

[1]Unidad de Hortofruticultura. Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Zaragoza, Spain
[2]Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Zaragoza, Spain
[3]Departamento de Hortofruticultura, Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX), Instituto de Investigaciones Agrarias Finca La Orden, Badajoz, Spain

Corresponding Authors:
Brenda I. Guerrero (773401@unizar.es)

Email Addresses for All Authors:

guerrero.bren@gmail.com
773401@unizar.es
mariaengracia.guerra@juntaex.es
jrodrigo@aragon.es

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

   NOTE: Authors changed the choice for interview statements during the shoot. Now the statements are required to be read by VO.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes, same building but different laboratories on different floors and the plum orchard that is about 1 km from the building.**

Current Protocol Length

Number of Steps: 21
Number of Shots: 38

Introduction

1. Introductory Interview Statements

NOTE for VO: Please record the introductory interview and conclusion statements.

REQUIRED:

1.1. This protocol is becoming increasingly important due to the high number of new self-incompatible Japanese plum cultivars with unknown pollination requirements.

1.1.1. *2.3.2*

1.2. The main advantage of this technique is that it allows for determination of the pollination requirements of cultivars by combining two complementary approaches in field and laboratory conditions.

1.2.1. *2.6.1*

OPTIONAL:

1.3. This approach can also be applied to determine pollination requirements in other self-incompatible fruit tree species like apricot or sweet cherry.

1.3.1. *5.1.1*

Protocol

2. Hand-pollination in the field

2.1. For pollen extraction, collect flower buds at balloon stage, according to stage 57 on the BBCH *(B-B-C-H)* scale **[1-TXT]**. *Videographer: This step is important!*

2.1.1. Talent collecting flower buds as per the given stage. **TEXT: BBCH- Biologische Bundesanstalt, Bundessortenamt and CHemical industry**

2.2. Remove the anthers using a plastic mesh **[1-TXT]** and place them on paper at room temperature for 24 hours until anther dehiscence **[2]**.

2.2.1. Talent removing anthers using plastic mesh. **TEXT: 2 mm x 2 mm pore size**

2.2.2. Talent placing the anthers on paper.

2.3. Sieve the pollen grains through a fine mesh **[1-TXT]** and conserve them in a 10-milliliter glass tube with a cap at 4 degrees Celsius until use **[2]**.

2.3.1. Talent sieving the pollen grains through mesh. **TEXT: 0.26 mm x 0.26 mm pore size**

2.3.2. Talent placing the pollen grains in glass tube.

2.4. When 10 to 20% of the flowers are open, select and label several branches, leaving 1000 to 1500 flowers per pollination treatment **[1]**.

2.4.1. Talent labelling the branches for pollination treatment.

2.5. On the next day, when more flowers are open, pollinate each flower using a paintbrush with pollen from the same cultivar for self-pollination, and pollen from other cultivars as cross pollination control. Pollinate every day until all flowers are open **[1]**.

2.5.1. Talent pollinating flowers using paintbrush.

2.6. Record weekly counts of flowers and developing fruits from anthesis to harvest to characterize the fruit drop pattern and quantify the final fruit set in each pollination treatment **[1]**.

2.6.1. Talent recording counts of flowers and developing fruits.

3. Hand pollinations in the laboratory

3.1. Collect 50 to 100 flowers at balloon stage, according to stage 57 on the BBCH scale **[1]**. Emasculate 30 flowers per treatment in the laboratory for self- and cross-pollination **[2]**.

3.1.1. Talent collecting flowers at balloon stage.

3.1.2. Talent emasculating some flowers.

3.2. Make a fresh cut on the base of each flower pedicel underwater **[1]** and place it on a piece of wet florist foam. Use one piece of foam for each pollination treatment **[2]**.

3.2.1. Talent making fresh cut at the base of flower pedicel.

3.2.2. Talent placing the flower on wet florist foam.

3.3. After 24 hours, hand pollinate each pistil with the pollen collected previously using a fine paintbrush **[1]**. Pollinate one set of pistils with pollen from the same cultivar, and the other set with pollen from another compatible cultivar as control **[2]**.
Videographer: This step is difficult and important!

3.3.1. Talent hand pollinating each pistil using paint brush.

3.3.2. Talent self-pollinating and cross pollinating the pistils.

3.4. Fix these pollinated pistils in a fixative solution of ethanol and acetic acid for at least 24 hours at 4 degrees Celsius **[1-TXT]**.

3.4.1. Talent fixing the pistils in fixative solution. **TEXT: Ethanol:Acetic acid - 3:1**

~~3.4.2. Talent replacing the fixative solution with 75% ethanol~~

NOTE: 3.4.2 was not filmed and hence corresponding VO description is removed.

4. Microscopic Observations

4.1. Spread the pollen of each cultivar previously used as a pollen donor in the pollinations in two Petri dishes **[1]** and incubate them at 25 degrees Celsius for 24 hours **[2]**.

4.1.1. Talent spreading pollen on a petri dish.

4.1.2. Talent storing the petri dishes at 25 degrees for 24 hours.

4.2. To observe the pollen grains, add 2 to 3 drops of aniline blue solution to each Petri dish **[1]** and observe them under a UV epifluorescence microscope after 5 minutes **[2-TXT]**.

4.2.1. Talent adding aniline blue solution in the petri dish.

4.2.2. Talent observing the pollens under microscope. **TEXT: Use exciter filter BP340-390 and barrier filter LP425**

4.3. Count viable and non-viable pollen grains in three fields per Petri dish, with each field containing about 100 to 200 pollen grains in two Petri dishes for each cultivar **[1]**.

4.3.1. Talent counting the pollen grains under microscope.

4.4. To observe the pollen tubes, squash softened pistils in a drop of 1% aniline blue solution under a cover glass on a slide **[1]**. Observe the pollen tube growth along the style under a microscope with UV epifluorescence using exciter filter BP340-390 and barrier filter LP425 **[2]**. *Videographer: This step is important!*

4.4.1. Talent squashing softened pistils in aniline solution under cover glass.

4.4.2. Talent observing pollen tubes under microscope.

5. Determining incompatibility relationships and monitoring flower dates

5.1. Collect 3 to 4 young leaves from each cultivar in the field **[1]** and extract DNA from the leaves **[2]**.

5.1.1. Talent collecting leaves in the field.

5.1.2. Tube with extracted DNA.

5.2. Set up a master mix for each pair of primers in 1.5-milliliter microtubes **[1]**. Add all reagents and mix thoroughly **[2]**. *Videographer: This step is difficult!*

5.2.1. Talent setting up the volume of master mix in microtube. Author's NOTE: Text overlay has been transferred from 5.2.1 to 5.3.1

5.2.2. ~~Talent adding all reagents in the microtube.~~ Author's NOTE: 5.2.2 was not filmed.

5.2.3. Talent mixing the tube thoroughly.

5.3. Aliquot 16 microliters of this master mix into each 200-microliter PCR tube containing 4 microliters of DNA template or control **[1-TEXT]**. Mix them gently **[2]**, close the caps **[3]** and centrifuge at 2000 times g for 30 seconds **[4]**.

5.3.1. Talent aliquoting the master mix in PCR tubes. **TEXT: 16 µL per reaction.**

5.3.2. Talent mixing the tube.

5.3.3. Talent closing the caps of the tube.

5.3.4. Talent centrifuging the tubes.

NOTE: Text overlay is added at 5.3.1

5.4. Place the tube in the thermocycler **[1]** and run the PCR program as mentioned in the text manuscript **[2]**.

5.4.1. Talent placing the tubes in thermocycler.

5.4.2. Talent performing PCR.

5.5. For estimating the fragment size by gel electrophoresis, load the first and last wells with 2 microliters of the DNA ladder **[1]** and 3 microliters of the PCR product in the other wells **[2]**. Close the chamber **[3]** and run the gel at 100 Volts for 30 minutes **[4]**.

5.5.1. Talent loading the DNA ladder.

5.5.2. Talent loading the PCR products.

5.5.3. Talent closing the chamber of electrophoresis unit.

5.5.4. Talent turning the power on.

5.6. Observe the gel under UV light using a gel imaging system. Use the DNA ladder to estimate the size of the amplified fragments and compare it with the positive and negative controls **[1]**. *Videographer: This step is important!*

5.6.1. Talent observing the gel.

5.7. Monitor the phenology of different trees of each cultivar over different years of flowering. Establish the length of the flowering period from the first to the last open flowers. Full bloom is considered when at least 50% of flowers are at stage F, according to stage 65 on the BBCH **[1]**. *Videographer: This step is important!*

5.7.1. Talent looking at a calendar of flowering times.

Results

6. Results: Determination of Pollination Requirements in Japanese Plum-type Hybrids

6.1. The pattern of fruit drop of hand-pollinated flowers after emasculation **[1]** or supplementary pollination **[2]** showed that most flowers dropped 2 to 3 weeks after pollination **[3]**, but 4% of self-pollinated flowers remained in the tree, indicating that this cultivar is self-compatible **[4]**.

6.1.1. LAB MEDIA: Figure 2 A and B.

6.1.2. LAB MEDIA: Figure 2 C and D.

6.1.3. LAB MEDIA: Figure 3.

6.1.4. LAB MEDIA: Figure 3. *Video Editor: Emphasize the purple line.*

6.2. Viable pollen grains showed a pollen tube longer than its length after 24 hours in the culture medium **[1]** and non-viable pollen grains did not germinate. **[2]**.

6.2.1. LAB MEDIA: Figure 4 A.

6.2.2. LAB MEDIA: Figure 4 B.

6.3. During pollination, pollen grains were transferred to the stigma. Each germinating pollen grain produced a pollen tube, which grew through the pistil structures **[1]**.

6.3.1. LAB MEDIA: Figure 4 C.

6.4. In self-incompatible cultivars, the pollen tube stopped growing in the upper third of the style **[1]**. However, in self-compatible cultivars the pollen tube could grow through the style **[2]**, reach the ovary, and fertilize an ovule **[3]**.

6.4.1. LAB MEDIA: Figure 4 D.

6.4.2. LAB MEDIA: Figure 4 E.

6.4.3. LAB MEDIA: Figure 4 F.

6.5. PCR analysis was carried out using primers from the conserved regions of *S-RNase* of sweet cherry and Japanese plum, making it possible to determine the size of *S*-alleles in each cultivar **[1]**.

6.5.1. LAB MEDIA: Figure 5.

6.6. The length of the flowering period for a total of four years was calculated. Flowering in orchard conditions **[1]** allowed the determination of which cultivars are coincident at flowering time each year **[2]**.

6.6.1. LAB MEDIA: Figure 6.

6.6.2. LAB MEDIA: Figure 7.

Conclusion

7. Conclusion Interview Statements

7.1. When attempting this protocol, make sure to use a higher number of flowers of Japanese plum for hand-pollinations since the percentage of fruit set is very low compared to other *Prunus* species.

7.1.1. *2.5.1, 2.1.1.*

7.2. It is not possible determine self-incompatibility by molecular approaches. Further work of *S*-allele sequencing is required to assess if some *S*-alleles are associated with self-compatibility.

7.2.1. *LAB MEDIA: Figure 5*

7.3. The combination of field and laboratory approaches has resulted in valuable information for the design of new commercial orchards and can be used in other fruit tree species.

7.3.1. *2.2.1 and 3.3.1*