COST
EUROPEAN COOPERATION
IN SCIENCE & TECHNOLOGY

Report on the outcomes of a Short-Term Scientific Mission[1]

Action number: CA18201

Grantee name: Joana Filipa Martinho da Costa

Details of the STSM

Title: Finding Bermuda buttercup – Floral biology

Start and end date: 11/05/2023 to 19/05/2023

Description of the work carried out during the STSM

Description of the activities carried out during the STSM. Any deviations from the initial working plan shall also be described in this section.

(max. 500 words)

Three main activities were planned under this STSM as described in the STSM application. Below, a description of the work carried out under each of the three main activities is provided.

1. Lab work contributing for two tasks of the 'Finding Bermuda buttercup' project – i) flower morphometric analyses and ii) pollen germination and pollen tube growth along the style.

This was the core activity of this STSM. The grantee worked closely with the researchers from the Department of Biodiversity at UP FAMNIT for setting the protocol for i) flower morphometric analyses and ii) pollen germination and pollen tube growth along the style. At first, tests were made using an extra population sampled for this purpose: 30 flowers of the S-morph were used for flower morphometric analyses (photos and measurements using the software Image J) and for investigating pollen germination and pollen tube growth along the style. The protocols described in Costa et al. (2014, 2016) were followed.

Once this was done, laboratory work progressed and 3 mixed populations, 2 dimorphic (S- and M-morphs, S- and L-morphs) and 1 trimorphic populations, were processed. This accounts for 66 flowers for flower morphometric analyses (16 L-, 20 M-, 30 S-morph flowers) and 86 pistils (21 L-, 20 M-, 45 S-morph flowers) for investigating pollen germination and pollen tube growth along the style (test population not considered). In addition, the tables for entering data and a protocol (Appendix 1) were prepared.

[1] This report is submitted by the grantee to the Action MC for approval and for claiming payment of the awarded grant. The Grant Awarding Coordinator coordinates the evaluation of this report on behalf of the Action MC and instructs the GH for payment of the Grant.

2. Knowledge exchange through a seminar on the study system *Oxalis pes-caprae*

A lecture entitled 'Reproductive traits and population structure of the invasive *Oxalis pes-caprae* in the Mediterranean area' was delivered by the grantee on 12 May 2023, in Izola, Slovenia. This was the perfect timing for knowledge exchange between researchers and the grantee, not only under the topic of the lecture, but also about conservation strategies to deal with biological invasions in Slovenia.

3. Short course on scientific writing in biological sciences (7,5h) for MSc students (also open for PhD students and researchers).

Finally, the initially planned 7,5h became a 10h course, which was mainly attended by MSc students, but also by young researchers. The objectives of this course were as follow: a) demystify the process of paper writing, b) brief review of the process of paper writing, and c) provide the attendees with tools for paper writing → scientific writing made easy. All materials used for the classes were made available to the students, including 2 commented papers.

Costa et al., 2014. Plant Biol 16:208-214

Costa et al., 2016. Plant Biosyst 150:923-931

Description of the STSM main achievements and planned follow-up activities

Description and assessment of whether the STSM achieved its planned goals and expected outcomes, including specific contribution to Action objective and deliverables, or publications resulting from the STSM. Agreed plans for future follow-up collaborations shall also be described in this section.

(max. 500 words)

This STSM has contributed to the objectives of the COST Action 18201 Conserve Plants. More specifically, this STSM was planned under the WG1 - *Improving knowledge in plant biology for appropriate in situ conservation*, task 1.1 *Evaluation of species-based approaches aimed at providing plant conservation actions.*

Main goals and expected outcomes of this STSM were fully achieved. The lab protocol jointly established during this STSM will allow researchers from the Department of Biodiversity at UP FAMNIT to proceed with the following tasks under 'Finding Bermuda buttercup' project: a) flower morphometric analyses and b) pollen germination and pollen tube growth along the style. More importantly, comparability with previous data (Castro et al. 2007, 2013) is ensured, this being a major contribution to the study of reproductive strategies and population structure of this invasive plant species in the Mediterranean area.

During the following months, researchers from the Department of Biodiversity at UP FAMNIT will proceed with lab work and stay in close contact with the grantee. Short Zoom calls are planned to follow-up on the work.

This STSM is expected to contribute to the following deliverables: a) final reports (2) from the Bachelor students working in this project at the Department of Biodiversity, UP FAMNIT, b) presentation of preliminary results at the MC planned for August 2023, in Coimbra, Portugal, and c) a scientific paper with the data collected under this project.

For the reasons stated above, I consider this STSM to have been successful in achieving the main objectives initially planned.

APPENDIX 1

Protocol for flower morphometrics

For each population, **10 flowers** per floral morph will be used for measurements of a) corolla length, b) height of the anthers and c) stigma whorls. This task is divided in two subtasks: a) photographing the flowers and b) measurements of sex organs using Image J.

a) Material

Make sure that you have all the material you need before starting. For your convenience, you can find it in a tray in the lab (Fig. 1).

Fig. 1. Tray with the material at the lab.

For this protocol, you will need:

- Labels and pencil (do not use pen)
- Camera and support for the camera
- Stereomicroscope
- Computer
- Millimeter paper
- Tweezers and blade
- Tissue paper
- Glass slide

Since the flowers used for morphometric analyses will also be used for pollen loads and pollen tubes growth, you will also need:

- Tray with Eppendorf tubes
- Glycerol 50% (prepared in distilled water) → for your convenience, 2mL Eppendorf tubes can be used for storing glycerol
- Pasteur pipet

b) Photographs of the flowers

1. Prepare the table where you will be working: i) place the camera at the height marked with a white line, ii) turn on the lights, iii) adjust the millimeter paper under the camera so the white frames appear in the photograph (Fig. 2), iv) place an Eppendorf tray with Eppendorf tubes and a drop of glycerol 50%.

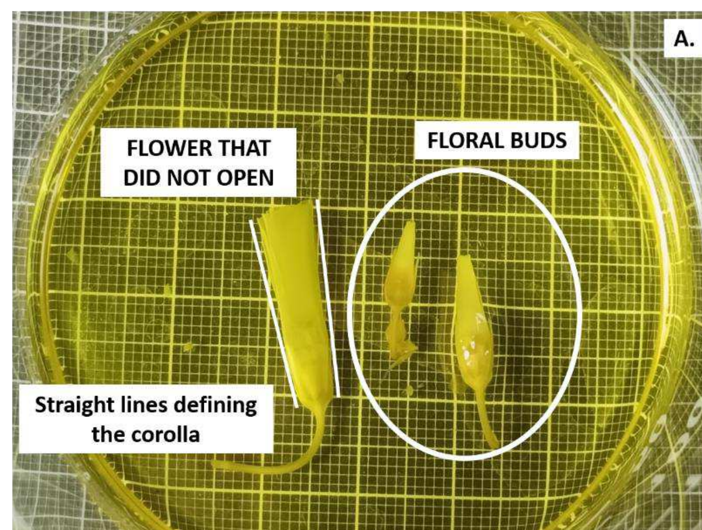
Fig. 2. Material for making the photographs of the flowers.

2. Photographs will be made in groups of 5 flowers and 5 stigmas/anthers. Thus, after selecting the population you will be working with write down two labels i) one for identifying the population in the photos and the ii) other for the Eppendorf tray.
3. The label is made of the complete name of the population plus the style morph and the number of the first and last flower in that group as such (Fig. 3):

Code of the population	IT-AT-01
Floral morph to be photographed	S-morph
Number of the flowers	6-10
Label	IT-AT-01 S6-10

Fig. 3. Corollas for photographs with the label. You should be able to see all these three elements through the camera: label, corollas/sex organs and the white frames.

4. Make sure to get all the labels before starting – this will save you time throughout the process.
5. Carefully select one flower from the vial and make sure that the flower was not too young nor too old at the time of collection. This is easy to check while looking at the flowers in ethanol (Fig. 4).
    a. Please, be aware that young flowers (not flower buds) are perfect for morphometric analyses but these were not exposed to pollinators, so cannot be used for stigmatic pollen loads and pollen tubes growth along the style.



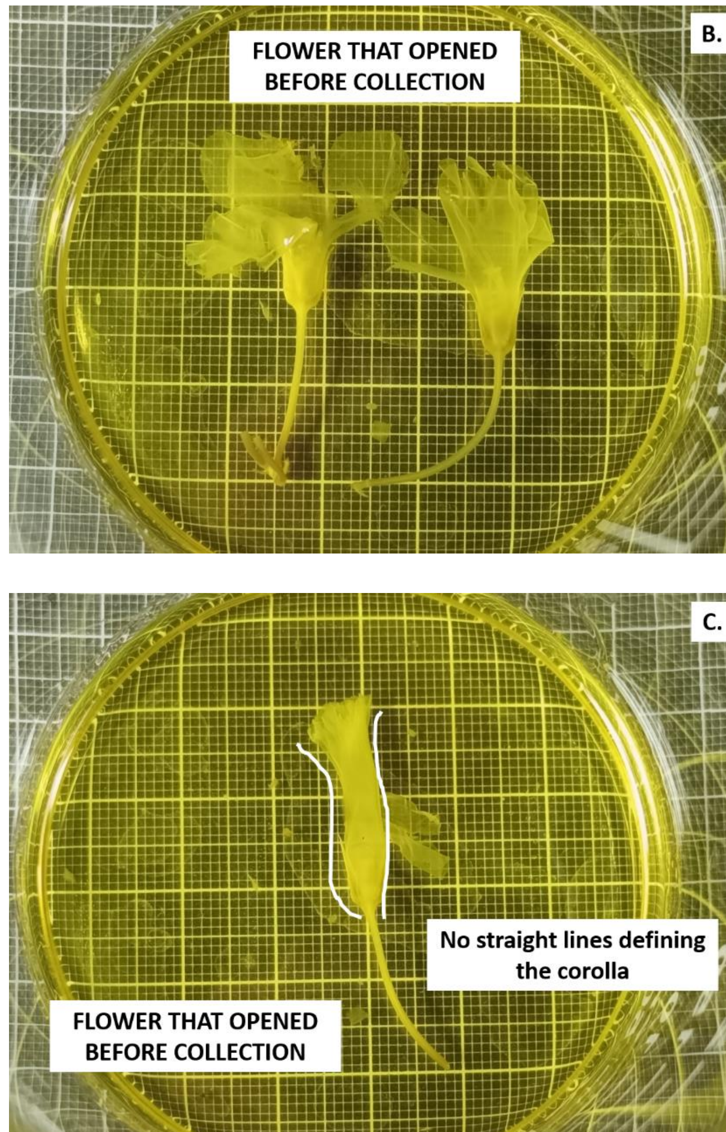
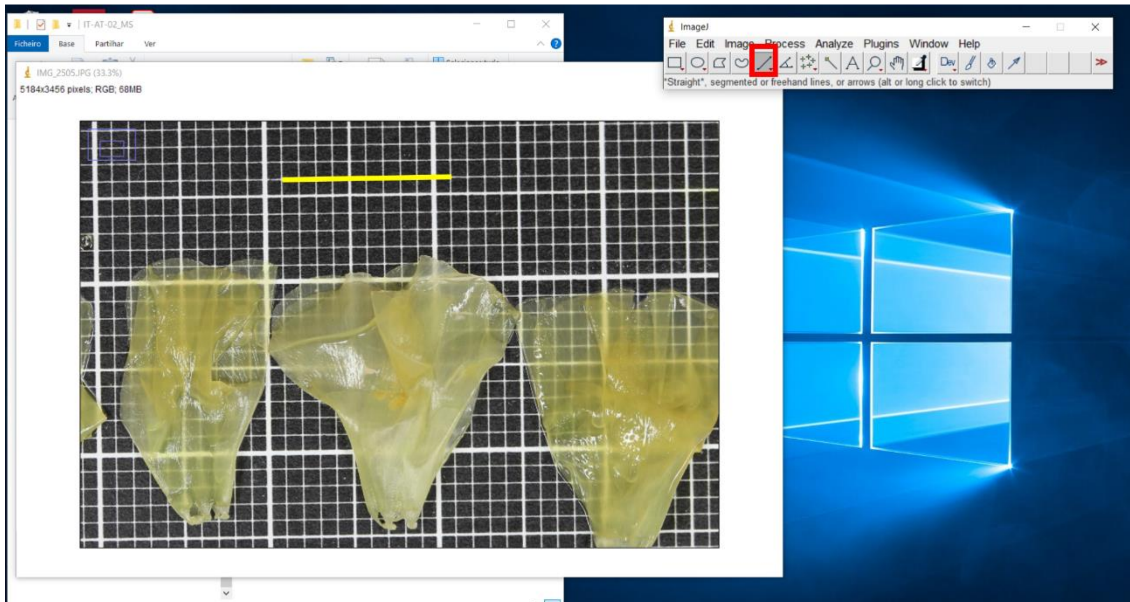


Fig. 4. Flowers that were not open at the time of collection (Fig. 4A) and flowers that were already open at the time of collection (Fig. 4B-C).

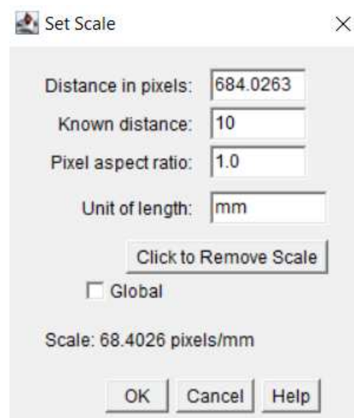
6. By using the tweezers, hold the flower by the calyx and use the other to hold it by the corolla, right above the higher whorl of anthers or stigma. Gently push the calyx with the tweezers until it separates from the corolla. This procedure can be done under the stereomicroscope to make it easier for you.
7. Transfer the corolla and the sex organs of the flower to the millimeter paper and place the flowers in order from 1→5 or 6→10.
8. After photographing the flowers, you will use the pistils from these flowers to look at pollen loads and pollen tubes → check point 2b).

c) Measurements with Image J

1. Open Image J and click *File* → *Open…* to select the image to work with.
2. Before start measuring, you need to calibrate the photo. Select the *Straight* tool and trace a line of 10 mm in the photo as follows:



3. Click *Analyze* → *Set scale* and set the details as follows:



4. For measuring the weight of the corolla and of the sex organs, select the *Straight* tool, draw a straight line for the length that you want to measure and click *ctrl + M*. This command will make the measurement and save it to the *Results* window as follows:

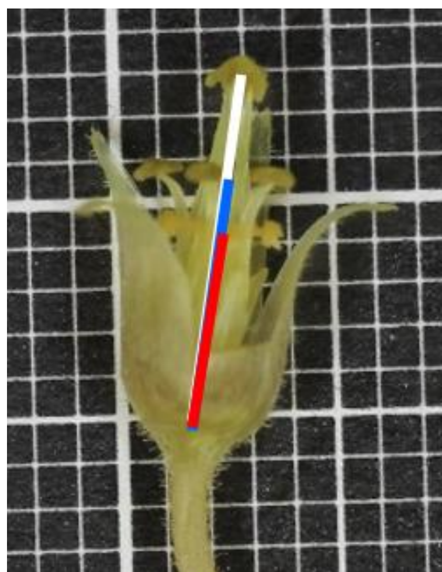
Results

File Edit Font Results

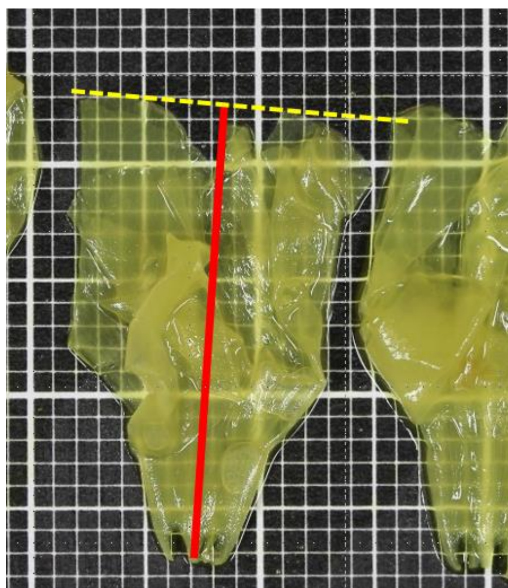
	Area	Mean	Min	Max	Angle	Length
1	0.225	154.413	28.667	222.333	90.327	15.351

For making it easier for you, follow this order for making the measurements: corolla, lower sex organs level (1st level), mid sex organs level (2nd level) and highest sex organs level (3rd level).

5. Measurements of the sex organs should be made from the base of the ovary until the mid-point of the anther and the mid-point of the stigmas as follows:



6. Measurements of the corolla should be done from the basis of the corolla to the top:



7. Make sure to avoid zoom in and zoom out after setting the scale for making the measurements.
8. After finishing the measurements in one photo, copy the results from Image J to your Excel file. Make sure to take any notes in the Excel file that you might think are important.

2. Protocol for observing pollen loads and pollen tubes growth under the fluorescence microscope

For each population, **15 flowers** per floral morph will be used for pollen loads and pollen tubes growth along the style, 10 of these are the flowers used for morphometric analyses plus 5 additional flowers. For the additional flowers, select older flowers if possible.

a) Material

Make sure that you have all the material you need before starting. For your convenience, you can find it in a tray in the lab (Fig. 1).

For this protocol, you will need:

- Labels and pencil (do not use pen)
- Stereomicroscope
- Tweezers and blade
- Tissue paper
- Glass slide
- Tray with Eppendorf tubes
- Trays (2/person) for protecting the i) Eppendorf tubes with pistils in aniline blue and ii) the microscope slides from light
- Glycerol 50% (prepared in distilled water) → for your convenience, 2mL Eppendorf tubes can be used for storing glycerol
- Pasteur pipet
- Petri dish
- Distilled water
- 8N NaOH
- 0.05% (w/v) aniline blue prepared in 0.1 N potassium phosphate → for your convenience, 2mL Eppendorf tubes protected with foil can be used for storing the aniline blue (protocol for preparing it in appendix)
- Nail polish
- Box for storing microscope slide preparations

b) Preparation of the pistils for observation under the fluorescence microscope

1. Gently separate the pistil from the anthers under the stereomicroscope. For L- and M-morphs, there is no need to cut the ovary out; for the S-morph, you should cut the ovary. To avoid disturbing pollen tubes in the lower part of the style, cut the ovary after the first line of ovules as showed in Fig. 5.

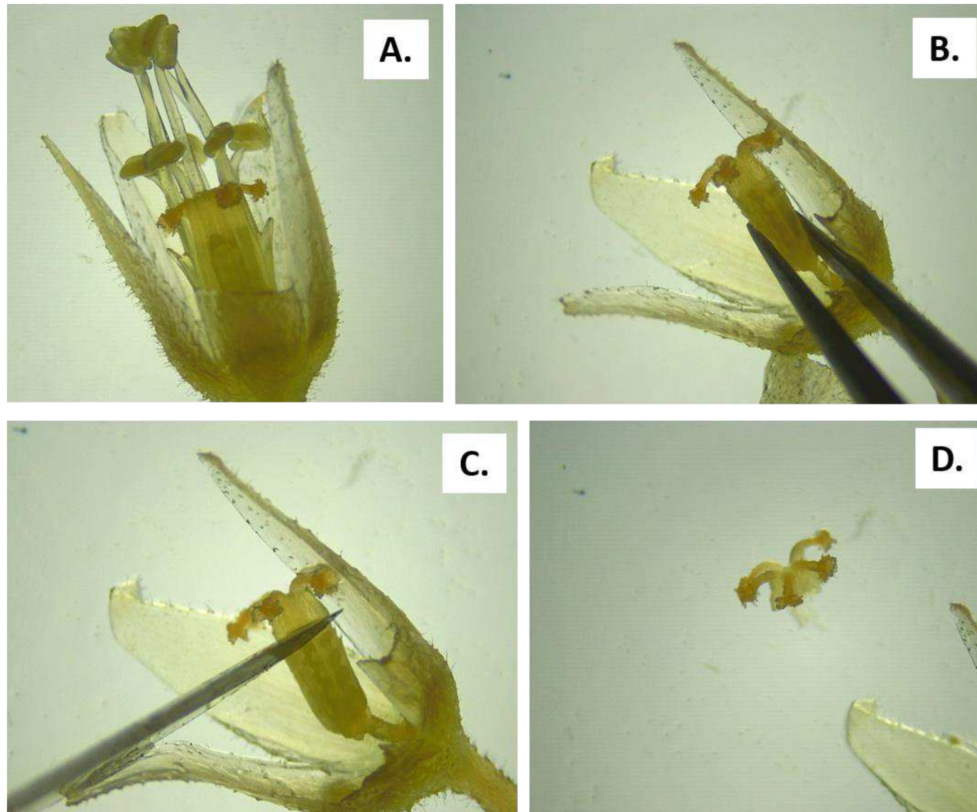


Fig. 5. Removing the ovary from S-morph pistils.

2. Transfer the pistil to an Eppendorf with a drop of glycerol 50%.
3. After you finish one population, pipette 1 mL of 8N sodium hydroxide or the volume that covers the pistils and let them soften for 3h.
4. Wash the pistils in distilled water (use a petri dish with distilled water for this step) and place them overnight in 0.05% (w/v) aniline blue prepared in 0.1 N potassium phosphate. Keep them in the dark.
5. For your convenience, prepare all labels in advance before starting making the preparations. Labels are made of the population code followed by style morph and number of the flowers (e.g., IT-AT-01 S1-2 for population IT-AT-01, S-morph, flowers no. 3 and 4). Pistils 3 and 4 should be placed from left to right in the microscope slide.
6. In the following morning, wash the pistils in distilled water (use a petri dish with distilled water for this step), place them on a microscope slide with a drop of glycerol

50% and squash them beneath a coverslip. Seal the preparation with nail polish and protect it from light.

Fig. 6. Working space for making the microscope slide preparations.

c) Observation under the fluorescence microscope

1. The following variables will be measured under the fluorescence microscope: a) number of germinated pollen grains and b) number of pollen tubes growing along the style at 3 heights: upper part of the style, middle part of the style and lower part of the style.
2. A hand counter is available at the lab → use it for making counts under the fluorescence microscope.
3. Take a photograph of each of the 5 stigmas for stigmatic pollen loads and label the photo with population code followed by style morph and number of the flower (e.g., IT-AT-01 S6 for population IT-AT-01, S-morph, flower no. 6).

d) Counts with Image J

1. Open Image J and click *File* → *Open…* to select the image you will be working with.
2. For counts, there is no need to calibrate the photo. Double click on the *Multi-point* tool to open the *Point* tool and select the color, size and other features of your labels.
3. After selecting the features, you can start counting by clicking on the pollen grains that you identified as pollen grains from *Oxalis pes-caprae* (Fig. 7).
4. For each flower, there will be 5 counts of stigmatic pollen loads.

5. In the box for storing microscope slide preparations, you will find 3 preparations of *Oxalis pes-caprae* pollen. Some images are also found in Figs. 8 and 9. Pollen from *O. pes-caprae* has three longitudinal openings (i.e., colpus) and is finely reticulated. Any deviation from this should not be considered pollen from our target species.

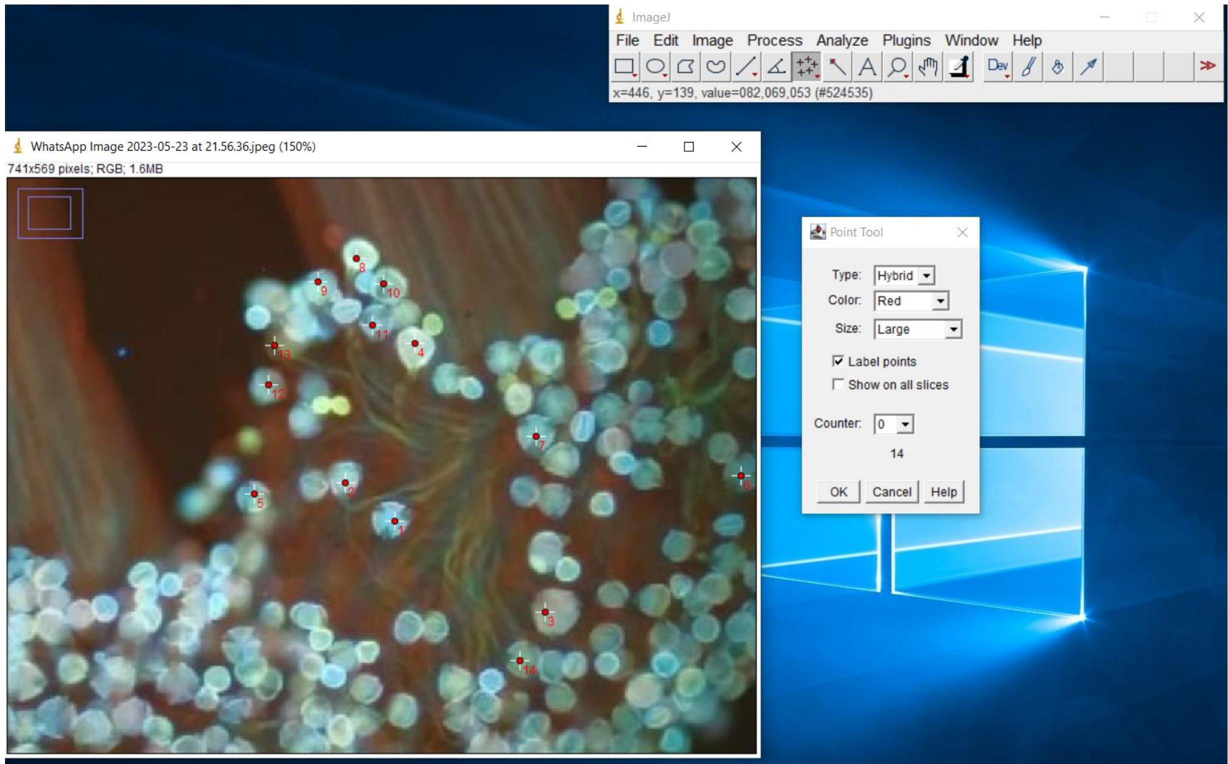


Fig. 7. Pollen counts using Image J. Pollen grains already counted will appear in the color you selected, so you know that you have already counted them.

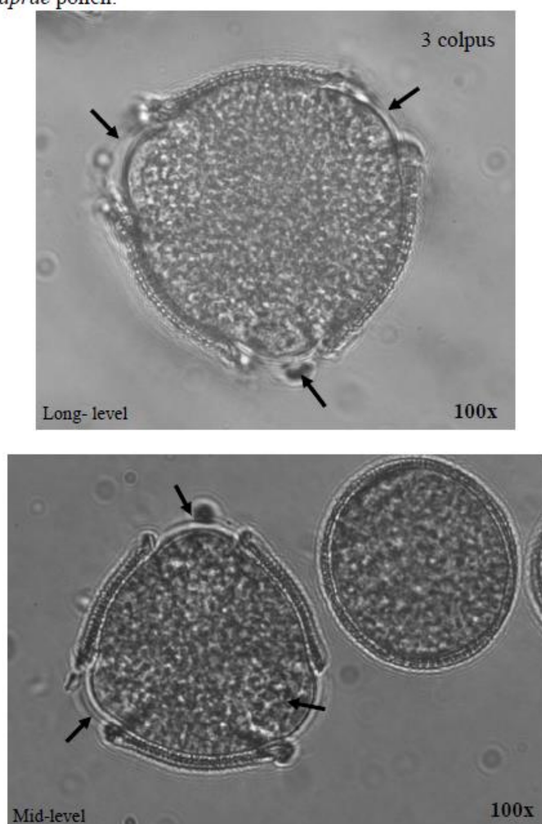


Fig. 8. Pollen grains of the S-morph produced by long- and mid-level anthers, showing the colpus.

Fig. 9. Pollen grains of *Oxalis pes-caprae* in different views at the light microscope (1400x)

Note: very slight differences in pollen grains size might be found, with pollen produced by long-level anthers being larger in size than pollen produced by mid- and short-level anthers. Pollen trimorphism is not characteristic of this species, and there is overlap in size of pollen produced by different anther whorls. Thus, it is not possible to distinguish the origin of pollen grains by size. Nevertheless, please be

aware that there is some variation in pollen size and take it into consideration while you are making the counts.

References

1. For the protocol on pistils' preparation for fluorescence microscopy:

Dafni A., Pacini E., Nepi M. (2005) Pollen and stigma biology. In: Dafni A., Kevan P., Husband B. (Eds), Practical pollination biology. Enviroquest, Ontario, ON, Canada, pp 83–142.

2. For knowing more about the species, reproductive biology and population structure in the Mediterranean area:

Castro S., Loureiro J., Santos C., Ater M., Ayensa G., Navarro L. (2007) Distribution of flower morphs, ploidy level and sexual reproduction of the invasive weed *Oxalis pes-caprae* in the western area of the Mediterranean region. *Annals of Botany*, 99, 507– 517.

Castro S., Ferrero V., Costa J., Sousa A.J., Navarro L., Loureiro J. (2013) Reproductive strategy of the invasive *Oxalis pes-caprae*: distribution patterns of flower morphs, ploidy levels and sexual reproduction. *Biological Invasions*, doi: 10.1007/s10530-013-0414-2.

Costa J., Ferrero V., Loureiro J., Castro M., Navarro L., Castro S. (2013) Sexual reproduction of the pentaploid, short-styled *Oxalis pes-caprae* allows the production of viable offspring. *Plant Biology*, doi: 10.1111/plb.12010.

3. For getting familiarized with heterostyly:

Barrett S.C.H., Shore J.S. (2008) New insights on heterostyly: comparative biology, ecology and genetics. In: Franklin-Tong V.E. (Ed), Self-incompatibility in flowering plants – Evolution, diversity and mechanisms. Springer-Verlag, Berlin, pp 3-32.

Ganders F.R. (1979) The biology of heterostyly. *New Zealand Journal of Botany*, 17, 607-635.

Webb C.J., Barrett S.C.H. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms. II. Herkogamy. *New Zealand Journal of Botany*, 24, 163-178.

APPENDIX 1 - Preparation of 0.05 Aniline blue in 0.1 M K3PO4 (Dafni *et al*., 2005)

1. Preparation of 0.1M K3PO4 solution (K3PO4 1.5 H20)

- Add 21.228 g of K3PO4 in 1 L of H2O in a bottle.

2. Preparation of the Aniline blue solution in 0.1 M K3PO4

- Dissolve 0.05 g of Aniline blue in the solution prepared in step 1.
- For this, dissolve the aniline in small doses at a time or it will not dissolve. The solution will turn an intense dark red color.
- Leave to shake in the dark (use aluminum foil around the bottle) for 1-2 hours until the solution is colorless.

Note: Test the solution before starting the counts.