



Finding Bermuda buttercup

Dear Researchers,

In 2023 we gathered a network of researchers from the Mediterranean Basin collaborating to a common experimental protocol to comprehensively study sexual reproductive strategies of the invasive Bermuda buttercup (*Oxalis pes-caprae* L.) in this region. Bermuda buttercup is native to the western part of South Africa and has been introduced to all areas with Mediterranean climate of the world, where it became an invasive species. This plant has a complex flower biology consisting of three floral morphs (tristily) – short- (SS), mid- (MS) and long-styled (LS) morph (Figure 1) – and different ploidy levels (di-, tetra- and pentaploid). It has been considered that invasive populations are largely composed solely of pentaploid short-styled morph (SS). Consequently, the species has completely switched to asexual reproduction via the production of bulbils due to a self- and morph-incompatibility system. However, other floral morphs (MS and LS) were recently detected in western Mediterranean Basin, along with the production of fruits and seeds. This opens the possibility for broad-scale changes in sexual ability with consequences for heterostyly maintenance during invasion.

Call for collaboration

After surveys in the Mediterranean Basin during 2023, we are now extending the sampling for one more year to cover unsampled areas in the Mediterranean Basin and improve sampling in areas with new floral morphs. Thus, a protocol has been prepared and is provided below. **If you or any of your colleagues are interested in participating in this project, please contact Sílvia Castro (scaastro@bot.uc.pt) and Peter Glasnović (peter.glasnovic@upr.si).** All the participants actively contributing to the sampling will be included as co-authors in the manuscript describing current patterns of Bermuda buttercup in the Mediterranean basin.



PROTOCOL (version 1.3 for 2024)

The protocol involves the sampling of flowers, leaves, and fruits of Bermuda buttercup throughout the Mediterranean Basin. Because the plant starts to set fruits during flower, **only one visit** to each locality is necessary and it should be made at the **peak flowering period** (or slightly later).

Step-by step procedure:

1. Population characterization:

- Population information: Locate a population and record geographical coordinates in World Geodetic System 84 system (WGS84). Estimated population size. Classify the habitat in the categories provided in Table 1, provide crop identity (if applicable) and indicate if there is soil movement (e.g., soil tillage). Record co-flowering native species. In notes, please provide additional details that you find relevant for the spread of the plant.
- Floral morph proportions: assess floral morph proportion in each population in longitudinal transects across the entire population, recording floral morph of 100 individuals (when possible) with 5-m distance between two consecutive sampled plants. Floral morphs should be identified as SS, MS and LS morph or St for the double-flowered sterile form (Figure 1). Additionally, semi-homostylous (SHS) flowers have been detected and refer to flower forms where one of the anther levels overlaps with the stigmatic level (Figure 1) – this floral morph should be considered.
- Database: Enter the data in the Excel file provided.

NOTE: Participants in the **limits of the distribution of *Oxalis pes-caprae*** or in **areas where *Oxalis pes-caprae* is more sporadic** should not constrain their sampling because of population size. In these cases, **sample less plants (up to maximum possible) keeping the 5 m rule.**



2. Plant sampling for cytotype and fitness analyses: locate **10 plants from each morph** detected in the population (**separated at least 5 m apart**) and collect the following samples in every plant (in a block design):

For this component, please note that **selected plants need to have both, flowers and fruits**. This is possible if you sample at the **peak flowering period (or slightly later)** as from this moment onwards the plant starts to set fruits during flower.

- Morph voucher: collect 1-2 flowers (recently open) per plant in separate microtubes with 70% ethanol (1 microtube per plant) labelled inside with the population and plant ID code (SEE coding instructions below).
(OPTIONAL – ALTERNATIVELY YOU CAN PHOTOGRAPH THE FLOWERS IN LATERAL VIEW WITH THE SEXUAL ORGANS VISIBLE REMOVING ONE PETAL AND SEND THEM BY E-MAIL)
- Reproductive fitness – here you can proceed in one of two ways:
 - i. 1) identify a fruiting stem per plant and count the number of scars (i.e., aborted flowers), fruits, flowers and flower buds (if applicable); collect 3 non-dehiscent fruits (when available) into a paper bag identified with the population and plant codes; place the bags to dry at 30 °C until completely dried – send them by mail to Sílvia Castro;

OR
 - ii. 2) collect a fruiting stem per plant into a paper bag identified with the population and plant codes; place the bags to dry at 30 °C until completely dried. NOTE: If the fruit production is zero, you can but do not need to collect the stem; if you decide not to collect it, you need to provide the number of scars and zero fruits in the Excel file.



- Cytotype analyses: collect the plant with some root system, prune the old leaves, store it in a small plastic bag identified with the population and plant codes and maintain at 4 °C until shipping. (**OPTIONAL – COLLECT THIS MATERIAL ONLY WHEN YOUR REGION PRESENT DIVERSITY OF FLORAL MORPHS**)

IMPORTANT: **fresh samples** need to be **sent as fast** as possible for flow cytometric analyses to: **Sílvia Castro**, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas s/n, 3000-456 Coimbra, Portugal. [See shipping notes for further details.](#)

Instructions for population and plant coding:

To have a homogenous and easily understandable code, please follow the following instructions to code the samples:

Population codes:

Country code_Researcher code_population number

e.g., **PT-SC-25** refers to population 25 sampled by Sílvia Castro in Portugal.

Plant codes:

Country code_Researcher code_population number_plant number

e.g., **PT-SC-25-03** refers to the plant number 3 from the population 25 sampled by Sílvia Castro in Portugal.



Excel file for field data:

An excel file has been prepared for all participant to introduce the information collected in the field and will be send upon receiving your contact of interest. The excel file includes the following four sheets: 'Read me' – with detailed explanation of the remaining sheets; 'Table 1 HABITAT' – with the table of habitat categories; 'POPULATION Dataset' – to fill with population information, and 'FITNESS Dataset' – to fill with data on reproductive fitness registered in the field.

Sample shipping:

Sample	Destinate
2. Plant sampling for cytotype and fitness analyses	Sílvia Castro
Morph voucher – 1-2 flowers in microtubes per plant	
Reproductive fitness – up to 3 fruits per plant	
Cytotype analyses – 10 plants per morph URGENT	

Address: Sílvia Castro, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas s/n, 3000-456 Coimbra, Portugal

Instructions are being prepared to obtain the permits to ship the samples, in particular to Portugal as they involve live propagules.

Contacts

Sílvia Castro (scaastro@bot.uc.pt), +351 968654306

Peter Glasnović (peter.glasnovic@upr.si), +386 40384602



Timetable

- **February – March 2024:** Sample collection to fill gaps and improve sampling in areas with diversity of floral forms
- **February – March 2024:** FCM analyses as samples are received
- **Until December 2023:** Morphometric analyses of flowers collected in 2023
- **Until March 2024:** Pollen load analyses of flowers collected in 2023
- **April – July 2024:** Data analyses and results, first draft of the manuscript
- **July 2024:** Presentation in International Botanical Congress (Madrid)
- **September – October 2024:** manuscript finalization and submission



Table 1. Habitat categories considered here.

Category	Description
Natural or semi-natural areas	Natural areas refer to an area of land dominated by native vegetation and relatively undisturbed by human activity. Such areas could include grasslands, forests, wetlands, peatlands or riparian areas.
Agricultural areas	
- Intensive permanent crops	Crops which last for many seasons, rather than being replanted after each harvest, cultivated in intensive systems to maximize production on a given area of land with inputs such as labour, fertilizer and machinery.
- Traditional permanent crops	Crops which last for many seasons, rather than being replanted after each harvest, cultivated in systems that use traditional knowledge, tools, natural resources, organic fertilizer, among others.
- Annual crops	Crops which last for one season, being replanted after each harvest.
Managed forest areas	Planted stands of trees, usually productive strands (excludes natural forests, tree stands in agricultural production systems and trees in urban parks and gardens).
Managed urban areas	Green areas that are managed by municipalities in urban area defined as human settlements with a high population density and infrastructure of built environment.
Non-managed urban areas	Green areas that are abandoned (non-managed) in urban area defined as human settlements with a high population density and infrastructure of built environment.
Managed peri-urban areas	Green areas that are managed by municipalities in peri-urban areas defined as areas with scattered and dispersive urban growth that create hybrid landscapes of fragmented and mixed urban and rural characteristics.
Non-managed peri-urban areas	Green areas that are abandoned (non-managed) in peri-urban areas defined as areas with scattered and dispersive urban growth that create hybrid landscapes of fragmented and mixed urban and rural characteristics.
Rural areas	Areas that are located outside towns and cities, typically having a low population density and small settlements, and with fragments of agricultural areas and areas with forestry.
Road edges	Green areas along road edges.
Other	Please specify.



Figure 1. *Oxalis pes-caprae* floral forms: short- (SS), mid- (MS) and long-styled (LS) morph, multipedal sterile form (St) and semi-homostylous morph (SHS).