Preliminary actions for *in vitro* propagation of *Adonis distorta*, *Androsace mathildae* and *Cypripedium calceolus*, target species of the LIFE project Floranet

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The main objective is to protect and improve the status of plant species of Community importance (Annexes II-IV to Directive 92/43/EEC) in Natura 2000 areas that are included in three parks of the Central Apennines: Majella National Park, National Park of Abruzzo, Lazio and Molise, Sirente-Velino Regional Park.

The species of Community interest at the heart of the project are: *Cypripedium calceolus, Adonis distorta, Androsace mathildae, Iris marsica, Astragalus aquilanus, Klasea lycopifolia*, *Jacobaea vulgaris* subsp. *gotlandica.*

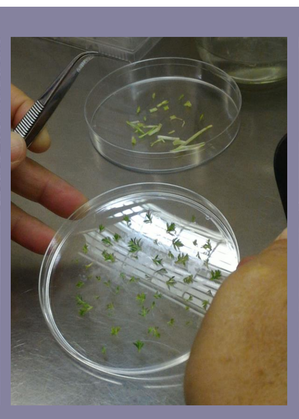
Action C2: *In vitro* propagation

This action aims to micropropagate the plant material resulted from seeds and/or seedlings picked in the wild of those species for which the seed propagation can be unsuccessful.

The traditional tissue culture methods (a bud or another explant is used for vegetative propagation after being isolated from the mother plant) will be applied. They enable us to grow several individuals in reduced spaces, as well as to have fast cycles of propagation, to maintain the germplasm for a long time and to propagate even those species that hardly propagate *in vivo*.

MICROPROPAGATION

- Leaves and shoot tips from rhizome will be used for micropropagation.
- Pinnules were cut from leaves and sterilized with a 50% Plant Preservative Mixture (PPM) solution for 30 min; then they were vertically placed on Murashige and Skoog (MS) **[1]** medium containing 15.34 mg/L of 6-benzylaminopurine (BAP), 3 mg/L of indole-3-acetic acid (IAA), 3% sucrose, 0.8 % agar and 0.1 % PPM with pH 5.8; cultures were incubated at T = 25°C with a 12/12 h photoperiod **[2, 3]**



- Shoot tips from rhizomes will be washed with tap water and sterilized as described above. They will be placed on the same MS medium with pH 5.6 and then incubated at T = 25°C with a 16/8 h photoperiod **[2]**.

SEED GERMINATION

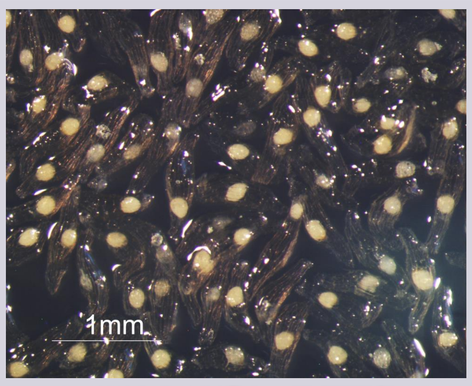
- Seeds will be surface sterilized in a 5% NaOCl solution with Tween 80, then, rinsed 3 times with sterile distilled water.
- MS basal medium will be used as a culture medium, supplemented with 3% sucrose and 0.7% agar, with pH 5.7. Plates will be placed in a growth chamber at T = 22°C in the dark **[4]**.

MICROPROPAGATION

- Seedlings will be used for micropropagation.
- Cotyledons will be placed on MS medium supplemented with 0.5 mg/L of BAP and 0.25 mg/L of IAA to induce cell differentiation and callus induction **[4]**.
- Plates will be placed at T = 22°C with a 14/10h photoperiod.

ASYMBIOTIC SEED GERMINATION

- Seeds were surface sterilized/scarified in a 5% NaOCl solution with Tween 80, until bleaching. Then, they were rinsed 3 times with sterile distilled water and centrifuged at 6,000 rpm for 3 min between rinses **[5]**.
- BM-1 and BM-2 Terrestrial Orchid Media **[6]** were used, with the addiction of 0.6% agar.
- Plates with seeds (300-500 per plate) were incubated at T = +24 ± 1°C in darkness until protocorm and seedling development.



MICROPROPAGATION

Both leaves and protocorms obtained from *in vitro* asymbiotic germination will be used for micropropagation according to the following protocols.

- Protocorms will be cut approximately in half with a sterile blade and placed in sterile tubes containing 1/4 MS salts, 1 mg/L of IAA, 0.25 mg/L of BAP, 3% sucrose, and 0.7 % agar, with pH 6.0 **[7, 3]**; cultures will be incubated at T = 22°C in the dark.
- Seedling leaves will be cut were into horizontal pieces (0.5 cm) and vertically placed in the MS medium supplemented with 1 mg/L of IAA, 2 mg/L of BAP, 3% sucrose, and 0.8 % agar, with pH 5.6 **[8]**; cultures will be incubated at T = 25°C with a 12 h photoperiod.

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