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Mini Review

Low Germination Success in *Phyteuma cordatum* Balb and *Empetrum hermaphroditum* Hagerup

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Introduction

This study aimed at carrying out experimental laboratory activities on some spontaneous plant species present within areas of the Piedmontese Natura 2000 Network, with the aim of identifying the regeneration protocol for their *ex situ* conservation. In Europe and around the world, climate change and human actions are compromising the conservation status of natural habitats, spontaneous plant and animal species. To cope with these changes, the European Union has set up the Natura 2000 Network, a network of sites of community interest (SIC) and special protection areas (SPA) created in the regulatory framework of the "Habitats Directive (92/43/EEC) and the Birds Directive" (79/409/EEC), for the protection and conservation of habitats, animal and plant species identified as priorities by the Member States of the European Union. Conservation methodologies for the protection of biological resources include conservation strategies *ex situ* that is, out of the natural environment. Micropropagation is a multiplication technique that allows to obtain a clone of the plant itself, or a set of individuals with the same genetic heritage, through the use of *in vitro* culture methods of cells and plant tissues. These techniques have been used for the propagation of rare and/or endemic species of the Ligurian and Maritime Alps. The study concerned *Phyteuma cordatum* Balb and *Empetrum hermaphroditum* Hagerup.

Phyteuma Cordatum

The species is included among the most significant endemics of the Ligurian and French-Italian Maritime Alps [1]. It has a fragmented area in a small number of stations, mostly distant from each other, a clear testimony of its antiquity. Stress-tolerant competitor species [2] occupies habitat 8210 "Limestone rocky walls with chasmophytic vegetation", on limestone cliffs at altitudes of 1800-2200 meters [3].

Empetrum Hermaphroditum

It is an evergreen shrub with a prostrate and branchy habit, capable of occupying different habitats. Although it presents a circumboreal distribution, in the Ligurian and Maritime Alps it occupies a single station at 2000 meters above sea level and is considered a rare species. Prostrate habit and dense ground cover inhibit seed germination of some competing species, while creating conditions for seed germination and conifer seedling development [4,5].

Materials and Methods

Adult plants of *Phyteuma cordatum* Balb were collected in their natural environment in an area within the Marguareis Natural Park. Explants about 8/10cm long were collected from few adult plants. To ensure adequate genetic variability and maintain the right humidity, the material taken from some individuals has been stored in plastic bags with little water inside. This material was stored in a refrigerator for a few days before being used in laboratory. Here we proceeded with the preparation of leaves and small explants 1-2 centimeters long, each containing a vegetative apex (Figure 1). These explants were placed in containers for washing in cold running water (10min). Subsequently, sterilization operations were carried out for

the *in vitro* culture. Sodium hypochlorite was tested by varying time between 30' and 1h.

Figure 1: Stages of leaves and explants preparation in *Phyteuma cordatum* Balb plants (I. Pace, 2019).

a. 30min 0.5% NaOCl+Tween 20

b. 60min 0.5% NaOCl+Tween 20

After the sterilization treatments, several rinses were carried out with sterile distilled water to completely eliminate both the surfactant (Tween 20) and the sterilizing agent. MS was used as culture medium, in presence of 2.4D plant growth regulator at different concentrations:

a. MS+2.4D 0.5mg/L

b. MS+2.4D 1mg/L

c. MS+2.4D 2mg/L

The explants were placed in falcon tubes containing culture medium and plant growth regulator and placed in the growth chamber with artificial white light (photoperiod of 12 hours light/dark) at 25°C. A strong contamination was present and, to contain the problem, we tried to use Plant Preservative Mixture (PPM 3ml/l) by adding it to the culture medium, with scarce results. Using MS culture medium, leaf or explant sections showed no responsiveness for callus formation at plant growth regulator concentrations used.

Sterilization Procedures on Seeds of Alpine Species

Seeds of *Empetrum hermaphroditum* and *Phyteuma cordatum* were subjected to sterilization tests, which was followed by the vitality test (TTC) to verify that the same did not compromise their germination. The test took place on (Table 1).

Table 1: NaOCl treatment results.

Species	Treatment	Result
Empetrum Hermaphroditum	5% NaOCl 2' + etanolo 96% 2' + tween	Presence of fungal and bacterial pollution
Empetrum Hermaphroditum	1% NaOCl 30' + tween	Absence of pollution
Phyteuma Cordatum	1% NaOCl 30' + tween	Absence of pollution

Viability Test on Seeds of Alpine Species

The chemical test TTC (2,3,5-Triphenyl Tetrazolium Chloride), takes its name from the chemical reagent used which, in contact with the oxygen present in the vital cells, converts into a product called "Formazan", producing a color reaction observable in still vital tissues. The resulting color at the end of the test can be a more or less marked shade of red for healthy and vital tissues, while the dead or damaged parts do not stain [6]. To evaluate the viability of the seeds of our alpine species, tests were carried out with tetrazolium chloride. The seeds were soaked with a 1% v/v TTC solution, incubated in the dark in an oven at 25 ° C for 1 hour. The sample was then checked under the stereoscope. Based on the tissue response to TTC, seeds are divided into several categories:

a. **Reactive**, if both the embryo and the endosperm are uniformly colored red;

b. **Partially reactive**, if only the endosperm or only the embryo or both take on a spotted or uniform pink color;

c. **Non-reactive**, if neither the embryo nor the endosperm stain;

d. underdeveloped, in the case of incompletely formed seeds;

e. contaminated, if they have loose and rotting tissues;

f. compartments, if they are empty.

This method, widely used by Germplasm Banks [7], is extremely effective, rapid (as the results can be observed after a few hours from the application of the reagent) and advantageous (Table 2), as it allows the use of a reduced number of randomly extracted seeds from the batch to be analyzed.

TTC was tested on a sample of the available seeds of *Empetrum hermaphroditum* and *Phyteuma cordatum* collected in 2012 and 2018. The ideal conditions for the conservation of orthodox seeds foresee their dehydration and subsequent storage at low temperatures [6]. On the Seed Information Database (SID) of the Royal Botanic Gardens of Kew (UK), the conservation conditions of the two species are indicated as follows:

Table 2: Empty seeds are not included in the final percentage counting*.

Species	Harvesting	Seeds	Reactive or Partially Reactive Seeds	Percentage*
Empetrum Hermaphroditum	2012	25	19(+5 empty)	70
Empetrum Hermaphroditum	2018	34	22(+9 empty)	65
Phyteuma Cordatum	2012	25	19(+2 empty)	82
Phyteuma Cordatum	2018	25	22(+0 empty)	88

a. ***Empetrum hermaphroditum*:** Dehydration up to 15% relative humidity (RH) and freezing at -20 °C (orthodox, i.e. able to tolerate dehydration);

b. ***Phyteuma cordatum*:** Despite the lack of data available for the species, of 7 taxa belonging to the genus *Phyteuma*, 71% are orthodox, while 29% are indeterminate. Although the seeds of both species have been stored for years in an air-conditioned chamber at 10°C and RH 30%, they return good percentages of vitality (Figure 2).

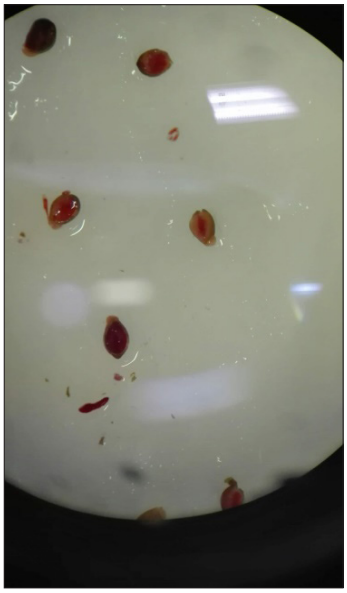

Figure 2: Reactive seeds of *Empetrum hermaphroditum* where both embryo and endosperm are uniformly coloured in red (I. Pace, 2019).

Germination Test on *Empetrum Hermaphroditum* and *Phyteuma Cordatum* Seeds

Our aim was to obtain sterile *Phyteuma cordatum* and *Empetrum hermaphroditum* seedlings, to be used as aseptic material for subsequent propagation. For *Empetrum hermaphroditum* we based on an already existing study [8], while for *Phyteuma cordatum* we had no data available regarding germination. It was chosen to use only agar (test) and agar with the addition of gibberellins (GA), hormones capable of eliminating seed dormancy, at concentrations of 250 and 1000mg/L (Table 3). The material was incubated under different conditions:

a. culture chamber with thermoperiod 25/15 °C and photoperiod 14h light/10h dark;

b. dark culture chamber with thermoperiod 20 °C.

The freshly germinated seedling was transferred in MS+AG medium, with a photoperiod of 14h light/10h dark and T 20 °C (Figure 3).

Table 3: Germination of one seed of *Phyteuma cordatum*, maintained for 3 months at T 20°C in the dark.

Species	Harvesting	Medium	Temperature and Light Period	Germinated Seeds
E. Hermaphroditum	2012	AG+1000 GA	T 25/15°C 14h light/10h dark	0/75
		AG+250 GA	T 25/15°C 14h light/10h dark	0/25
		AG	T 25/15°C 14h light/10h dark	0/75
	2018	AG+1000 GA	T 25/15°C 14h light/10h dark	0/100
		AG	T 25/15°C 14h light/10h dark	0/100
P. Cordatum	2012	AG+250 GA	T 25/15°C 14h light/10h dark	0/25
			T 20°C dark	0/25
			T 20°C 14h light/10h dark	0/25
	2018	AG+250 GA	T 25/15°C 14h light/10h dark	0/25
			T 20°C dark	1/25
			T 20°C 14h light/10h dark	0/25

Figure 3: *Phyteuma cordatum* germinated seed (I. Pace, 2019).

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