IN VITRO TREATMENTS TO IMPROVE THE GERMINATION RESPONSE IN OROPHYTIC-BOREAL SPECIES FROM THE NORTHERN APENNINES – CASENTINE FOREST NATIONAL PARK

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ABSTRACT

The Botanic Garden of Modena and the Casentine Forest National Park recently promoted an integrated *in situ/ex situ* conservation project. Researchers aimed to propagate certain plant species that grow in the Park, which have specific protection requirements on account of their phyto-geographic importance. These species are: *Hieracium villosum* L., *Saxifraga oppositifolia* L., *Saxifraga paniculata* Miller, *Saxifraga moschata* Wulfen. In this work, we present some useful treatments for bypassing the natural dormancy of the seeds/achenes and for improving *in vitro* germination and growth.

KEY WORDS

Conservation, *in vitro* germination, seed dormancy, gibberellic acid, chilling.

INTRODUCTION

The Botanic Garden of Modena, in line with the guidelines adopted on a worldwide scale by Botanic Gardens, has carried out a number of important conservation programs regarding certain rare and/or endangered plant species, simultaneously promoting multitasking actions for public awareness. Although these projects only involved a limited number of endangered and/or vulnerable species/populations, some interesting and noticeable results were achieved (Sgarbi et al., 2001; Bonafede et al., 2003; Dallai & Sgarbi, 2005; Del Prete et al., 2006). Integrated *in situ/ex situ* plant conservation practices are among the primary objectives of Botanic Gardens and these activities are developed both inside the Garden by *in vitro* and *in vivo* growing practices and museum-type educational activities and outside the Garden by means of numerous co-operations through partnerships with Parks, natural reserves and other administrative bodies or institutions (AA.VV., 1995; BGCI-IABG, 2000; Wyse Jackson, 2001).

An integrated *in situ/ex situ* conservation project is currently being conducted in conjunction with the Casentine Forest National Park. The Park, which was established in 1993, stretches along the Apennine ridge between the regions of Tuscany and Emilia-Romagna, covering an area of 36 000 hectares. The Park consists of an area of wooded land, of which over 5000 hectares of the Casentine Forest National Park and the neighbouring La Verna Franciscan Sanctuary are covered with centuries-old forest. Arctic-alpine plants grow on the peak of Mount Falco (1658 m above s.l.) and on the northern slope below the mountain, because of the elevate altitude, macro- and micro- climate and specific edaphic factors. These plants are not found elsewhere in the Romagna region and so Mount Falco therefore represents the southernmost point on which they grow. In this area, some plants must be considered rare and endangered as small populations or a limited number of specimens only are present. Certain orophytic boreal species growing in the Park, i.e *Hieracium*

villosum L., *Saxifraga oppositifolia* L., *Saxifraga paniculata* Miller, *Saxifraga moschata* Wulfen, were studied in order to prepare protocols to effectively propagate them from seed.

Seed germination consists in uptake of water by quiescent dry seeds and in a embryonic axis formation. Generally speaking, the first visible sign of germination is considered the protrusion of a radicle through integuments (Bewley, 1997). If in an intact viable seed germination does not occur, even under favourable environmental conditions, the seed is regarded as dormant. Dormancy can be regarded as an adaptive strategy to bypass and avoid adverse weather for plant development and establishment (Finch-Savage & Leubner-Metzger, 2006).

In this work, we present the results of *in vitro* germination tests carried out to establish experimental protocols for wild species above reported. *In vitro* cultures make it possible to control environmental and cultural parameters, such as light, temperature, humidity, salt concentration and growth occurs in aseptic conditions. This approach enables the establishment of cultures regardless of the seasons and facilitates the implementation of stimulating germination treatments, which proved very useful for wild species forming the focus of conservation projects (Fay & Chase, 1998; Ronse, 1989).

MATERIALS AND METHODS

The samples were collected by the Park's forest rangers during the summers of 2005 and 2006. The seeds/achenes were cleaned and counted under stereomicroscope and stored at +4 °C in the dark for 2 months, before the start of treatment. The samples underwent different trials to test germinability and viability, according the approaches reported in literature (AA.VV, 2006).

Germination test: for every species 10 seeds/achenes were placed on Petri dishes on three layers of filter paper soaked with 3 ml distilled water. The Petri dishes were placed inside a Vitro Vent box (Duchefa) with 200 ml of distilled water to maintain a constant level of moisture. The samples were kept inside a climatic chamber (CTL 700 LX, AHSI) at +23 °C, under artificial light (1,9 W/m^2), subject to a photoperiod of 12 hours. The tests were carried out in triplicate.

Viability test: this test was conducted by applying the TTC test (International seed testing Association, 1976). Thirteen seeds/achenes were bathed in 1% (w/v) aqueous solution of 2,3,5 triphenyl tetrazolium chloride and incubated at +30 °C for 24 hours. Lastly, the samples were soaked in water and examined and counted under the stereomicroscope. Seed tissue viability was indicated by the appearance of red colour.

In vitro *germination*: All seed/achenes were sterilised/scarified with an NaOCl solution (1.5 % available Chlorine + 0.1% Tween 80) before being rinsed three times in sterile water. The different times of sterilisation are listed in Table 1. Seeds/achenes of the various species were treated differently in order to improve the germination response. In all tests, 10 seeds/achenes for Petri dish were sown and the tests were carried out in triplicate. Petri dishes were examined 2-3 times a week for 8 months.

Asteraceae – achenes of *Hieracium villosum* underwent pre-chilling treatment for 8 weeks (kept in the dark at + 4 °C) on MS culture medium (Murashige & Skoog, 1962), at half strength of salts (MS½). Another set of achenes were cultured on MS½, without any pre-treatment, as a control test. The pre-treated and control cultures were placed inside the climatic chamber, at + 23 °C, under artificial light (1,9 W/m^2) and subject to a photoperiod of 12 hours. The tests were carried out in triplicate.

Saxifragaceae – For all species of *Saxifraga*, *S. oppositifolia, S.paniculata, S. moschata,* seeds were cultured on two different media: MS½ culture medium and MS½ to which GA3 was added at 100 ppm; other seeds were cultured on MS ½ and subjected to pre-chilling treatment (kept in the dark at +4 °C). Because of the very limited number of seeds collected during summer 2006, the pre-chilling treatment was not carried out for these latter samples.

All *Saxifraga* seeds not submitted to pre-chilling treatments were exposed to red light (λ = 650-680 nm). These tests were carried out in duplicate.

Table 1. Times of sterilisation

Species	Mean time of sterilization (min)
Hieracium villosum	27
Saxifraga oppositifolia	20
Saxifraga paniculata	25
Saxifraga moschata	25

RESULTS

Germination test: *Hieracium villosum* germinated with a germination percentage of 6.66 %. For all other species the test gave negative results.

Viability test: results are listed in tables 2 and 3.

Table 2. Viability test (Summer 2005)

Species	**1st test**	**2nd test**
Hieracium villosum	833%	60%
Saxifraga moschata	533%	333%
Saxifraga oppositifolia	733%	-*
Saxifraga paniculata	333%	466%

* seed not available

Table 3. Viability test (Summer 2006)

Species	**1st test**	**2nd test**	**3rd test**
Hieracium villosum	633%	10%	10%
Saxifraga oppositifolia	90%	666%	733%
Saxifraga paniculata	666%	666	20%

In vitro *germination*: **Asteraceae** – *Hieracium villosum* germinated after 2 weeks with a germination percentage of approximately 30 %. No difference was observed between achenes pre-treated with chilling and untreated achenes. **Saxifragaceae** – All *Saxifraga* species germinated within 9 - 15 days. No germination

response occurred in pre-chilled samples. The germination pattern is shown in Fig. 1. The results are listed in Tabs. 4 and 5.

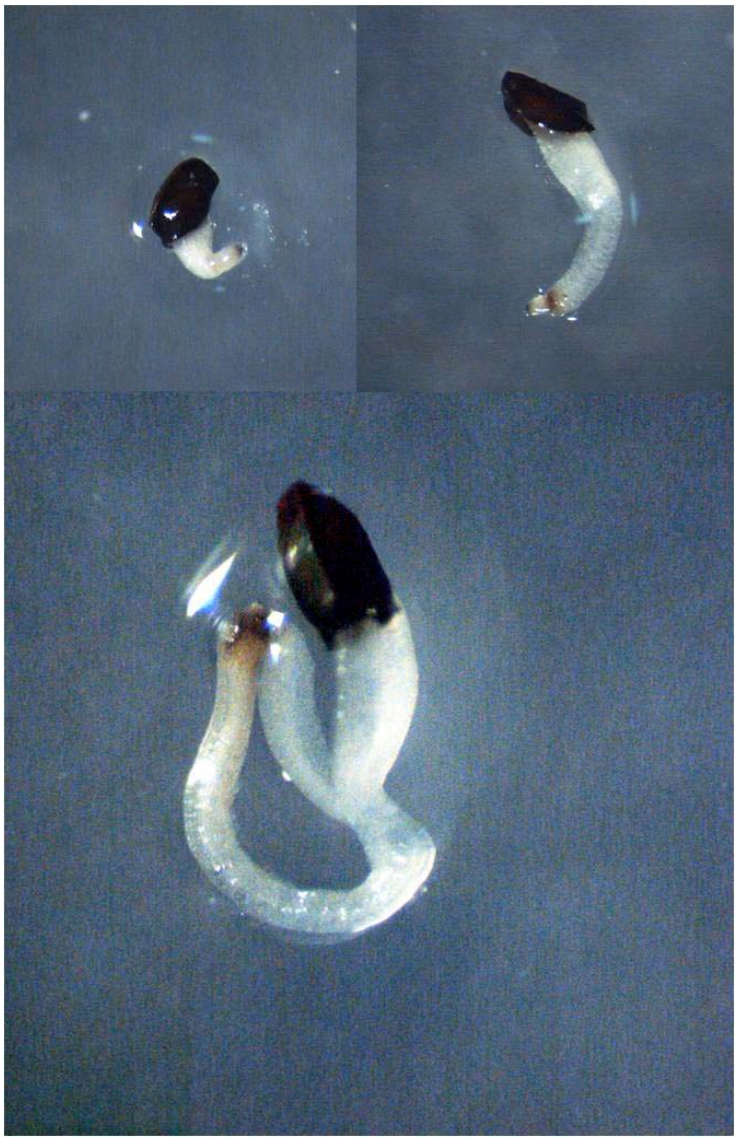

Fig. 1. Germination pattern of *Saxifraga moschata* Wulfen.

Table 4. In vitro Germination percentages (Summer 2005)

Species	MS 1\2 + GA_3 100 ppm - red light	Pre-chill
Saxifraga moschata	40% ± 44%	0%
Saxifraga oppositifolia	6 % ± 9%	0%
Saxifraga paniculata	3.3% ± 5%	0%

Table 5. In vitro Germination percentages (Summer 2006)

Species	MS 1\2 + GA_3 100 ppm - red light	MS 1\2 – red light
Saxifraga oppositifolia	5% ± 4%	3.75% ± 4.7%
Saxifraga paniculata	13.75% ± 7.5 %	2.5% ± 5%

Saxifraga moschata seeds were not available

DISCUSSION

Conservation practices are supported and favoured by seed propagation as high heterozygosis is maintained, thus preserving the biodiversity of wild population (Fay & Chase 1998; Ronse, 1989). Nevertheless, the seeds of many plants that grow in temperate regions are dormant seeds and do not germinate even when environmental conditions are favourable (Finch-Savage & Leubner-Metzger, 2006). Dormancy intrinsically blocks seed germination and as the function of a seed is to create a new plant, it might seem oddish that dormancy exists, however, it responds to the requirements of many plants, for which making seed germination possible in a unfavourable season could compromise the population's survival. The seeds of certain species are prevented from completing germination because the embryo is constrained by its surroundings structures (coat-enhanced dormancy); in other species a second category of dormancy is found, in which the embryo itself is dormant (embryo dormancy) (Bewley, 1997).

Atwater (1980) graded **Asteraceae** seeds into just one morphological category: no endospermic seeds with an external fibrous tegument that covers the achenes and a semi-permeable membranous tegument surrounding the embryo. The embryo occupies the largest part of seed; the cotyledons are large and the endosperm is reduced to one or more cellular layers. The main obstacle to germination in this family would appear to be constituted by the internal semi-permeable teguments. We could suppose that *Hieracium villosum* seeds are not dormant or that storage at + 4 °C for 2 months is adequate pre-treatment to stimulate the germination process in this specie. Nevertheless, despite the high percentage of viable seeds, the treatments applied in our experiments did not appear to be optimal and it may be necessary to apply different experimental conditions to stimulate germination in this species, for example alternating temperatures, ageing and repeated soaking.

Saxifragaceae seeds are classified and described as endospermic seeds, with an axillary miniature embryo protected by fine teguments that require exposure to red light to increase oxygen permeability. Dormancy would appear to be sensitive to light exposure and gibberellic acid is also helpful (Atwater, 1980). Generally speaking, all phoblastic seeds are sensitive to cold temperatures and GA and the need for light can be bypassed by chilling or GA treatments (Srivastava, 2001).

The *Saxifraga* species seeds considered in this case are dormant seeds as germination tests were unsuccessful. Of the pre-treatments applied to interrupt dormancy, chilling gave negative results. Conversely, we observed successful germination by applying GA3 plus red light and red light only, although the results obtained were sporadic and scarce.

CONCLUSIONS

A number of important dormancy breaking and germination improving guidelines, all regarding wild plants, are reported in literature (Atwater, 1980; Ellis et al, 1985; AA.VV, 2001; AA.VV, 2006). Nevertheless, attention may be warranted by certain wild species with specific conservation requirements, which have different needs from the treatments recommended for the genus or family they belong to.

One important factor affecting germination experiments regards the limited availability of seeds from a natural habitat. In *Saxifraga* species, the few hundred seeds harvested and the high number of preliminary trials required, left a reduced number of seeds for performing germination tests. This prevented us from being able to carry out tests many times and to confirm the preliminary results obtained.

Significant success was achieved for *Hieracium villosum* the good germination percentages obtained by applying *in vitro* techniques suggest that by conducting further experiments, it could be possible to develop more effective *ex situ* conservation practices.

ACKNOWLEDGEMENTS – We are grateful to Dr. Alessandro Bottacci and Guido Crudele, Corps of Forest Rangers - Casentine Forest National Park for seed and fruit harvesting.

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