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Research Article

Collecting Times and Sterilization Methods Affect Tissue Culture of Rare and Endangered Species from Western Alps

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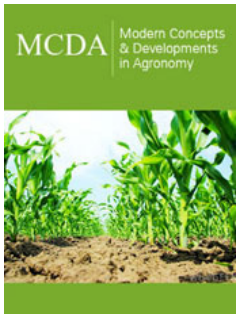

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Abstract

Climate change and human actions are compromising the conservation status of natural habitats, spontaneous plants, 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 and special protection areas created for the protection and conservation of habitats, animal and plant species biodiversity. Among the methods used to conserve biodiversity, micropropagation is an *in vitro* culture method of plant tissues. The aim of this study was to examine the effect of different collecting times and sterilization methods with the potential to enhance *in vitro* performance for ex situ conservation of six rare and endangered plant species of the Ligurian and Maritime Alps (*Clematis alpina* (L.) Miller, *Dracocephalum ruyschiana* L., *Gentiana asclepiadea* L., *Hyssopus officinalis* L., *Phyteuma cordatum* Balb. and *Ruscus hypoglossum* L.). Results showed that for both *C. alpina* and *D. ruyschiana*, as later in the summer were collected explants as higher values in established explants with shoot induction. While the use of plant preservative mixture in tissue culture media was not effective in increasing explant establishment in the studied species. The findings raise concerns regarding the most promosing *in vitro* protocols for the multiplication of rare and endangered alpine species.

Keywords: Endangered plants; GD medium; *In vitro*; Micropropagation; Rare plants; Red lists plants; Regeneration

Introduction

The loss of gene pool of any species is an irreversible damage to the biological diversity of the Earth. Therefore, the conservation of wild rare and endangered plant species is a priority task. At the same time, many plant species that are under threat of extintion are considered as sink for pharmaceuticals, nutraceuticals, cosmetic, and perfumery products [1]. Climate change and human actions are compromising the conservation status of natural habitats and spontaneous plant species, particularly from alpine areas. 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 biodiversity [2].

Biotechnological methods are becoming increasingly important for the conservation and exploitation of plant species. Among the methods used to conserve biodiversity, micropropagation is a multiplication technique that allows to obtain a clone of the plant or a set of individuals with the same genetic heritage, using *in vitro* culture methods of plant tissues. The most important part of any experiment involving plant tissue culture is the seasonal source of the plants and the target surface-sterilization method; wastage of plant

cultures, labour and time may result from errors performed at this stage [3]. In fact, the season of explant collection can play a pivotal role in the *in vitro* establishment of initial cultures starting from adult mother plants [4]. Another major concern in plant tissue culture is the occurrence of *in vitro* contamination due to the natural presence of bacteria or fungi upon the surfaces and natural openings of the target tissue [3]. In order to reduce the occurrence of such contaminations and boost plant survival, the highest priority is placed on the development and pairing of both surface sterilization recipes and efficient aseptic techniques prior to their execution on the target explant [5]. Isothiazolones are a class of industrial biocides that have been used in the form of plant preservative mixture (PPM™) in tissue culture media to control microbial contamination [6].

Therefore, this study aims to examine the effect of different collecting times and sterilization methods with the potential to enhance *in vitro* performance for *ex situ* conservation of six rare and endangered plant species of the Ligurian and Maritime Alps (*Clematis alpina* (L.) Miller, *Dracocephalum ruyschiana* L., *Gentiana asclepiadea* L., *Hyssopus officinalis* L., *Phyteuma cordatum* Balb. and *Ruscus hypoglossum* L.) present within Natura 2000 Network.

Materials and Methods

Plant material

Adult plants of *Clematis alpina* (L.) Miller, *Dracocephalum ruyschiana* L., *Gentiana asclepiadea* L., *Hyssopus officinalis* L., *Phyteuma cordatum* Balb. and *Ruscus hypoglossum* L. were collected in their natural environment (Table 1).

Table 1: Collecting site of the studied species.

Species	Location
Clematis alpina	Maritime Alps Natural Park, Valdieri (CN), Italy
Gentiana asclepiadea	Maritime Alps Natural Park, Valdieri (CN), Italy
Hyssopus officinalis	Maritime Alps Natural Park, Valdieri (CN), Italy
Dracocephalum ruyschiana	Marguareis Natural Park, Chiusa di Pesio (CN), Italy
Ruscus hypoglossum	Marguareis Natural Park, Chiusa di Pesio (CN), Italy
Phyteuma cordatum	Marguareis Natural Park, Briga Alta (CN), Italy

Explants about 8/10cm long were collected from a 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. This material was stored at 4 °C for a few days before being used in the laboratory. Explants of 1-2 centimeters long, each containing a vegetative apex were placed in containers for washing in cold running water (10 min). Subsequently, sterilization operations were carried out for the *in vitro* culture. To face fungal pollution, a fungicide (Enovit Methyl FL) was applied in association with NaOCl. To identify the right concentrations, various tests were carried out at different concentrations and exposure times. In Table 2 are reported the applied sterilization protocols.

Table 2: Sterilization protocols used for each species in the different trials.

Species	Data	Protocols		
Clematis alpina	08/17/2023	H_2O 10 min	Fungicide 60 min 3.0ml/L	NaOCl 1.5% + Tween 30 min
	09/28/2023	H_2O 10 min	Fungicide 45 min 3.0ml/L	NaOCl 2.0% + Tween 15 min
Phyteuma cordatum	08/03/23	H_2O 10 min	Fungicide 45 min 1.3ml/L	NaOCl 1.0% + Tween 20 min
	08/09/23	H_2O 10 min	Fungicide 60 min 3.0ml/L	NaOCl 1.0% + Tween 15 min
Dracocephalum ruyschiana	08/03/23	H_2O 10 min	Fungicide 45 min 1.3ml/L	NaOCl 1.0% + Tween 45 min
	08/17/2023	H_2O 10 min	Fungicide 60 min 3.0ml/L	NaOCl 1.5% + Tween 30 min
	09/28/2023	H_2O 10 min	Fungicide 45 min 3.0ml/L	NaOCl 1.0% + Tween 15 min
Ruscus hypoglossum	09/21/2023	H_2O 10 min	Fungicide 45 min 1.3ml/L	NaOCl 1.0% + Tween 30 min
Hyssopus officinalis	09/21/2023	H_2O 10 min	Fungicide 45 min 1.3ml/L	NaOCl 1.0% + Tween 30 min
Gentiana asclepiadea	09/21/2023	H_2O 10 min	Fungicide 45 min 1.3ml/L	NaOCl 1.0% + Tween 30 min

Collecting time and sterilization methods

Two different trials were conducted. First, explants of *C. alpina*, *P. cordatum* and *D. ruyschiana* were collected in different times during august and september 2023 and placed in a Greesshoff and Doy (GD) medium (Table 3). While, in the second, the sterilization of explants of *R. hypoglossum*, *H. officinalis* and *G. asclepiadea* was conducted by applying or not PPM™ at the concentration of $2mlL^{-1}$ in addition to the protocolos described in Table 2. Explants were maintained at 24 °C with a photoperiod of 16h light/8h dark under 25-30$\mu molm^{-1}$ s^{-2} under cool, fluorescent white lamps. In both trials, after 35 days from the beginning of the *in vitro* experiment, the percentage of established explant, the percentage of shoot induction, the number and the lenght of shoot produced per plant, and the percentage of browning explants were measured.

Table 3: Micro and macro elements containing in the used micropropagation medium (Gresshoff and Doy medium; GD; mgL^{-1}).

Micro Elements	$CoCl_2.6H_2O$	0.025
	$CuSO_4.5H_2O$	0.025
	FeNaEDTA	36.7
	H_3BO_3	0.3
	KI	0.8
	$MnSO_4.H_2O$	1
	$Na_2MoO_4.2H_2O$	0.025
	$ZnSO_4.7H_2O$	0.3

Macro Elements	$Ca(NO_3)2.2H_2O$	208.81
	KCl	65
	KH_2PO_4	300
	KNO_3	1000
	$MgSO_4$	17.09
	NH_4NO_3	1000

Statistical analysis

Significant differences were verified with the t-test ($p < 0.05$) after checking the data for normality (Shapiro-Wilk's test, $p \geq 0.05$) and homoscedasticity (Levene's test, $p \geq 0.05$). Moreover, a one-way ANOVA test by applying Ryan–Einot–Gabriel–Welsch Studentized Range Q (REGW-Q) post hoc test ($p < 0.05$) was performed to note differences between the collecting time of *D. ruyschiana*. These statistical analyses were computed by SPSS software (version 26.0, SPSS Inc., Chicago, IL, USA).

Results and Discussion

The anthropogenic load and the low competitiveness of the plant species in phytocenoses are the main reasons for the rarity of some plant species [7]. Moreover, these rare plant species are low seed germination or vegetative reproduction, relict species, torn areas, harsh climatic conditions, eaten by animals and birds [8]. *In vitro* culture protocols are widely used to solve these problems and to restore the gene pool of rare and endangered plant species. In the present study, for the first time, the importance of explant collecting times and the use of PPM in the first phases of micropropagation of *Clematis alpina*, *Dracocephalum ruyschiana*, *Gentiana asclepiadea*, *Hyssopus officinalis*, *Phyteuma cordatum* and *Ruscus hypoglossum* were tested.

The effect of different collecting times on *in vitro* development of *C. alpina* and *D. ruyschiana* explants are reported in Table 4. No parameters were affected in *P. cordatum* with 0.0 % of established explant. While, for both *C. alpina* and *D. ruyschiana*, collected explants had higher values in established explants and shoot induction were observed (Figure 1). Regarding the number of shoots produced per explant, an opposite dynamic was highlighted with a significant increase in *C. alpina* and a decrease in *D. ruyschiana*. In this last species was observed also a reduction in the percentage of browning. These findings indicated that, for the studied species, a later collection time in late august and september was most favourable because the mother plants are still in full activity with probably high levels of growth promoting substances and low growth inhibitors [9]. Furthermore, it was found that some genotypes responded well whereas, others are recalcitrant, which suggests that regeneration is genetically controlled. Regarding *D. ruyschiana*, low browning may be due to low *in vivo* phenolic content as the short-day length prevailing during this period has been reported to reduce the *in vivo* phenolics [10].

Figure 1: *In vitro* established explants of *Clematis alpina* (left) and *Dracocephalum ruyschiana* (right).

Table 4: Effect of different collecting times on the percentage of established explants (%), percentage of explants with shoot induction (%), number of shoots per explant (n.), mean lenght of shoot per explant (cm) and percentage of browning explants (%) of *Clematis alpina, Phyteuma cordatum* and *Dracocephalum ruyschiana* after 35 days from the beginning of the experiment.

Note: Mean values showing the same letter are not statistically different at $p < 0.05$, according to the REGWF post hoc test. The statistical relevance is provided (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant).

Species	Date	Established	Shoot Induction	Shoot per Explant	Mean Shoot Lenght	Browning
Clematis alpina	08/17/2023	6.6	6.6	1	1.5	20
	09/28/2023	25	8.3	3	1	16.6
	p	**	*	*	ns	ns
Phyteuma cordatum	08-03-23	0	-	-	-	26.3
	08/17/2023	0	-	-	-	34.6
	p	ns				ns
Dracocephalum ruyschiana	08-03-23	6.2c	6.2c	3.0a	0.9	37.5a
	08/17/2023	32.1b	32.1b	2.1b	1.5	10.7b
	09/28/2023	90.9a	42.4a	1.8b	0.4	9.1b
	p	**	***	*	ns	**

The effective sterilization of biological material (e.g., initial explant) is required for successful *in vitro* culture initiation. Table 5 reports the effects of two sterilization protocols for micropropagation of *R. hypoglossum* and *G. asclepiadea*. Only *R. hypoglossum* explants were established in used GD medium. A significant reduction of this parameter was observed in the presence of PPM™. In the same medium an increase in browning was measured. Although no explants of *G. asclepiadea* were established, a reduction in browning was seen in the medium with PPM™. The data presented does not show a positive effect of the use of PPM™ in the sterilization protocols. Likewise, Compton et al. [11] reported reduced embryo formation in *Cucumis melo* and poor shoot organogenesis of *Petunia* × *hybrida* even at low concentrations of PPM™. The use of PPM™ is generally limited to species that present contamination problems in the establishment stage such as *Guadua angustifolia* and *Malus domestica* [12]. Finally, as reported by Ledo et al. (2019) PPM™ has never been evaluated as a substitute for autoclaving. Apart from contamination, browning of excised plant tissues and nutrient media occurs frequently and remains a major basis for recalcitrance *in vitro*. The severity of browning has varied according to species, tissue or organ, developmental phase of plant, age of tissue or organ, nutrient medium and other tissue culture variables [13].

Table 5: Effect of sterilization with the presence or not of plant preservative mixture (PPM™) at the concentration of 2mlL^{-1} in addition to the protocolos described in Table 2, on the percentage of established explants (%), percentage of explants with shoot induction (%), number of shoots per explant (n.), mean length of shoot per explant (cm) and percentage of browning explants (%) of *Ruscus hypoglossum, Hyssopus officinalis* and *Gentiana asclepiadea* after 35 days from the beginning of the experiment.

Note: The statistical relevance is provided (* $p < 0.05$; ** $p < 0.01$; ns = not significant).

Species	Sterilization	Established	Shoot Induction	Shoot per Explant	Mean Shoot Lenght	Browning
Ruscus hypoglossum	No PPM™	33.3	-	-	-	0
	PPM™	25	-	-	-	25
	p	*				**
Hyssopus officinalis	No PPM™	0	-	-	-	0
	PPM™	0	-	-	-	0
	p	ns				ns
Gentiana asclepiadea	No PPM™	0	-	-	-	66.7
	PPM™	0				40
	p	ns				**

Figure 2: Division of elongated shoots of *Dracocephalum ruyschiana* in aspetic conditions.

Conclusion

Up to date, there was no information regarding the micropropagation protocols for *C. alpina*, *D. ruyschiana*, *G. asclepiadea*, *H. officinalis*, *P. cordatum* and *R. hypoglossum*. In the present study, explant establishment and shoot multiplication technique was standardized mainly for *D. ruyschiana* (Figure 2).

Nodal segments explants proved to be useful for initiating micropropagation of this species. When the explants were collected during late summer season there was higher explant establishment and lower loss of the cultures due to explant browning and endogenic contamination. Regarding sterilization, the used protocols were not able to increase establishment in *R. hypoglossum*, *H. officinalis* and *G. asclepiadea*.

Taking all these considerations together, the direction of micropropagation research will enable a number of rare endangered species to be saved and in a number of cases to achieve a measure of success in ecological *ex vitro* repatriation of some species.

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