IN VITRO PROPAGATION OF *Campanula polymorpha* Witas. – AN ENDEMIC PLANT OF CARPATHIAN MOUNTAINS

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Abstract. *Campanula polymorpha* is an endemic plant of Carpathian Mountains, included in the Carpathian List of Endangered Species. An efficient micropropagation system was developed for regeneration and *in vitro* conservation of *C. polymorpha*. Single-node aerial stem segments from mature flowering plants collected from natural growing populations was sterile inoculated on regeneration media. For an efficient regeneration a range of 12 variant of basal MS (Murashige - Skoog) media formulation was tested, with different growth regulators combinations. Histological investigation shows that axillary bud dormancy was broken after 5 days of culture. The best rate of shoot production (average 14.8 shoot/explant) was achieved after 5 weeks of culture on media supplemented with 0.1 mg/l NAA (1-naphthylacetic acid) and 1 mg/l BAP (6-benzylaminopurine). Shoots of about 4-5 cm high, developed roots after 3-4 weeks on media without growth regulators. The established protocols offer a valuable micropropagation method that could be useful as a starting point for *in vitro* conservation of this endemic plant, or for mass propagation of other *Campanula* species of horticultural importance.

Keywords: *Campanula polymorpha* Witas., *in vitro* conservation

INTRODUCTION

According to the latest release of International Union for Conservation Nature (IUCN) approximately 22% of the world's assessed plants on the 2010 Red Lists are in jeopardy [29]. The loss of plant genetic resources has made necessary the development of new *ex situ* conservation methods. Advances in biotechnology, especially *in vitro* culture techniques and molecular biology, provide some important tools for assessment, conservation and management of plant genetic resources [1, 7, 9]. Several *in vitro* techniques have been developed, mostly for vegetatively propagated and recalcitrant seed producing species, with recent establishment of extensive germplasm collections [19, 23]. *In vitro* storage techniques include the medium-term storage (for a determined period - a few months up to a few years) using slow growth strategy or artificial seed production [22], and long-term storage (tentatively for an indeterminate period of time) using cryopreservation [8]. Slow growth storage strategy is used to preserve some endangered phytotaxa in the Institute of Biology from Bucharest (Romania) within *in vitro* regenerative lines collections. The main steps to establish a germplasm collection are *in vitro* regeneration, followed by multiplication and finally, maintaining the regenerative tissue, with constant care for preserving the genetic integrity of original donor plant [23]. Up to date, there are very few references concerning *in vitro* reactivity and complete regeneration of *Campanula* species [4, 12, 26, 27], and none regarding *Campanula polymorpha*. This is the first report of an efficient and reproducible protocol to establish regenerative lines for conservation purposes, and evaluation of morphogenetic reactivity to *in vitro* conditions of *C. polymorpha*.

C. polymorpha Witas. is an endemic phytotaxa with a limited distribution along the Carpathian Mountains. It is a perennial plant between 5 - 20 cm high with different shaped leaves (elliptic, lance-shaped, reniform) and deep blue-violet flowers (either solitarily or in loose spikes), having large corollas up to 25 mm long. The fruit is a nutant capsule with three basal openings, bearing small seeds, often infertile. It grows on rocky slopes, cliffs and scree. Because more than a half of the species populations occur in Romanian territory, it is evaluated as subendemic for Romanian Carpathians. Since 2003, *C. polymorpha* is included in the Carpathian List of Endangered Species [28].

MATERIALS AND METHODS

Plant material and regenerative lines induction

Mature plants in flowering stage were collected from Ceahlau Massif (Oriental Carpathians), in July 2009. Single node aerial defoliated stem segments (0.5-1cm) were surface disinfected by immersing in 70% ethanol for 20 seconds, followed by 15 minutes in 0.5% (w/v) of Sodium dichloroisocyanurate and washed three times in double distilled sterile water. The explants were aseptically inoculated in 80 mm Petri dishes containing 30 ml of basal MS (Murashige - Skoog) [16] modified medium, and sealed with self adhesive cotton tape. The 12 variant of basal MS media were formulated to stimulate regeneration via direct organogenesis (with 1/10 auxin/cytokinin ratio). The grows regulators content in culture media, were 1 mg/l cytokinin and 0.1 mg/l auxin, and could be followed in Table 1. Established cultures were maintained at 20°C with a 16 h photoperiod (3000 lux). When the initial culture vessels became insufficient large (within 4-5 weeks of culture) each shoot cluster was transferred in jars (67 mm diameter x 80 mm high) with self–sealing leads, each contained 40 ml medium. Individual regenerated shoots were placed for rooting in wide mouth 250 ml Erlenmeyer flasks, with 80 ml basal media without growth regulators, stoppered with cotton leads and sealed with aluminium foil. After 12 weeks onto rooting media the plantlets with well-established roots were transplanted to autoclaved soil in pots, in acclimatization chamber at 70% relative humidity, and watered for 10 days with a half-strength MS solution containing 2% sucrose. Micropropagated plantlets were successfully acclimatized within 4-5 weeks, with 85% survival rate.

Table 1. Growth regulators combinations of regeneration media.

Media variant	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Auxin (0.1mg/l)	NAA	NOA	IAA	IBA	2,4D	2,4,5T	NAA	NOA	IAA	IBA	2,4D	2,4,5T
Cytokinin(1mg/l)	BAP	BAP	BAP	BAP	BAP	BAP	Kin	Kin	Kin	Kin	Kin	Kin

Note: BAP (6- benzylaminopurine), IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), Kin (Kinetin), NAA (1-naphthylacetic acid), NOA (2-naphthyloxyacetic acid), 2,4D (2,4-dichlorophenoxyacetic acid), 2,4,5T (2,4,5-trichlorophenoxyacetic acid).

Histological studies

In order to estimate the timing of breaking dormancy, histological studies of the nodes, after 5 and 10 days post inoculation, were performed. Fresh tissue samples from reactive explants were fixed in formalin buffered solution. Dehydration was conducted in graded ethanol series, followed by clearing with xylene and embedding in paraffin. Serial sections (7-10 μm thick) were cut and transfer onto glass slides. The sections were stained with 0.12% (w/v) toluidine blue, in a water solution containing 5% (w/v) sodium tetraborate decahydrate and 0.1% (w/v) basic fuchsine [13] and finally mounted in synthetic resin (Entellan). Histological observations were made using bright-field light microscopy under a Nikon Eclipse E200 microscope and micrographs were recorded using a Nikon Coolpix 5400 digital camera.

RESULTS

After 2 weeks of culture the stem nodes appears visible swallowed. The histological studies show that within 10 days of culture the nodal meristems were activated and generate multiple bud primordia (Fig. 1).

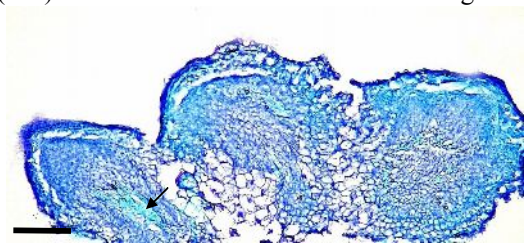

Figure 1. Multiple axillary buds primordia. (Arrow – traheid cells. Bar scale 100 μm).

After 5 weeks of culture most of the activated buds generate shoots (Fig. 2), with an average of 5.2 shoots/explant. The best rate of shoot formation (14.8 shoots/explant) was recorded on variant with BAP and NAA (Fig. 2a). A good rate of bud proliferation occurs also on media with BAP combined with NOA or IAA (Fig. 2b). Onto media with kinetin the number of developed shoots per node, varies between relatively low values (4.3 – 1.1).

The rate of shoot proliferation and elongation, as well as the percent of nodes producing shoots, could be followed in Table 2.

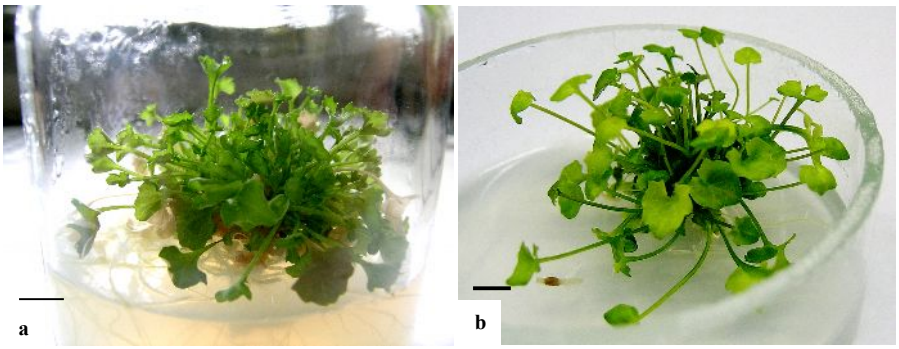

Figure 2. Multiple axillary shoots (**a** - on M1 media, **b** - on M2 media; Bar scale 0.5 cm).

Table 2. Explant response to hormonal content of culture media, after 5 weeks of culture.

Media variant	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Mean number of shoots/ explant[a]	14.8 ± 0.17	10.2 ± 0.12	11.3 ± 0.56	6.8 ± 0.14	1.2 ± 0.19	2.2 ± 0.12	3.1 ± 0.56	4.3 ± 0.98	3.5 ± 0.04	2.6 ± 0.16	1.1 ± 0.22	1.5 ± 0.32
Mean length of shoots (cm)	1.9 ± 0.12	2.4 ± 0.03	3.2 ± 0.20	1.7 ± 0.32	0.7 ± 0.14	0.9 ± 0.46	1.4 ± 0.44	1.6 ± 0.23	0.7 ± 0.74	0.2 ± 0.28	0.1 ± 0.49	0.2 ± 0.16
Nodes with shoots (%)	89.8 ± 0.15	91.2 ± 0.14	80.1 ± 0.34	61.4 ± 0.21	10.3 ± 0.18	22.3 ± 0.37	7.2 ± 0.39	2.6 ± 0.93	9.1 ± 0.13	6.3 ± 0.21	2.5 ± 0.28	5.2 ± 0.29

Note: [a]Values are means ± standard deviation of variants with three replicates (culture vessels), each variant consisting of a total of 5 explants.

When placed on basal MS media without growth regulators, solitary well developed shoots were first elongated and rooted within 3-4 weeks (Fig. 3a). When established in soil, the acclimatized plants developed flower buds and shows normal morphological characteristics (Fig. 3b).

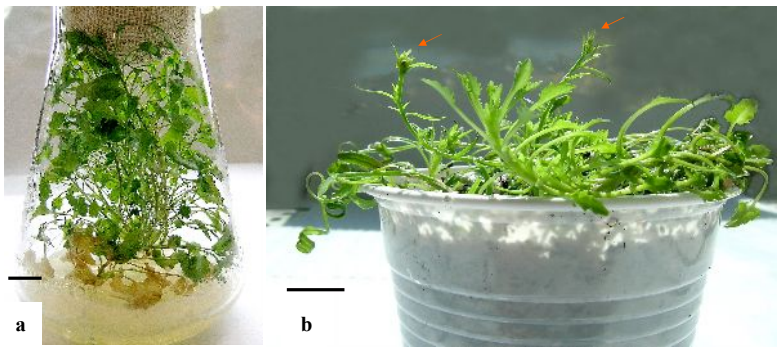

Figure 3. Regenerated plantlets (**a** – rooted plantlets, **b** – aclimatizated plants, arrow: flower buds. Bar scale 1 cm).

DISCUSSIONS

Generally, the mature plants are recalcitrant to adventitious organ formation and regeneration [26]. One of the most used method for direct regeneration from mature explants, involve axillary buds. It is commonly known that axillary buds are dormant structures, their dormancy being regulated by the endogenous auxin levels produced by the caulinar apical meristem. If the apical dominance is removed nodal meristems becomes activated and axillary bud dormancy is broken. When single nodal explants are cultivated *in vitro*, external addition of cytokinins to the medium was found to stimulate multiple axillary bud proliferation and direct shoot regeneration in a wide range of plant species: herbs [2, 3, 11, 14, 20, 24, 25], bushes [5, 6, 10, 17, 21], trees [18] or climbing plants [15]. The concentration and type of cytokinin used significantly affected the percentage of shoot regeneration, shoot number, and shoot length. It was found that BAP, single or in combination with auxin is one of the most efficient cytokinin to break bud dormancy and subsequent regeneration of multiple shoot [2, 14, 15, 25]. Present study confirm these findings, the best results being registered on media with BAP, in terms of bud proliferation (BAP/NAA combination), shoot elongation (BAP/IAA combination) and percent of nodes that produce shoots (BAP/NOA combination). The stimulative effect of BAP is confirmed by histological investigation of the nodes showing that within 5 days of culture the dormancy was broken by activation of the bud meristem which expand laterally by multiple periclinal cell divisions. Within 10 days of culture multiple bud primordia are developed, each having a distinct shape and a clear cytodifferentiation into a meristematic apical area and a parenchymatic one, with inner clusters of traheid cells (Fig. 1). In contrast, kinetin was not so efficient to stimulate the regenerative ability of the explants and promote adventitious shoot formation, the total percentage of nodes producing shoots being only 5.4%. Although high BAP content (10 mg/l) was reported to break axillary bud dormancy and multiple adventitious shoot formation in other *Campanula* species [26, 27], in *C. polymorpha* a content of 1 mg/l proved to be very efficient. It is likely that other *Campanula* species would have a different reactivity to BAP content of culture media, but the proposed concentration of 1 mg/l is recommended as a starting point to break axillary bud dormancy.

Monitoring the explants regenerative ability on different culture media allows the establishment of an optimal vitroculture protocol according to a specific purpose. If the target is multiple bud proliferation, M1 media is recommended, for shoot elongation M3 media, and for an efficient stimulation of nodal meristems and higher rate of nodes producing shoots, M2 media is the most suitable.

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