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Propagation and antioxidative enzymes of *Campanula velebitica* Borbás grown *in vitro*

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Abbreviations:

APX	– ascorbate peroxidase
BA	– 6-benzylaminopurine
CAT	– catalase
GA_3	– gibberellic acid
GPOD	– guaiacol peroxidase
IAA	– indole-3-acetic acid
IBA	– indole-3-butyric acid
MS medium	– Murashige and Skoog medium
PAGE	– polyacrylamide gel electrophoresis
ROS	– reactive oxygen species
IUCN	– International Union for Conservation of Nature
NBT	– nitro blue tetrazolium

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Abstract

Background and purpose: *Micropropagation possibility for* Campanula velebitica *Borbás (*Campanulaceae*), a rare Croatian endemic plant was studied. Additionally, the activity of several antioxidative enzymes, as biomarkers of stress conditions, was investigated in plants grown* in vitro.

Materials and methods: *Shoots from aseptically germinated seeds, collected from natural habitat, were used for culture initiation. Multiplication rate of shoots was monitored after six weeks on half-strength Murashige and Skoog (MS) medium supplemented with 0, 0.2, 0.5 and 1.0 μM 6-benzylaminopurine (BA). Rooting was induced on half-strength MS medium supplemented with 2.9 or 5.7 μM indole-3-acetic acid (IAA); or 2.5 or 4.9 μM indole-3-butyric acid (IBA). Activity of ascorbat (APX) and guaiacol (GPOD) peroxidases as well as catalase (CAT) was determined in shoot extracts spectrophotometrically and in gel.*

Results and conclusions: *The highest multiplication rate, 7.7 shoots per explant, was achieved in the 3rd subculture on the medium containing 1.0 μM BA. Very good rooting (>90%) of excised shoots was achieved on media containing either indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). On the medium containing 4.9 μM IBA flowering was observed. Activities of APX and GPOD were significantly increased on media containing lower concentrations of BA (0.2 and 0.5 μM). Native PAGE analysis revealed one CAT, three GPOD and two APX isoenzymes. We conclude that* C. velebitica *is easily propagated in* in vitro *conditions, without substantial stress response. With further studies of plantlet acclimation, this endemic plant species could be efficiently preserved through culture* in vitro.

INTRODUCTION

The Velebit bellflower, *Campanula velebitica* Borbás (*Campanulaceae*) is a Balkan endemic plant species growing in several mountains of the southwestern and Adriatic Croatia, with most findings in the Velebit Mountain range. It has been assigned International Union for Conservation of Nature (IUCN) 'near threatened' status *(1)* due to its relatively difficult-to-access habitats (rock crevices and frequently within montane and subalpine beech stands), and since 2006 it has been provided strict statutory protection. *C. velebitica* is a perennial with many ascending or erect glabrous stems up to 35 cm in height. Basal leaves are cordate to reniform, serrate, and cauline lanceolate to linear, remotely serrate to entire. The lax inflorescence carries many erect buds and many light purple flowers, with corolla 16–20 mm long

(2). This highly decorative endemic species is rare in botanical collections and relatively unfamiliar to professional collectors. To ensure the survival of *C. velebitica*, permanent garden populations need to be established. Conventional vegetative methods of propagation of most *Campanula* species and cultivars include division of the shoots and rooting of stem cuttings. Those techniques are time-consuming and require many stock plants and, due to relatively limited size of wild population of *C. velebitica* and its protection, micropropagation will meet the need to produce sufficient number of wild-type plants, without harming the natural populations.

Some difficulties in otherwise beneficial micropropagation techniques are well known: increased stress conditions due to artificial environment in culture *in vitro* have been shown to cause high production and accumulation of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2). ROS are regularly formed in living cells by the partial reduction of molecular oxygen and are normally controlled by plant *(3, 4)*. Higher-than-usual concentrations of ROS can cause oxidative damage at cellular and molecular level (4) which can affect the morphogenesis of plant cells and tissues and slow or even inhibit development during *in vitro* cultivation *(5)*. To scavenge ROS, plants have developed complex antioxidative systems, including highly efficient antioxidative enzymes like catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOD). There have been some studies on how different hormonal conditions in *in vitro* culture affect antioxidative enzyme activity in reference to plant tissue growth and multiplication or root formation *(6, 7, 8)*.

In this experiment the objective was to investigate micropropagation possibilities for *C. velebitica* which could improve its cultivation and successful large-scale production. Although there have been several reports on micropropagation of different *Campanula* species *(9, 10, 11, 12),* there is no information on tissue culture of *C. velebitica* or any other Croatian endemic bellflower to our knowledge. We extended our research to measurement of activity of antioxidative enzymes as biomarkers of stress conditions in culture *in vitro*.

MATERIALS AND METHODS

C. velebitica seeds were collected in 2007 from a natural habitat in northern Velebit. Prior to inoculation, the seeds were rinsed in tap water and cold stratified on moist sterilized fine-quartz sand at 4 °C for at least 20 days. The sterilization was carried out with 2% (w/v) water solution of a commercial chlorine product Izosan-G (99% sodium dichloroisocyanurate dihydrate, Pliva, Zagreb) for five minutes, and then, after three five-minute rinses in sterile distilled water, with 6% solution of hydrogen peroxide. After three finishing washes in sterile distilled water (five minutes each), one to three seeds were inoculated per test tube (30 x 120 mm) filled with 10–12 mL of basal nutrient medium. After inoculation, test tubes were capped with cotton plugs and aluminum foil. The basal nutrient medium contained MS *(13)* mineral salts with half concentration of macroelements (half-strength MS, MS½), 100 mg L^{-1} *myo*-inositol, 0.1 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} pyridoxine-HCl, 0.5 mg L^{-1} nicotinic acid, 30 g L^{-1} sucrose and 8 g L^{-1} agar. The pH value of all media used was adjusted to 5.8 before autoclaving at 118 kPa and 120 °C for 15 minutes. The seedlings developed in this culture were separated from their roots and inoculated on shoot multiplication media containing MS½ medium supplemented with 2.9 μM gibberellic acid (GA_3) and three different concentrations of 6-benzylaminopurine (0.2, 0.5 or 1.0 μM BA) or without it. The number of shoots was estimated after six weeks in culture on 12 explants per medium through three subcultures. After six weeks in culture, well-developed shoots were cut off and rooted on MS½ medium supplemented with 2.5 or 4.9 μM indole-3-butyric acid (IBA); or 2.9 or 5.7 μM indole-3-acetic acid (IAA). The rooting of shoots was recorded after six weeks in culture. All cultures were incubated at 22 ± 2 °C under a 16 hour photoperiod (40 W fluorescent light, 80 μE m^{-2} s^{-1}).

For enzyme isolation leaves were excised and stored in deep-freezer at –20 °C until analysis. Frozen samples (0.1 g) were ground in ice-cold 50 mM potassium phosphate buffer (pH 7.0) with addition of 50 μM ethylenediaminetetraacetate, 2 mM ascorbate and polyvinylpyrrolidone. Extracts were centrifuged for 20 minutes at 30 000*g* at 4 °C. The supernatant obtained was used for enzyme activity measurements and soluble protein determination according to Bradford *(14),* using bovine serum albumin as a protein standard. CAT (EC 1.11.1.6) was assayed according to Aebi *(15)*. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and 50 μL of enzyme extract. Activity was determined spectophotometrically by following the decomposition of H_2O_2 in 1 mL reaction mixture at 240 nm and expressed as μmol of decomposed H_2O_2 mg^{-1} protein min^{-1} using the molar extinction coefficient of 40 mM^{-1} cm^{-1}. APX (EC 1.11.1.11) activity was determined in 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 50 mM ascorbate, 12 mM H_2O_2 and 100 μL of enzyme extract, by following the H_2O_2 dependent decomposition of ascorbate at 290 nm, as described by Nakano and Asada *(16)*. Enzyme activity was defined as μmol of oxidized ascorbate mg^{-1} protein min^{-1} using molar extinction coefficient of 2.8 mM^{-1} cm^{-1}. GPOD (EC 1.11.1.7) activity was determined according to Chance and Maehly *(17)* in 1 mL reaction mixture composed of 50 mM potassium phosphate buffer (pH 7.0), 18 mM guaiacol, 5 mM H_2O_2 and 50 μL of enzyme extract. The enzyme activity was measured by monitoring the increase of absorbance at 470 nm, and defined as μmol of tetraguaiacol mg^{-1} protein min^{-1} using molar extinction of coefficient 26.6 mM^{-1} cm^{-1}.

For isoenzyme analysis, polyacrylamide gel electrophoresis (PAGE) without sodium dodecylsulfate was performed on 8% (w/v) polyacrylamide gels *(18)*. Approximately equal amounts of proteins, 35–45 μg per well,

were loaded and electrophoresis was performed at 4 °C. For APX separation, the gel was pre-run for 30 min before the samples were loaded and ascorbate was added to the electrode buffer. After electrophoresis the detection of isoenzymes was achieved as following: for CAT detection, the gels were incubated in distilled water for 45 min and then in H_2O_2 solution (0.012%, v/v), for 10 min. The gels were then washed in distilled water and stained in 1:1 mixture of 2% (w/v) $FeCl_3$ and 2% (w/v) $K_3Fe(CN)_6$ for 10 min *(19)*. For GPOD detection, the gels were equilibrated with 200 mM potassium phosphate buffer (pH 5.8) supplemented with 20 mM guaiacol and 4 mM H_2O_2, until the appearance of brown bands *(17)*. For APX staining, the gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate, for 30 min (3 times 10 min). Thereafter, the gels were incubated in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2, for 20 min. The gels were then briefly washed in 50 mM potassium phosphate buffer (pH 7.0) and stained in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N',N'-tetramethylethylenediamine and 2 mM nitro blue tetrazolium (NBT), for 10 min, until clear bands appeared on an intensely blue background, due to NBT reduction by ascorbate *(18)*.

All results were expressed as mean values followed by corresponding standard errors. For each exposure treatment, data were compared by analysis of variance (Duncan New Multiple Range Test), using STATISTICA 7.1 (StatSoft, Inc., USA) software package, and differences were considered statistically significant at $P \leq 0.05$.

RESULTS

The seed sterilization with the chlorine preparation and hydrogen peroxide was successful. After six weeks of incubation on MS basal medium, 93% of the cultures remained sterile. Germination of inoculated seeds was relatively low, only 30% of seeds germinated.

For multiplication experiments, germinated plantlets (2–4 cm) were cut off from primary roots and transferred to the multiplication medium. High concentrations of BA stimulated the shoot multiplication rate. The highest multiplication rate (7.72 shoots per explant) was achieved on MS½ medium with 1.0 μM BA in the third subculture (Table 1). The number of shoots induced on this medium was statistically higher than the number of shoots induced on other media in the same subculture. The subculturing had a positive effect on shoot multiplication; it was higher in the third subculture in comparison to the first and second subcultures on media with 1.0 μM BA. Mild induction of yellow to green colored callus and some adventitious roots were occasionally observed on all media tested.

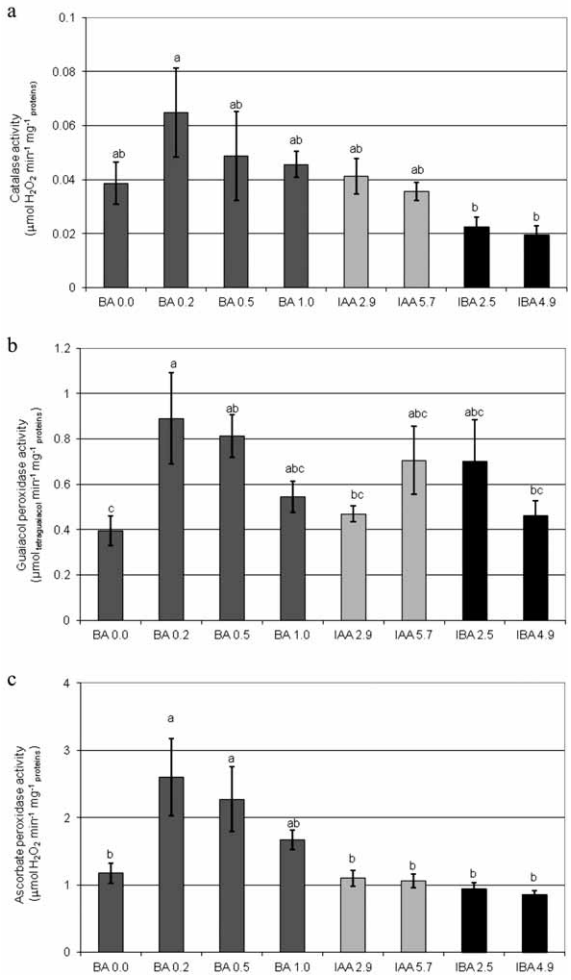

Figure 1. *Influence of different concentrations (in μM) of BA and auxins (IAA and IBA) on catalase (a), guaiacol peroxidase (b) and ascorbate peroxidase (c) activity measured in* C. velebitica *shoots grown* in vitro. *The columns marked with different letters are statistically different at* $P \leq 0.05$.

TABLE 1

Effect of cytokinin BA on shoot multiplication of Campanula velebitica during three subcultures. Values are means ± standard error. Values accompanied with different letters indicate significant differences between means at $P \leq 0.05$.

Medium	BA (μM)	No. of shoots per explant		
		1st subculture	2nd subculture	3rd subculture
MS 1/2	0	0.40 ± 0.16 [b]	0.5 ± 0.22 [b]	1.9 ± 0.53 [c]
	0.2	2.10 ± 0.61 [ab]	2.6 ± 0.73 [a]	4.6 ± 0.82 [b]
	0.5	2.73 ± 0.78 [a]	4.09 ± 0.86 [a]	4.55 ± 0.77 [b]
	1.0	2.45 ± 0.61 [a]	4.27 ±0.65 [a]	7.73 ± 0.98 [a]

To investigate rooting, elongated shoots (1–2 cm) were excised from multiple-shoot cultures and inoculated on MS½ basal medium supplemented with IBA (2.5 or 4.9 μM) or IAA (2.9 or 5.7 μM). The percentage of rooted shoots was 90.1% on the media with 2.5 μM IBA and 100% on the media with 4.9 μM IBA and on both media containing IAA. The roots developed on a medium supplemented with high concentration of IAA were more abundant and shorter in comparison to the roots developed on other media. Incubation on rooting media also favored shoot multiplication, which was significantly high on the medium with 4.9 μM IBA (data not shown). Slight formation of callus as well as flowering were occasionally observed. The highest flowering rate (30%) occurred on the medium with 4.9 μM IBA.

The activities of antioxidative enzymes (CAT, APX and GPOD) were measured in protein extracts obtained from the shoots grown *in vitro*. The highest CAT activity was observed on the medium with the lowest concentration of BA (0.2 μM) but it was not statistically significant in comparison to the medium without BA. Increasing BA also did not significantly influence CAT activity. The lowest CAT activity was noticed on the media complemented with auxins, especially IBA, but it was still not significantly different from untreated control (Fig 1a). Native PAGE analysis showed one slow migrating acidic CAT isoenzyme (C1) in all samples (Fig 2a). The activity of GPOD was significantly increased on the medium with lower BA concentration (0.2 and 0.5 μM) in comparison to activity on the medium without cytokinin. The highest IAA concentration (5.7 μM) as well as 2.5 μM IBA slightly increased GPOD activity, but it was not significantly different from control (Fig 1b). Native PAGE revealed one slow migrating (G1), and two fast migrating (G2 and G3), GPOD isoenzymes in all samples (Fig 2b).

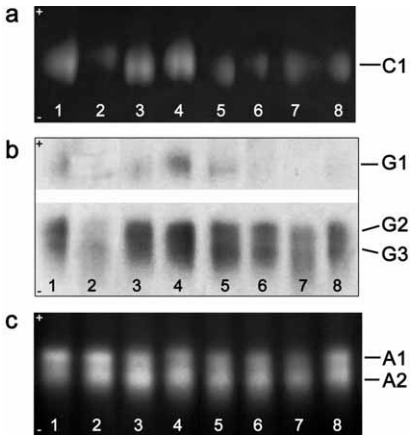

Figure 2. *Isoenzyme patterns of CAT (a), GPOD (b) and APX (c) in* C. velebitica *shoots grown on media with different concentrations of BA and auxins (IAA and IBA). Equal amounts of protein (40 μg for GPOD and CAT; 45 μg for APX) were loaded in each lane. Lane 1: 0 μM BA, Lane 2: 0.2 μM BA, Lane 3: 0.5 μM BA, Lane 4: 1 μM BA, Lane 5: 2.9 μM IAA, Lane 6: 5.7 μM IAA, Lane 7: 2.5 μM IBA, Lane 8: 4.9 μM IBA. Different isoforms are marked with letters: C for CAT, G for GPOD and A for APX.*

The activity of APX increased significantly in shoots grown on the media with 0.2 or 0.5 μM BA in comparison to other media. Auxins did not have effect on APX activity (Fig 1c). After native PAGE analysis, two fast migrating APX isoenzymes (A1 and A2) were detected in all samples (Fig 2c).

DISCUSSION

In this study we investigated the possibility of *in vitro* propagation of *Campanula velebitica* Borbás, a Balkan endemic 'near threatened' plant species which enjoys strict statutory protection. Throughout the experiment, half-strength MS media were used according to previous studies that suggested that plants growing in rock crevices and on pour soils prefer lower concentrations of mineral salts *(19, 20, 21)*. The pH value of all media used for *C. velebitica* culture was adjusted to 5.8, which was found to be optimal for several Croatian endemic species growing on washed-out, skeletal lime or dolomite rich soils of neutral to lightly acid pH value *(22)* and for *C. isophylla* culture *(9)*. Low germination rate was observed (only 30%), which could partly be explained by non-representative seed sample collected from natural populations, as well as *C. velebitica* seed morphology; those are less than 0.7 mm long and 0.3 mm wide, thus very difficult to handle during the sterilization procedure. Other specific properties concerning *Campanula* seed ecology and physiology are of significance as well; the seeds of most alpine species, including the genus *Campanula*, require moist cold stratification to germinate *(23)* but there are no specific data regarding the length of stratification period. It is possible that longer-than-tested (8–12 weeks) chilling treatment would favor germination. Another complication is presented by collecting seeds from wild populations. The maturity of seed capsules is difficult to establish for the high-altitude *Campanula* species, which can lead to collecting of underripe fruit with most seeds immature and unable to germinate, as reported by Blionis and Vokou *(24)*.

The highest multiplication rate (7.72 shoots per explant) was achieved on MS½ medium with 1.0 μM BA in the third subculture. High cytokinin levels obviously stimulated sprouting from axillary buds. Stimulative effect of high BA concentrations on shoot multiplication was observed earlier for *C. isophylla (9, 10)* as well as for *Centaurea ragusina (20)* and *Fibigia triquetra (19)*. The increase of BA concentration above 4.4 μM did not seem to significantly improve the multiplication rate *(9, 12)*. The shoots of *Campanula velebitica* grown on the media with 1.0 μM BA were shorter and more robust in comparison to elongated, slender shoots growing on the media with lower concentrations of BA, which is a typical growth response to different cytokinin concentrations *(25)*. The significant increase in multiplication rate observed during subculturing, especially on the medium with the highest BA concentration, is in accordance with the results reported for *C. isophylla (9)*, as well as for some other Croatian endemic species *(19, 20)* and is often considered a general feature of *in vitro* cultures propa-

gated from axillary shoots. IAA and IBA were both good root-inducing auxins for *C. velebitica*; unlike in *F. triquetra (19), C. ragusina (20)* and *Centaurea rupestris (26)* where IBA proved to be the best root-inducing auxin. Concentrations of IAA and IBA similar to the ones tested in this experiment were successfully used in culture of *Alyssum montanum* subsp. *pluscanescens*, another Croatian endemic plant species *(22)* and reported for other plants as well (27, 28, 29). On the other hand, these results differ greatly from low rooting percentages of endemic *Degenia velebitica (21)* that grows in hydro-edaphic conditions alike those of *C. velebitica*.

Formation of callus which was occasionally observed on multiplication as well as on rooting media is a phenomenon usually found during root induction on NAA-supplemented medium *(26)*. Flowering was observed on most rooting media but the highest flowering rate occurred on the medium with 4.9 μM IBA. Flowering *in vitro* is not a common phenomenon and occurs spontaneously or deliberately with variable cytokinin-auxin combinations, as reported for many plant species, e.g. *Kalanchoe blossfeldiana (30), Ceropega bulbosa* var. *bulbosa* (28), *Melia azedarach (27)* etc. The presence of auxins alone for flower induction and development has been observed in *Torenia (31)* and *Vigna radiata (32)*. Even though flowers showed distinctive *C. velebitica* morphology, they were smaller in comparison to normal *in vivo* ones. Changes in flower morphology due to *in vitro* culture observed in this experiment have been described for a number of different plants *(33, 34, 35)*.

All investigated antioxidative enzymes tended to have higher activities in shoots grown on the media with BA, with the highest activity observed on the media with lower concentrations of BA (0.2 and 0.5 μM). However, the effect was significant for ascorbate and guaiacol peroxidise activity and not catalase. Stimulative effect of cytokinin BA on peroxidase activity has already been noticed in explants of *Cymbidium ensifolium (36)* as well as in *in vitro* culture of *Rosa hybrida (6)*. Auxins, on the other hand, did not have significant effect on enzyme activities. Statistically significant increase in peroxidase activity was not observed in the shoots grown on rooting media. This was somewhat unexpected because it has been shown that peroxidase activity increased during rooting of various plants cultured *in vitro (37, 38)*. The role of auxins in relation to the peroxidase activity in the rooting of plants is well-known *(7)*. It was assumed that peroxidases and oxidases may have an important role in metabolic regulation of auxins and cytokinins *(39)* but more recently it was shown that IAA levels did not depend on peroxidase levels *(36)*. On the other hand, shoots propagated *in vitro* are considered to be under continuous stress conditions *(40)*. It has been shown that oxidative stress is induced in *in vitro* culture causing hyperhydricity and dedifferentiation *(41)*. In this experiment, activities of GPOD and APX were stimulated by BA indicating that *C. velebitica* was able to induce antioxidative defense against ROS and overcome possible oxidative stress due to *in vitro* conditions. This was in accordance with the morphology of the plants tested; there was only slight-to-none formation of callus and no hyperhydricity observed. Since increased activity of peroxidase has already been noticed *(6, 36),* we suppose that cytokinins might make enzymes active, thus controlling the level of ROS and oxidative stress.

In conclusion, successful multiplication as well as excellent rooting of *C. velebitica* shoots through micropropagation, without substantial stress response, provides an alternative to classical propagation methods, thus enabling large-scale cultivation and preservation of wild populations of this rare and endangered Croatian endemic plant species.

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