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In vitro propagation of the Balkan endemic species *Stachys leucoglossa* Griseb.

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Abstract

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In vitro shoot culture of the Balkan endemic species *Stachys leucoglossa* Griseb. was initiated by successful sterilization of seeds with 70% ethanol. Regenerated plants on hormone free MS medium had low growth index, shortened internodes and did not form a root system. Then the effect of different concentrations of cytokinine 6-benzylaminopourine (BA) and auxins – indole-3-butyric acid (IBA) and naphthyl acetic acid (NAA) on the *in vitro* multiplication of *S. leucoglossa* was studied. MS medium supplemented with 0.5 mg/L BA was most effective for shoot development and approximately 75% of the explants showed shoot proliferation and produced 5.61±1.15 shoots per explant. For stimulating root development *in vitro* cultivated *S. leucoglossa* plants were cultivated on WPM medium with different concentrations of IBA and NAA. Root formation was observed in 60% of the explants cultivated on WPM media supplemented with 0.5 mg/L IBA and 40% with WPM media supplemented with 0.5 mg/L NAA. A collection of *in vitro* tissue culture was successfully established, which is an alternative approach for preservation of *S. leucoglossa* Griseb.

Keywords: woundwort; *in vitro* multiplication; shoot culture; auxin; cytokinin

Introduction

The genus *Stachys* includes more than 300 species of flowering plants and it is considered one of the largest genera from the *Lamiaceae* family. There are 22 species in Bulgaria, 5 of which are under the protection of Bulgarian biodiversity law. *Stachys leucoglossa* Griseb. (The Plant List) is a Balkan endemic plant which is included in the Red Data Book of Bulgaria with conservation status: endangered. In Bulgaria the populations of *Stachys leucoglossa* are comprised of very few individuals located in areas which are under high anthropogenic impact.

For centuries many species of this genus have been used in ethnomedicine under the form of extracts, decoctions, ointments for treatment of genital tumors, sclerosis of the spleen, inflammatory diseases, cough, ulcers and infected wounds (Skaltsa et al., 2007; Conforti et al., 2009). Externally herbs were applied for the treatment of festering wounds, slashed, rheumatic swellings, and snakebites and internally for abdominal pain, cramps, dizziness, fever, gout and menstrual disorders (Haznagy-Radnai et al., 2012).

Nowadays most of the medicinal plants which are sources of valuable pharmacological properties are threatened with extinction. The Global Strategy for Plant Conservation (IUCN, 2002, updated 2011–2020) is the world's authority on biodiversity and conservation, which aims to prevent extinction of endangered species. The Strategy provides a framework to facilitate cooperation between the existing initiatives aimed at plant conservation, and to promote the development of *ex situ* conservation methods related with *in situ* conservation of rare and vulnerable species (Bunn et al., 2007; Engelmann, 2011). The biotechnological approaches are successfully applied for *ex situ* conservation of rare and valuable plant genotypes. The *in vitro* cultivation (micropropagation) of plants allows their

cultivation in controlled environment on nutrient medium in fully sterile state. This process can be carried out through the whole year regardless of the season and the climatic conditions (Kapchina et al., 2000). The method allows mass multiplication of plants which are sources of valuable secondary metabolites and their subsequent isolation without disturbing the ecological equilibrium and biodiversity.

The present study aims to develop an effective protocol for initiation of *in vitro* shoot culture and examination the effect of cytokinine 6-aminobenzylopourine (BA) and the auxins indole-3-butyric acid (IBA) and naphthyl acetic acid (NAA) on the *in vitro* multiplication of the Balkan endemic species *Stachys leucoglossa* Griseb.

Material and Methods

Stachys leucoglossa seeds were collected from its natural habitat – Pobiti kamani protected area, near the city of Varna with the permission of the Ministry of Environment and Water of Bulgaria. *In vitro* shoot culture was initiated by sterilization of dried ripe seeds with 70% ethanol (v/v) for 5 min, followed by washing with 96% ethanol (v/v) for 10 s. Under aseptic conditions, sterilized seeds were inoculated on half-strength MS medium (Murashige & Skoog, 1962) and on 0.7% water agar (w/v) without and with growth regulators in the following concentrations 250 μM GA (gibberellic acid), 50 μM Kin (kinetin) and 250 μM GA + 50 μM кинетин. After four weeks of germination the sprouting seedlings were transferred on basal MS medium, supplemented with 3% (w/v) sucrose and 0.7 g/L agar. Seedlings were *in vitro* cultivated under controlled environmental conditions (16 h light/8 h dark, 60 μmol/(m^2s) photosynthetic photon flux density, Philips TLD-33, temperature 25°C and 60-70% relative air humidity) and after 30 days explants from regenerated plants were inoculated on MS medium supplemented with different concentrations of BA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L), IBA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L) and NAA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L). Further, after 25 days regenerated plants were transferred on hormone-free basal MS medium and cultivated under controlled environmental conditions for 25 days.

The presented data for all experiments are average values from at least three independent experiments and are compared by standard error of the means (S.E.M).

Results and Discussion

Sterilization of seeds and initiation of *in vitro* culture of *S. leucoglossa*

The population of *S. leucoglossa* has been dramatically reduced from last decades of the 20th century till today due to the strong anthropogenic pressure on which this plant species is exposed. Despite the measures taken by the governmental institutions and recent inclusion of *S. leucoglossa* habitats to protected areas of the European ecological network "NATURA 2000" in Bulgaria, the rapid decrease of its numbers requires application of other approaches in order to ensure *S. leucoglossa* conservation. There are no available data on *ex situ* conservation of *S. leucoglossa* and little is known about its chemical composition. The limited distribution and pharmacological significance of this species in Bulgaria forces the development of alternative approaches for plant growth and conservation. The micropropagation is essential method for *ex situ* conservation of rare and threatened plant species, species with reduced populations and low fertility, and for fast propagation of valuable medicinal plants, as well.

S. leucoglossa shoot culture was induced by sterilization of 120 ripe dry seeds with 70% ethanol and subsequent washing with 96% ethanol. After 30 days, only 13% of 15 seeds cultivated on water agar supplemented with 50μM Kin germinated, while the rest of the seeds (105 seeds) inoculated on other variants nutrition medium (described in Material and methods) did not germinate. The sprouting seedlings were then transferred on basal MS medium, supplemented with 3% (w/v) sucrose and 0.7 g/L agar and cultivated under controlled environmental conditions. After one month of cultivation the regenerated *S. leucoglossa* plants had low growth index, shortened internodes and did not form root system (Figure 1C). Contrary to our results, Panayotova et al. (2008) reported successful initiation of *in vitro* culture of *Stachys maritime*, which has vigorous growth on basal MS medium.

Influence of growth regulators on in vitro multiplication of *S. leucoglossa* Griseb.

Mono-nodal segments of regenerated *S. leucoglossa* plants were inoculated on MS medium supplemented with different concentrations of BA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L) in order to study the effect of the cytokinin on the multiplication *in vitro*. Within 25 days, on all tested media, shoots developed directly from explants. Media supplemented with 0.5 mg/L BA were more effective in promoting shoot development than those supplemented with other concentrations of BA, and approximately 75% of the explants showed shoot proliferation and produced 5.61±1.15 shoots per explant (Table 1, Figure 2D). All concentrations of BA suppressed root formation and stimulated callus formation at the base of shoot tips.

MS media supplemented with different concentrations of IBA did not show any effect on root formation. There-

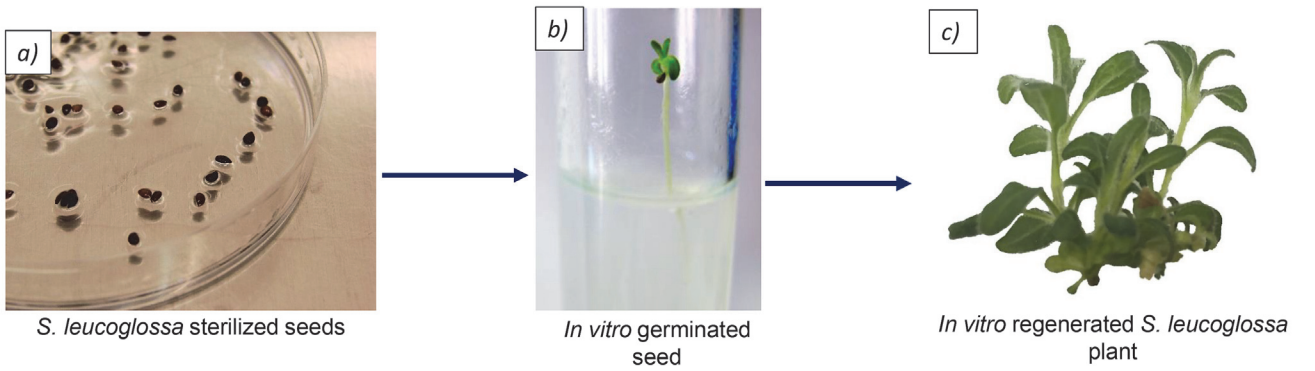

Fig. 1. Initiation of *in vitro* culture of *S. leucoglossa*

Table 1. Influence of different concentrations of BA on length and number of shoots, root formation, and callogenesis of *in vitro* propagated *S. leucoglossa* Griseb.

Variants	Length of shoots, cm	Number of shoots	Root formation	Degree of callus formation
Control MS medium	1.15 ± 0.39	2.64 ± 1.14	–	–
MS+BA 0.1 mg/L	1.59 ± 0.60	3.21 ± 1.45	–	1
MS+BA 0.5 mg/L	1.95 ± 0.43	5.61 ± 1.15	–	1
MS+BA 1.0 mg/L	1.28 ± 0.54	2.61 ± 1.14	–	2

[1]Week callus formation; [2]Significant callus formation

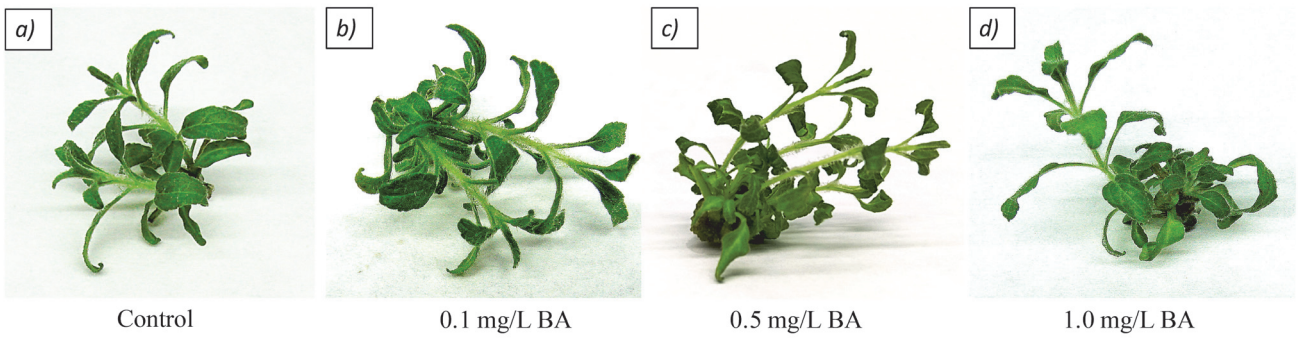

Fig. 2. *In vitro* propagated *S. leucoglossa* plants. *a*) Control plant, *in vitro* cultivated on MS medium; *b*) *In vitro* cultivated plant on MS medium supplemented with 0.1 mg/L BA; *c*) *In vitro* cultivated plant on MS medium supplemented with 0.5 mg/L BA; *d*) *In vitro* cultivated plant on MS medium supplemented with 1.0 mg/L BA

fore, mono-nodal segments of regenerated *S. leucoglossa* plants were inoculated on WPM (McCown Woody plant medium) with different concentrations of IBA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L) and NAA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L). All tested concentations of IBA stimulated root formation and suppressed shoot development (Table 2, Figure 3). IBA in concentration 0.5 mg/L induced development of roots in 60% of the explants. Higher concentrations of IBA (1.0 mg/L) also stimulated root formation but only in 36% of the explants. The cultivation of explants on WPM medium supplemented with NAA also enhanced root formation (Table 3, Figure 4). NAA in concentrations of 0.5 mg/L was most effective in root development and approximately 40% of the explants managed to form roots. Successful induction of *in vitro* cultures of *S. sieboldii and S. ocymastrum* was achieved by Legkobit et al. (2004) on B5 medium supple-

Table 2. Influence of different concentrations of IBA on length and number of shoots, root formation and callogenesis of *in vitro* propagated *S. leucoglossa* Giseb.

Variants	Length of shoots, cm	Number of shoots	Root formation	Length of roots (cm)	Degree of callus formation
Control WPM medium	1.82 ± 0.35	1.51 ± 0.14	–	–	–
WPM + IBA 0.1 mg/L	1.92 ± 0.23	1.63 ± 0.19	+	1.10 ± 0.14	–
WPM + IBA 0.5 mg/L	1.61 ± 0.35	1.56 ± 0.09	+	1.56 ± 0.27	+[1]
WPM + IBA 1.0 mg/L	1.50 ± 0.31	1.40 ± 0.10	+	2.03 ± 0.30	+[2]

[1]Week callus formation; [2]Significant callus formatio

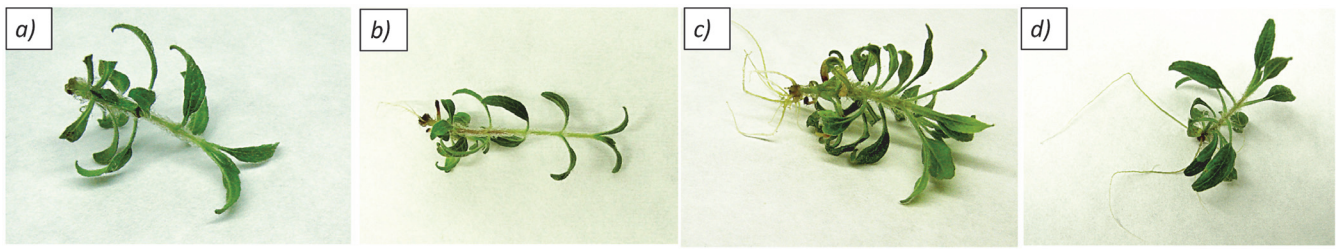

Fig. 3. *In vitro* propagated *S. leucoglossa* plants. *a*) Control plant, *in vitro* cultivated on WPM medium; *b*) *In vitro* cultivated plant on WPM medium supplemented with 0.1 mg/L IBA; *c*) *In vitro* cultivated plant on WPM medium supplemented with 0.5 mg/L IBA; *d*) *In vitro* cultivated plant on WPM medium supplemented with 1.0 mg/L IBA

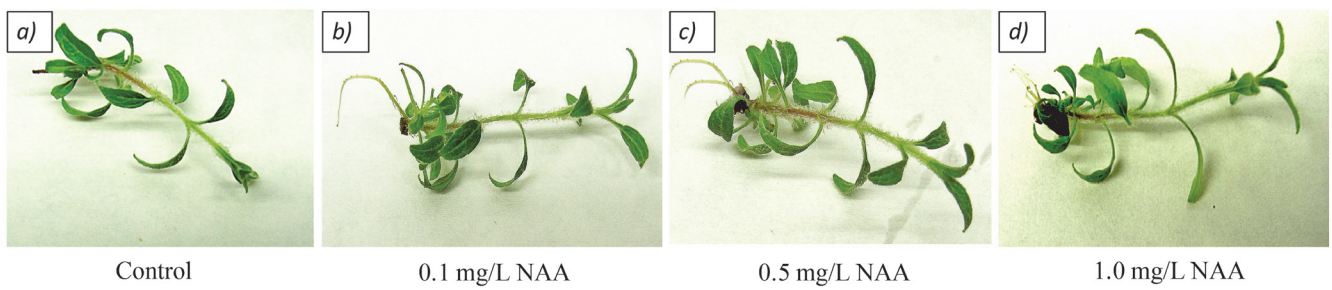

Fig. 4. *In vitro* propagated *S. leucoglossa* plants. *a*) Control plant, *in vitro* cultivated on WPM medium; *b*) *In vitro* cultivated plant on WPM medium supplemented with 0.1 mg/L NAA; *c*) *In vitro* cultivated plant on WPM medium supplemented with 0.5 mg/L NAA; *d*) *In vitro* cultivated plant on WPM medium supplemented with 1.0 mg/L NAA

mented with different concentration of BA and NAA. They also established that high phytohormone concentrations (over 10 mg/L) demonstrated qualitative and quantitative changes suggesting the appearance of somaclonal variants even during plant regeneration directly from nodal segments, bypassing callus formation.

Generally, the optimized protocol for *in vitro* multiplication of *S. leucoglossa* comprises germination of sterilized ripe dry seeds on water agar supplemented with 50 μM Kin and cultivation of sprouting seedlings on hormone free MS medium for 30 days. For induction of shoot proliferation the procedure involves inoculation of mono-nodal segments on MS medium supplemented with 0.5 mg/L BA for a period of 25 days and subsequent transferring of regenerated plants on WPM medium supplemented with 0.5 mg/L IBA for root development for 15 days. Then the plants were cultivated on hormon free MS medium for shoot development and accumulation of leaf biomass for a period of 25 days.

Table 3. Influence of different concentrations of IBA on length and number of shoots, root formation and callogenesis of *in vitro* propagated *S. leucoglossa* Giseb.

Variants	Length of shoots (cm)	Number of shoots	Root formation	Length of roots (cm)	Degree of callus formation
Control WPM medium	2.04 ± 0.30	1.51 ± 0.13	–	–	–
WPM + NAA 0.1 mg/L	2.37 ± 0.22	1.55 ± 0.18	+	2.17 ± 0.66	+[1]
WPM + NAA 0.5 mg/L	2.35 ± 0.35	1.33 ± 0.18	+	2.65 ± 0.65	+[1]
WPM + NAA 1.0 mg/L	2.70 ± 0.54	1.33 ± 0.06	+	1.47 ± 0.52	+[2]

[1]Week callus formation; [2]Significant callus formation

Conclusion

Woundworts are used in traditional medicine and represent a source of valuable bioactive molecules. The successful initiation of *in vitro* culture is an alternative biotechnological approch for preservation of *S. leucoglossa* and would allow protection of this species which is with conservation significance for Bulgaria and Europe, further analysis of metabolite profile and selection of lines with high production of valuable secondary metabolites.

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