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IN VITRO PROPAGATION OF THE BALKAN ENDEMIC SPECIES *VERBASCUM ERIOPHORUM* GODR.

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Abstract

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In vitro shoot culture of the Balkan endemic species *Verbascum eriophorum* Godr. was induced by successful sterilization of seeds with 70% ethanol. Regenerated plants on hormone free MS medium had low growth index, shortened internodes and well developed root system. Then the effect of different concentrations of cytokinin BA (6-benzylaminopurine) and auxin IBA (indole-3-butyric acid) on the *in vitro* multiplication of *V. eriophorum* was examined. All tested concentrations of IBA (0.1; 0.5; 1.0 mg/L) stimulated root formation but suppressed shoot development and leaf formation. More effective in promoting shoot development was MS medium supplemented with 0.5 mg/L BA and approximately 98% of explants showed shoot proliferation and produced 14.22±0.2 shoots per explant. Subsequently shoots were transferred on hormone free basal MS medium for root development and accumulation of leaf biomass. A collection from *in vitro* tissue cultures, which is an approach for preservation of *V. eriophorum* has been established.

Key words: mullein, shoot culture, *in vitro* multiplication, auxin, cytokinin

Introduction

The genus *Verbascum* (mullein) (Family: Scrophulariaceae) comprises about 360 species of flowering plant native to Europe and Asia, with the highest diversity in the Mediterranean. *Verbascum eriophorum* Godr. (The Plant List) is a Balkan endemic plant under the protection of the Bulgarian biodiversity law with national conservation status: Rare and is included in the Red Data Book of Bulgaria (1984). The species is distributed in northern Greece, Bulgaria and the former Yugoslavia. In traditional medicine, leaves, flowers and roots of plants of the genus *Verbascum* are widely used for the treatment of various inflammatory and respiratory disorders (incl. asthma and spasmodic cough), eczema, rheumatism, wounds and anal fistula (Alipieva et al., 2014).

Threatened plant species with pharmacological properties are at high risk of being extinct in the near future. 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 *in vitro* cultivation (micropropagation) of plants allows their growing in controlled environment on nutrient medium in closed sterile containers. This process can be carried out through the whole year regardless of the season and the climatic conditions (Kapchina et al., 2000). By varying the content of the nutrient media and environmental parameters it is possible to achieve optimal yield of the biologically active molecules that can then be tested for their pharmacological potential.

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The present study aims to develop a protocol for induction of *in vitro* shoot culture and investigation the influence of cytokinin 6-benzylaminopurine (BA) and auxin indole-3-butyric acid (IBA) on the *in vitro* multiplication of the Balkan endemic species *Verbascum eriophorum* Godr.

Material and Methods

Verbascum eriophorum seeds were collected from Medicinal Plant Garden, Department of Pharmacognosy with Medicinal Plant Unit (Medical University of Lublin, Poland). The plant material originates from Jardin botanique de Nantes, France (accession number 123). *In vitro* shoot culture was initiated by sterilization of *V. eriophorum* seeds with 70% ethanol (v/v) for 5 min, followed by washing with 96% ethanol (v/v) for 10 sec. Under aseptic conditions, sterilized seeds were inoculated on half-strength MS medium (Murashige and Skoog, 1962) and on 0.7% water agar (w/v). After two 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^2.s) 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) and IBA (0.1 mg/L; 0.5 mg/L and 1.0 mg/L). Further, after 20 days regenerated plants were transferred on hormone-free basal MS medium and cultivated under controlled environmental conditions for 45 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 induction of in vitro culture of V. eriophorum

Numerous plant species are vulnerable to extinction in the near future. The number of individuals in such species is continuously decreasing, mainly because of the impact of human activity – small-scale farming, pasture and infrastructure development. Most of threatened plant species have valuable pharmacological properties and are widely applied in the traditional phytotherapy, ethnomedicine, and in the contemporary therapeutic practices as well. The high importance of biological active molecules produced by mullein and the limited access to *V. eriophorum* forces the development of alternative approaches for plant growth and sustainable production of valuable secondary metabolites. 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.

V. eriophorum shoot culture was induced by sterilization of 60 ripe dry seeds with 70% ethanol and subsequent washing with 96% ethanol. After 10 days, 47% of 30 seeds cultivated on ½ MS medium germinated, while these (30 seeds) inoculated on 0.7% water agar showed lower germination rate (33%). 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 *V. eriophorum* plants had low growth index, shortened internodes and well developed root system (Figure 1C).

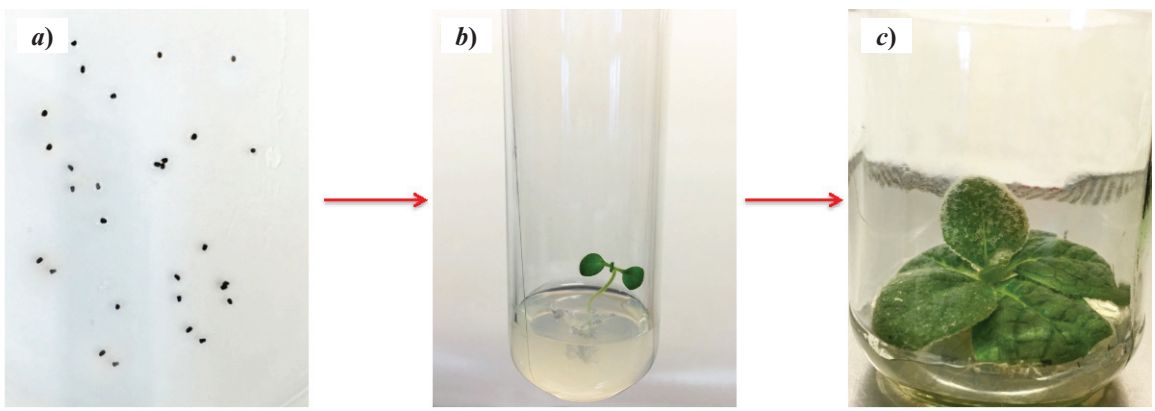

Fig. 1. Induction of *in vitro* culture of *V. eriophorum* Godr.

Table 1
Influence of different concentrations of BA on length and number of shoots, root formation, and callogenesis of *in vitro* propagated *V. eriophorum* Godr.

Variants	Length of shoots (cm)	Number of shoots	Root formation	Length of roots (cm)	Degree of callus formation
Control	1.56 ± 1.4	1.0 ± 0.3	+	7.62 ± 0.73	–
BA 0.1 mg/L	1.34 ± 2.1	6.27 ± 0.5	–	–	+[1]
BA 0.5 mg/L	0.96 ± 1.7	14.22 ± 0.2	–	–	+[1]
BA 1.0 mg/L	0.85 ± 0.8	18.06 ± 0.5	–	–	+[2]

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

Influence of BA and IBA on in vitro multiplication of V. eriophorum Godr.

Mono-nodal segments of regenerated *V. eriophorum* 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 20 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 98% of explants showed shoot proliferation and produced 14.22±0.2 shoots per explant (Table 1, Figure 2C). BA in concentration 1 mg/L significantly simulated the shoot development (18.06 ± 0.5), but the quality of explants was very low because of the strong vitrification and callus formation of regenerated plants (Figure 2D). All concentrations of BA suppressed root formation and stimulated callus formation at the base of shoot tips. The observed decrease in length of shoots at high concentration of BA (1.0 mg/L) might be due to the inhibition of organogenesis and induction of callogenesis (Table 1). The effective response of BA in stimulating shoot formation was reported early for *V. thapsus* (Turker et al., 2001).

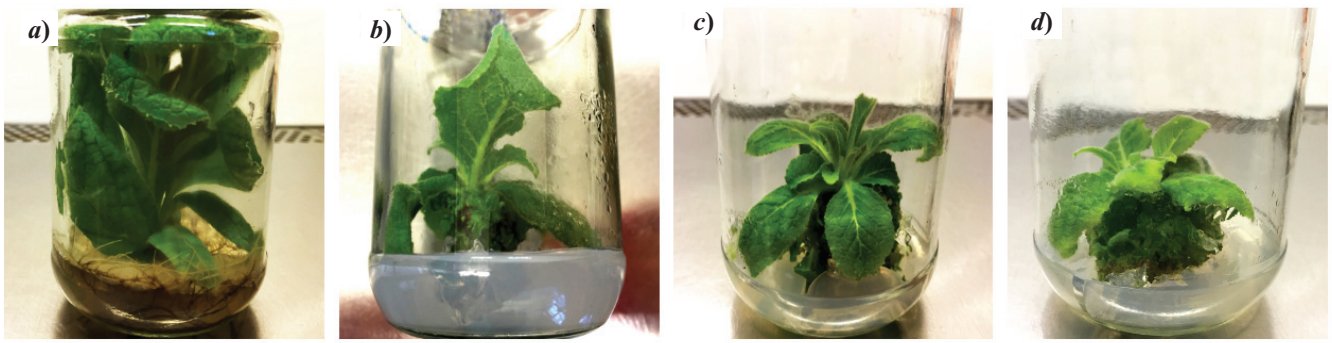

Fig. 2. *In vitro* propagated *V. eriophorum* plants. *a*) Control plant, *in vitro* cultivated on MS medium; *b*) *In vitro* cultivated plant on MS medium supplemented with 0.1 mg/LBA; *c*) *In vitro* cultivated plant on MS medium supplemented with 0.5 mg/LBA; *d*) *In vitro* cultivated plant on MS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

Table 2
Influence of different concentrations of IBA on length and number of shoots, root formation, and callogenesis of *in vitro* propagated *V. eriophorum* Godr.

Variants	Length of shoots (cm)	Number of shoots	Root formation	Length of roots (cm)	Degree of callus formation
Control	1.56 ± 1.4	1	+	7.62 ± 0.73	–
IBA 0.1 mg/L	0.54 ± 0.2	1	+	8.95 ± 0.43	–
IBA 0.5 mg/L	0.45 ± 0.3	1	+	11.80 ± 0.28	–
IBA 1.0 mg/L	0.47 ± 0.2	1	+	10.74 ± 0.35	–

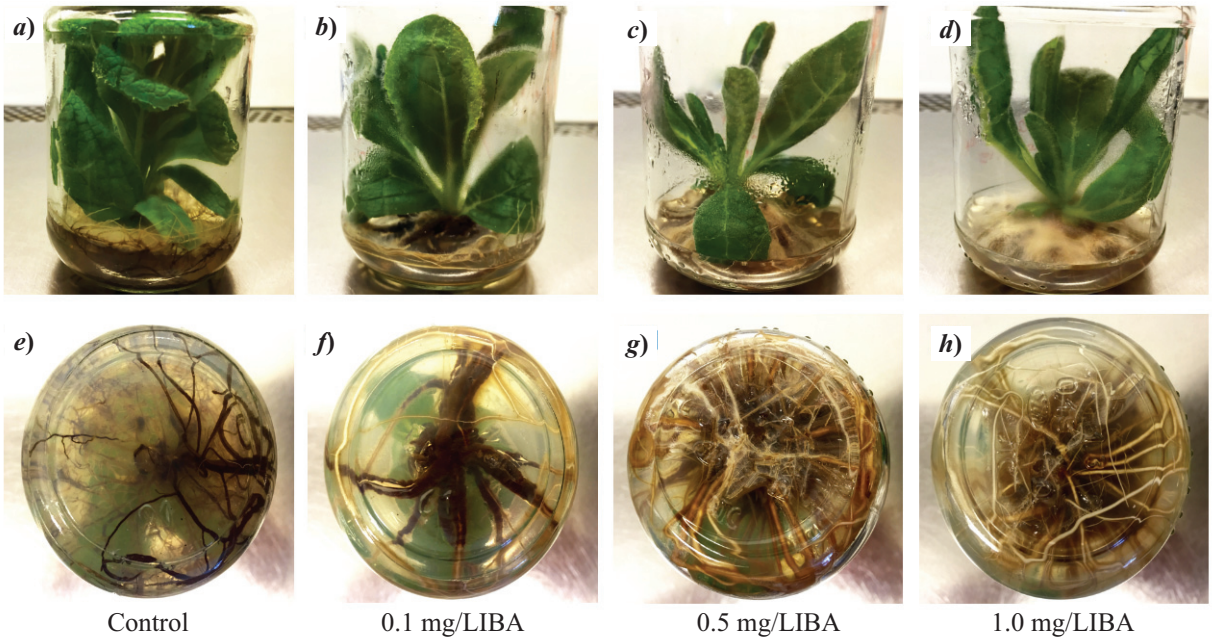

Fig. 3. *In vitro* propagated *V. eriophorum* plants. *a*) and *e*) Control plant, *in vitro* cultivated on MS medium; *b*) and *f*) *In vitro* cultivated plant on MS medium supplemented with 0.1 mg/LIBA; *c*) and g) *In vitro* cultivated plant on MS medium supplemented with 0.5 mg/LIBA. *d*) and *h*) *In vitro* cultivated plant on MS medium supplemented with 1.0 mg/LIBA

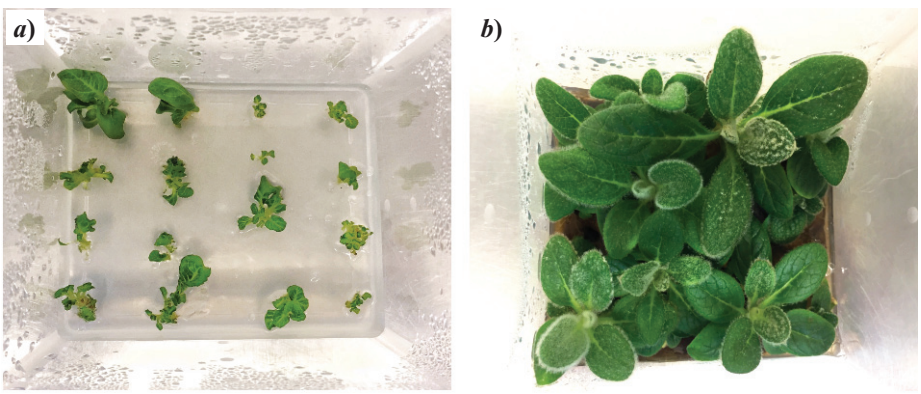

Fig. 4. *In vitro* multiplication of *V. eriophorum* Godr. *a*) Regenerated plants, transferred from MS medium supplemented with 0.5 mg/L BA on MS basal medium without any growth regulators; *b*) Regenerated plants on basal MS medium after 45 days of *in vitro* cultivation

The application of IBA in concentrations 0.1; 0.5; 1.0 mg/L stimulated significantly root formation, and suppressed shoot development and leaf formation (Table 2, Figure 3). IBA in concentration 0.5 mg/L had the strongest effect on root development in *in vitro* regenerated *V. eriophorum* plants. All concentrations of IBA did not induce callogenesis and vitrification. Turker et al. (2001) reported that NAA and 2.4-D stimulated development of root and shoots in *in vitro* cultivated *V. thapsus* plants. Successful rooting of micropropagated plants was achieved on hormone-free basal MS medium and 67% of them formed well-developed root system and plentiful leaf biomass (Fig. 3A).

The optimized protocol for *in vitro* multiplication of

V. eriophorum comprises cultivation of mono-nodal segments on MS medium supplemented with 0.5 mg/L BA for 20 days and subsequent transferring of regenerated plants on hormone free basal MS medium for root development and accumulation of leaf biomass for a period of 45 days (Figure 4).

Conclusions

Mulleins are used in traditional medicine and represent a source of valuable bioactive molecules. The application of biotechnological approach for the establishment of *in vitro* culture from *V. eriophorum* would allow the preservation 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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