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In vitro cultures initiation from seeds of Bulgarian localities of Glycyrrhiza glabra L. (Fabaceae)

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ABSTRACT
Glycyrrhiza glabra is a medicinal plant which is used in both traditional and official medicine for treatment of many diseases including asthma, renal calculus, ulcer, psoriasis, and rheumatism. Spasmolytic, anti-inflammatory, capillary-strengthening, anticancer, and antivirus activities of this perennial plant are due to its roots. Overexploitation of licorice led to decrease of its populations. The species is included in the Red Data Book of Bulgaria with conservation status "endangered" and is protected by the Biodiversity Act;furthermore its localities are in protected area of Natura 2000. In vitro propagation of licorice could be applied in order to establish commercial plantation and to produce roots for the pharmaceutic industry.
In vitro seedlings were obtained from three Bulgarian localities of Glycyrrhiza glabra near Danube River, close to the villages Dolni Vit, Koilovtsi, and Beltsov. Heavy fungal contamination and poor germination were noticed in winter. Seed survival and germination were successfully improved in summer by several consecutive short soaking in boiling and ice water (germination rate varied between 28 % and 78 %). After two-month cultivation on agar-solidified basal MS medium, about 70 seedlings reached height of 10 cm and were sub-cultured. Shoot and root cuttings were put on media containing different plant growth regulators. Best results were noticed for Beltsov locality: 84.6 % seedlings with long vigor roots and propagation coefficient 5.7 ± 1.4 explants per seedling. Most shoot cuttings rooted and developed new leaves while root segments ramified and formed callus..

Key words: Bulgarka Nature Park, algae, distribution, conservation status

Introduction

Glycyrrhiza glabra L. (licorice, or liquorice) is a perennial medicinal plant belonging to the family Fabaceae. Its healing properties are due to the roots (Radix Glycyrrhizae, Radix Liquiritiae) containing as main secondary metabolites glycyrrhizin, a triterpenoid saponin, and flavonoids. The name of the species means sweet root (from Greek: glukus sweet, and rhiza root) because glycyrrhizin is 30 to 50 times sweeter than sugar. Licorice has straight branched stem reaching 1 m height, and thick rhizome with long creeping suckers, up to 2 m. In nature, plant propagation occurs by seeds as well as vegetatively. Three-year or more old plants are used in both traditional and official medicine for treatment of many diseases including asthma, renal calculus, ulcer, psoriasis, and rheumatism. Spasmolytic, anti-inflammatory, capillary-strengthening, anticancer, antioxidant, antiradical, and antivirus activities of G. glabra are due to its roots. Different herb-tea recipes include roots of licorice cut into very small pieces, and many medical preparations are produced on the base of root extracts or root powder. There are many data in the literature concerning the active substances of licorice, its traditional use, pharmacological effects, clinical tests of the preparations, and toxicity levels of glycyrrhizin (Saxena, 2005; Anil & Jyotsna, 2012).

G. glabra is distributed in South and East Europe and Southeastern Asia and was naturalized in many places in Southeastern Europe. Overexploitation of licorice led to decrease of its populations. In Bulgaria, the species is limited to the Danube Plain, occurring along the Danube River and some of its feeders. G. glabra is included in the Red Data Book of Bulgaria (Evstatieva, 2015) with conservation status "endangered" and is protected by the Biodiversity Act (2000); furthermore its localities are in protected areas, some of them in areas of Natura 2000. The overall area of G. glabra decreased significantly between 1957 and 1996, from 66 dka to 9 dka (Genova & Valchev, 2005). In the last decade some of the species localities were no more detected. Besides in situ protection, additional measures are needed to prevent further decrease of the Bulgarian population: study of the reproductive biology of the species, ex situ seeds conservation, and field cultivation.

In vitro propagation of licorice could be applied in order to produce numerous plants in a short time which are needed for establishment of commercial plantation. The first trials in this direction in Bulgaria were carried out using seeds and nodal stem explants (Robeva-Davidova et al., 1992). Authors noticed intensive callogenesis and tissue necrosis as well as low seed germination. Phytochemical analyses of roots originating from five Bulgarian localities characterized with different glycyrrhizin and flavonoid content (Genova et al., 1998). Plants from Beltsov locality were found to be the most rich in these secondary metabolites, hence most perspective as initial material for in vitro cultivation. Authors recommended the use of plant biotechnologies as a reliable method for biodiversity conservation. Currently, a limited number of in vitro plants of G. glabra are maintained in the National gene bank in Sadovo; however, there is no effective technology for rapid in vitro propagation of the species (Krasteva et al., 2012). Investigations on in vitro cultivation concerning mainly the influence of the medium composition on the effectiveness of licorice propagation are in progress in many countries (Kukreja, 1998; Sharma et al., 2008; Sawaengsak et al., 2011) but there is still need of further optimization of the processes (Mousa et al., 2006).

The aim of the present study was to initiate in vitro cultures using seeds from three Bulgarian localities of Glycyrrhiza glabra, as a first step toward in vitro multiplication of plants from the local population of the species.

Materials and Methods

Seeds of G. glabra were gathered in December 2015 from three Bulgarian localities of the species sited near Danube River, close to the villages Dolni Vit, Koilovtsi (Pleven district), and Beltsov (Russe district), all of them in protected areas. The number of collected seeds was limited with respect to the permit received from the Ministry of Environment and Water in accordance with the Biodiversity Act (2000).

First trial on in vitro seed germination was performed with 50 seeds from Dolni Vit locality after 2-month storage in a cool room at 10 ± 5°C (control set). Seeds were disinfected by consecutive treating with 70 % ethanol for a minute and commercial bleach (chlorine < 5 %) for 10 min, followed by thrice rinse with distilled sterile water. To stimulate germination, stratification was applied to another set of 50 seeds gathered from the same locality, by 10-fold consecutive 5-second seed soaking in boiling and ice water, prior to their sterilization. Seeds were put on basal MS medium (Murashige & Scoog, 1962) solidified with 6,5 g/l Plant agar (Duchefa, NL), and supplemented with 30 g/l sucrose, 25 seeds per plastic container with grids (Duchefa, NL) (Fig. 1-A). Following the same procedure, two treatments, 75 seeds from each locality (3 repetitions of 25 seeds each) were carried out consecutively in summer 2016 (July and August) to test seed germination. In vitro obtained seedlings were cut to shoot and root segments during the first sub-cultivation. Shoot nodal segments with one leave each were put on three media in parallel: medium K1 (supplemented with 1 mg/l Kin), medium K1I5 (with 1 mg/l Kin and 0,5 mg/l IBA), and medium K1N5 (with 1 mg/l Kin and 0,5 mg/l NAA) while root segments were put on medium I1 (supplemented with 1 mg/l IBA). All cultures were grown in a culture room at temperature of 23 ± 2°C and 16 h/8 h light/dark regime daily, with light intensity of 2000 lx. Propagation coefficient of the first sub-cultivation was calculated as average number of shoot explants per seedling (means ± SD).

In addition, two sets of non-disinfected seeds from Beltsov locality, 15 seeds each, were tested for in vivo germination in petri dishes on wet filter paper, after the same stimulation with boiling and ice water and under the same ambient conditions; seedlings were potted in soil mixture.

Results

Heavy fungal contamination was noticed for the control set of 50 non-stimulated seeds, the rate of survival was 27,0 ± 0,1 % and that of germination – 12 ± 0,5 %; however, seedlings showed abnormal features and were unable to develop (Fig. 1-B). The consecutive repeated treating with boiling and ice water led to successful germination of several seeds which formed well shaped seedlings possessing leaves with typical laminas for the species (Fig. 1-C). Emergence of mauve exudates in the medium indicated the beginning of seed germination. The process was not simultaneous, about one third of all seeds developed hypocotyl during the first week while the overall germination period of the culture prolonged six weeks. Nevertheless the seed survival rate remained poor: 22 % due mainly to the microbial contamination. Additional seed storage for half year at room temperature resulted in both decrease of the fungal contamination and stimulation of seed germination. Sterilization efficiency was high although there were differences related to the localities, and some fluctuations were noticed between the two treatments (Fig. 2). Germination rate varied between 28 % and 78 % (Fig. 2) After two-month cultivation on agar-solidified basal MS medium, about 70 seedlings reached height of 10 cm and formed 10-12 leaves. Some individuals ramified and the number of their leaves increased up to 18; roots also differed by their growth and ramifications, the biggest ones reaching over 15 cm length (Fig. 3).

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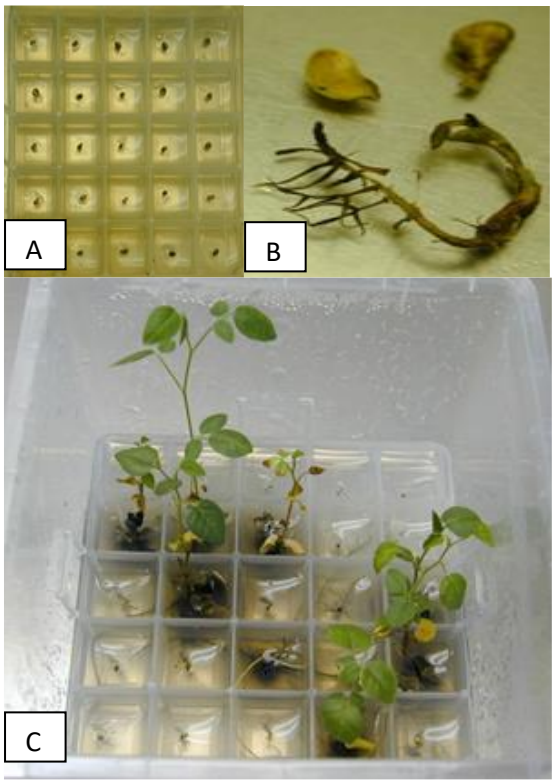

Fig. 1. Seed germination: A) Set of 25 seeds in a plastic container with grid; B) Abnormal seedling in necrosis; C) Container with well-shaped in vitro seedlings at different stage of development

Only the big seedlings were sub-cultured, the other were simply transferred to fresh MS medium. Best results were noticed for Beltsov locality, with 84.6 % seedlings with vigor roots, and propagation coefficient 5.7 ± 1.4 explants per seedling (Table 1). As a rule, seedlings with well-developed roots formed more nodes and leaves and yielded higher number of new explants. Thus, most of the seedlings originating from Beltsov locality formed 6 or more nodes, while among those originating from Koilovtsi and Dolni Vit localities smaller individuals with 2 to 4 nodes predominated over the bigger ones (Fig. 4).

Table 1. *Propagation coefficient (PC) of the first in vitro sub-cultivation and efficiency of vigor root formation, on medium MS free of plant growth regulators*

Locality	Number of sub-cultured seedlings	Seedlings with vigor roots [%]	PC
Dolni Vit	15	53,3	4,5 ± 2,5
Koilovtsi	26	65,4	4,6 ± 1,9
Beltsov	26	84,6	5,7 ± 1,4

Shoot and root cuttings were transferred to media containing different plant growth regulators. All shoot segments elongated and developed numerous new green leaflets while the initial leaves etiolated and died (Fig. 5-A). Most shoots formed callus below their base regardless medium composition. Root thickened and formed both new thin rootlets and pale yellowish callus (Fig. 5-B). Shoot-cultures' development was influenced by the presence of the plant growth regulators in the media. Thus, shoots growing on medium K1, containing only Kin, didn't root at all, and many of them raised one or two new shoots with well-shaped but small green leaflets (Fig. 5-C). On the other hand, older leaflets and stem bases turned brown. Shoots sub-cultured on medium K1I5 didn't produce new shoots but remained green and had normal leaflets; moreover, some of them formed vigor white roots (Fig. 6-A & B). Shoots growing on medium K1N5 seemed less viable whit their elongated thin stem covered with tiny leaflets, and larger calli below them (Fig. 6-C & D). Formation of short brownish roots was rare, along with callogenesis. Some shoots showed hipperhydricity, turned colorless, and died.

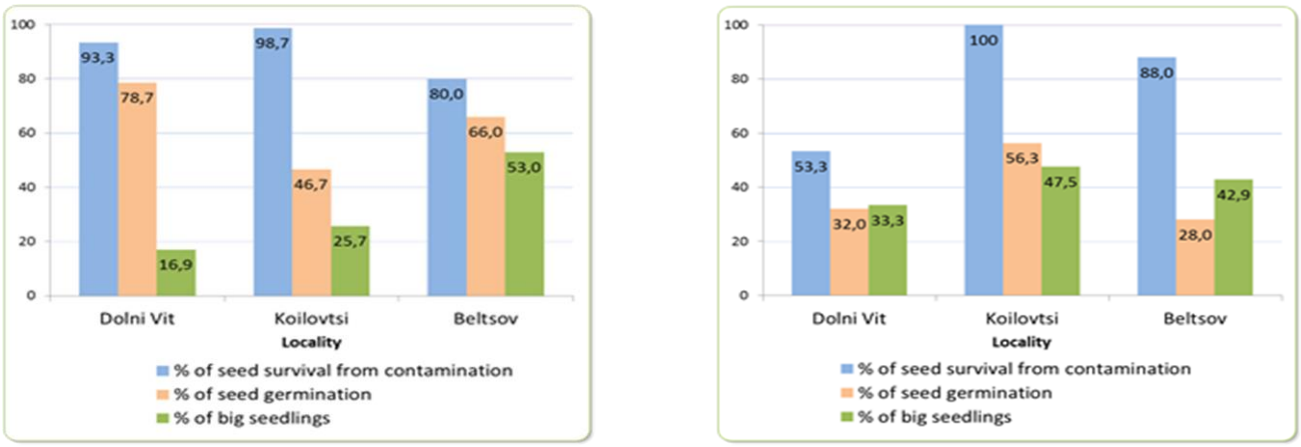

Figure 2. *Rates of seed sterilization efficiency, seed germination, and relative number of big seedlings for the three localities (Left: 1st treatment started on 29.07.2016; Right: 2nd treatment started on 08.08.2016)*

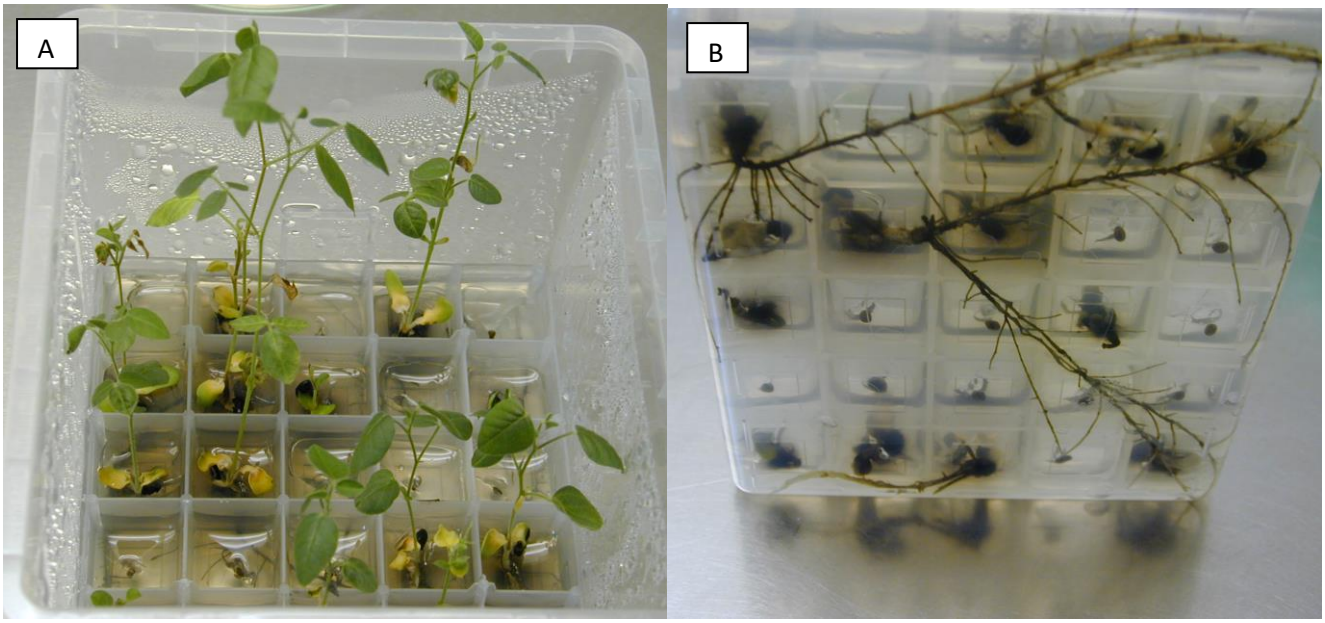

Figure 3. *Well-developed seedlings on MS medium at 6-week age: A) Upper part; B) Roots*

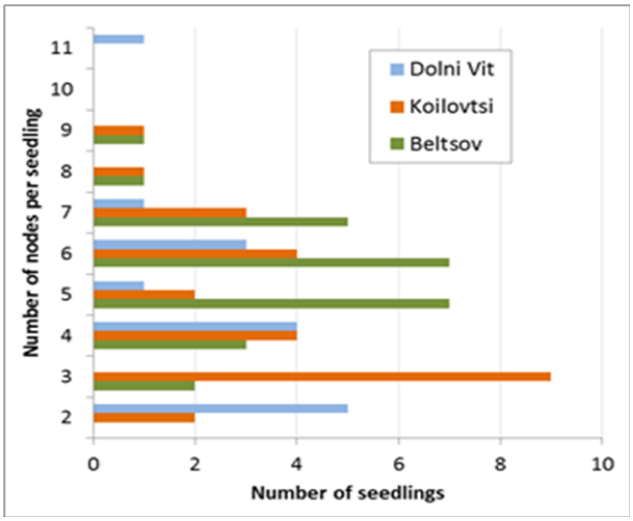

Figure 4. *Distribution of the seedlings with different origin according to the number of nodes formed in vitro for 6-week cultivation on MS medium*

Seed germination in vivo in petri dishes was weak: 13,3 % even if only 6,7 % dropped out due to fungal contamination. One from the four seedlings which were transferred to pots with soil mixture strengthened and continued its growth in the room phytotron.

Discussion

The heavy microbial contamination is usual for seeds and other plant material gathered directly from the wild. The long seed storage first in a cool room and then at normal room temperature, in combination with the stress caused by the consecutive soaking in boiling and ice water prior to sterilization, could explain the significant improvement of the disinfection efficiency in summer compared to the first trials in winter.

Variations in seed germination rate could be due to each of the following: seed dormancy due to the hard seed coat, low seed viability, and relatively small number of seeds in each treatment. The number of seeds gathered from the wild localities was in accordance with the protection measures foreseen in the Biodiversity Act of Bulgaria concerning species with conservation status “endangered”. Seed viability will be further tested. It is worth to mention that spring is the sowing-time for licorice. In December the pods with seeds were still attached to the plants; it is possible that they needed some more time to reach maturity. Problematic seed germination was previously reported for another Bulgarian locality of G. glabra situated near the town of Nikopol: on the best of three different basal media 42,2 % of the seeds formed epicotyl, but only 20 % developed cotyledons, and finally 7,8 % successfully grew into plants (Robeva-Davidova et al., 1992). Results were even poorer when plant growth regulators were added to the medium. Mousa et al. (2006) compared the effectiveness of G. glabra in vitro propagation starting from commercial hybrid seeds of exotic licorice and from shoots of one greenhouse plant. Authors concluded that seeds were very expensive, variable due to the cross pollination, and with decreasing viability in a short time; however, starting from seeds gathered from the natural localities has the advantage to preserve species biodiversity and to obtain plants appropriate for the local climate.

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Figure 5. *First in vitro sub-cultivation on media containing cytokinin or auxin alone: A) Shoot forming leaves and callus on medium K1; B) Roots sub-cultured on medium I1; C) Shoot multiplication on medium K1.*

Weak flowering and low seed viability had been indicated as the main obstacle for licorice cultivation in India, that's why the efforts were put on clonal shoot multiplication via nodal segments (Kukreja, 1998). Author reported best shoot sprouting on MS medium supplemented with 0,5 mg/l BAP and 1 mg/l IAA, giving about 6-8 shoots per initial explant, each with 2-4 nodes. In our preliminary tests BAP was found to cause tissue browning and necrosis when applied in low concentrations (0,2 to 0,5 mg/l) alone or in combination with IBA or NAA. To our experience Kin was much more appropriate cytokinin compared to BAP.

Comparison of the two media differing only by the auxin: K1I5 and K1N5 confirmed the rooting power of IBA. The presence of NAA, in contrast, hampered root development. Kukreja (1998) also mentioned the inhibitory effect of NAA concerning in vitro rooting of licorice. The application of this auxin was related to callus formation under the shoot base, up to 20 mm diameter (Sharma et al., 2008). Despite this observation authors recommended medium containing 1 mg/l BAP and 0,5 mg/l NAA as most suitable for shoot sprouting and the cheapest one. Furthermore, they obtained indirect organogenesis after sub-culturing of the callus on the same

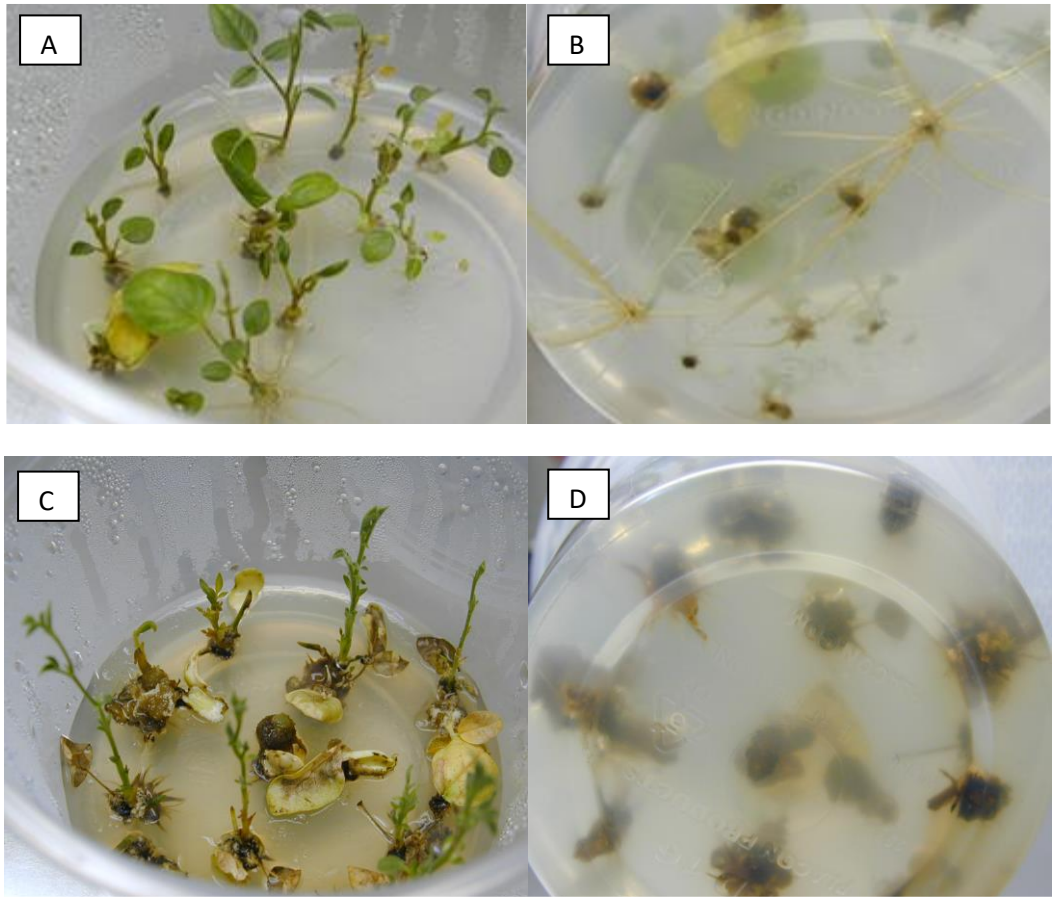

Figure 6. *First in vitro sub-cultivation on media containing combinations of cytokinin and auxin: A) Shoots on medium K1I5; B) Roots on medium K1I5 (view from the bottom of the container); C) Shoots on medium K1N5; D) Roots on medium K1N5 (view from the bottom of the vessel).*

medium. Other researchers reported 2,4-D as the most appropriate auxin for callus induction, especially in combination with BAP (0,5 : 2,0 mg/l) in order to obtain embryogenic callus (Fu et al., 2010). To produce somatic embryos they transferred calli to medium containing combination of BAP, kinetin, zeatin, and IBA.

Both auxins IAA and IBA are known as effective plant growth regulators for in vitro rooting of different plant species. According to Sharma et al. (2008) addition of 1 mg/l IAA alone to MS medium led to abundant root induction and high survival rate of the ex vitro adapted plants. Sawaengsak et al. (2011) noticed most effective root formation when IAA or IBA were added to B5 medium in a high concentration of 5 mg/l. In our study roots on IBA-containing K1I5 medium were vigor but occurred with less frequency, obviously because of the presence of the other plant growth regulator; still, kinetin limited callus formation. Interestingly, root culture growing on medium I1 containing 1 mg/l IBA as sole plant growth regulator, formed callus along with new roots. It will worth to test root growth on media supplemented with different concentrations of Kin and IBA. Moreover, medium containing 5 mg/l IBA was chosen as most suitable for glycyrrhizin production (Sawaengsak et al., 2011).

In conclusion, the data published by different authors concerning in vitro propagation of licorice is often contradictory in regard to the best basal medium as well as to the suitability of the plant growth regulators, their concentrations and combinations in the media. In our first study, seed dormancy in G. glabra due to the impermeable hard seed coat was successfully overcome by pretreatment with boiling and ice water, and germination rate was highly improved. As a result, in vitro cultures were initiated from three Bulgarian localities of the species. In vitro plants of best quality were regenerated from seedling shoots on medium K1I5 containing 1 mg/l Kin and 0,5 mg/l IBA. Further optimization of medium composition will be done aiming at additional shoot sprouting.

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