Editura AcademicPres

Available online: www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Horti Agrobo, 2018, 46(1):52-57. DOI:10.15835/nbha46110810

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Original Article

Micropropagation of *Inula germanica* L. from the Seedlings Explants

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Abstract

This is the first communication of micropropagation system for *Inula germanica* using seedling explants germinated *in vitro*. The development of this system gives the possibility of future reintroduction of *I. germanica* providing a way to stabilize or re-establish its population. Shoot tips and fragments of cotyledons, hypocotyls and roots were isolated from ten-day-old seedlings. Explants were put on MS medium containing 1.0 mg l^{-1} benzylaminopurine and 0.1 mg l^{-1} naphthaleneacetic acid and cultured under continuous white fluorescent light (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 26 ± 1 °C. The highest percentage of shoot organogenesis (83.3%) was recorded for hypocotyl, while the highest average number of shoots per explant (12.0) was recorded for shoot tips. In subsequent subcultures, multiplication rate decreased to 3.0-4.9 shoots per explant. Less than 19% shoots were able to root on the solid medium without auxins. The highest rooting efficiency (69.3%) was recorded for solid medium supplemented with indolebutyric acid, but growth of roots was inhibited. The percentage of rooted shoots (62.2%) and number of roots per shoot (2.4 per shoot) into the liquid medium were comparable to medium with 0.1 $mg \cdot l^{-1}$ indolebutyric acid. showing a positive impact on the process of acclimatization. The regenerated plants were able to flowering in the first year after acclimatization. Developed micropropagation system for *I. germanica* is efficient and can be a useful tool for the active protection of this species.

Keywords: *Asteraceae*, endangered species, solid and liquid medium, shoot multiplication, rooting *in vitro*

Introduction

Inula germanica is extremely endangered species and listed as critically endangered (CR) in Polish red lists (Mirek and Zarzycki, 2006). *I. germanica* has a wide natural distribution from the southern Ural Mountains through the southern part of Russia, the Caucasus, in southern and central Ukraine, the Balkan Peninsula, the Pannonian plain to eastern Austria and central Germany (Rutkowski and Paszek, 2000). In Poland, there is only one single site Bielinek on the Oder River (Kaźmierczakowa *et al*., 2014). However, natural succession occurs in this area (growth of shrubs and trees) and limits the size of the population of *I. germanica*, which is manifested by small flowering shoots, moreover, in some years, individuals do not bloom. Therefore, developing an effective system of regeneration would be a useful tool to active protection of this species. The technique of *in vitro* culture supports conservation of plant genetic resources without depleting their natural position, because it requires small fragments of plants or only a few seeds to initiate the regeneration process (Rybczyński and Mikuła, 2006). Additionally, *I. germanica* produces germanins (germacranolides, sesquiterpene lactones), that show a wide spectrum of biological activity, including anti-cancer properties (Liszewska, 2011) and plants micropropagated using *in vitro* techniques could be the source of these substances.

There are many research reports upon the tissue culture of *Asteraceae*. Most of these studies focused on obtaining an efficient system for *in vitro* propagation of crop or medical importance plants, e.g. *Echinacea purpurea* (Zobayed and Saxena, 2003) or *Carthamus tinctorius* (Kumar and Kumari, 2011) *via* somatic embryogenesis, while regeneration by organogenesis has been developed for *Heliantus annus* (Ozyigit *et al.*, 2007), *Launaea sarmentosa* (Mahesh *et al.*, 2012), and many others (Amin *et al.,* 2013). Moreover, efficient and rapid clonal regeneration method has been shown to be a great tool in the protection of rare and threatened species of the *Asteraceae* family e.g. *Centaurea ultreiae* (Mallón *et al.,* 2011), *Carlina onopordipholia* (Trejgell *et al.*, 2011), *Arnica montana* (Surmacz-Magdziak and Sugier, 2012), or *Taraxacum pieninicum* (Trejgell *et al.,* 2013) and others (Amin *et al.*, 2013).

The species of *Inula* genus, *I. viscose* (Romano, 1997), *I. royleana* (Stojakowska and Malarz, 2004), *I. verbascifolia*

Received: 31 Mar 2017. Received in revised form: 31 Apr 2017. Accepted: 06 June 2017. Published online: 19 Sep 2017.

(Perica *et al*., 2008), *I. japonica* (Bian *et al*., 2008) and *I. racemosa* (Kaur *et al*., 2010) have been successfully micropropagated. However, previous papers on *I. germanica* only describe the morphological, phytoecological and ecological studies (Rutkowski and Paszek, 2000). To our knowledge this is the first protocol for *in vitro* propagation of this species *via* shoot multiplication. Developing an effective system of regeneration would be a useful tool for conservation of this species and others members of the *Asteraceae* family.

Materials and Methods

Plant material

The seeds were picked from the collection of the Botanical Garden of UMCS in Lublin (Poland). They were sterilized with 70% (v/v) ethanol for 30 s and then 20 % (v/v) commercial bleach with sodium hypochloride (Domestos®) for 20 min. Then they were washed 4 times using sterile distilled water and transferred onto Petri dishes (6 cm in diameter) containing 10 ml of MS medium including vitamins (Duchefa) (Murashige and Skoog, 1962) supplemented with 3.0% sucrose, 1 mg l^{-1} gibberellic acid (GA3) and gelling with 0.7% agar (Sigma-Aldrich, Germany). The pH was adjusted to 5.8 and the medium was autoclaved for 20 min at 121 °C. The seeds were cultivated under continuous white fluorescent light (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 26 ± 1 °C in growth chambers.

Establishment of the culture and multiplication

Shoot tips cut under the cotyledonary node (2-3 mm in length), fragments of cotyledons (2-3 mm in length), hypocotyls (1 mm in length, cut out under the node), and roots (5 mm in length) were isolated from a ten-day-old seedlings and were used as initial explants. The explants (4 per flask) were cultivated in 100 ml Erlenmeyer flasks loaded with 30 ml of MS medium containing 1.0 mg l^{-1} benzylaminopurine (BAP) and 0.1 mg l^{-1} naphthaleneacetic acid (NAA), 3.0% sucrose, solidified with 0.7% agar and pH adjusted to 5.8 before autoclaving. They were maintained for 4 weeks under conditions described above. In our earlier studies on the regeneration of species of the *Asteraceae* family, the medium composition and the environmental parameters were optimized (Trejgell and Tretyn, 2010). The shoots were subcultured 4 times at 4 weeks intervals. After this period, the multiplication rate and percentage of explants producing shoot were analyzed.

Rooting and acclimatization

The shoots (1 cm and longer) obtained in the 3th subculture were excised and rooted into 50 ml tubes containing 10 ml solid or liquid MS medium solidified with 0.7% agar and supplemented with 0.1 mg·l^{-1} indole-3-butyric acid (IBA). Solid medium without IBA was used as a control. Furthermore, the liquid MS medium without auxins was tested, adding perlite (1.5 g) in order to maintain the shoot in a vertical position. The shoots were maintained for 4 weeks under conditions described above.The plantlets obtained after 4 weeks of culture were removed from the *in vitro* cultures, and then percentage of rooting, number of roots and length of roots were analyzed. The plantlets from solid medium were gently washed in sterile water and transferred into plastic pots filled with a sterile mixture of vermiculite and sand (1:1). The plantlets from liquid medium were directly transferred to pots filled with the same substrate. After 4 weeks of hardening, the microcuttings were transferred to pots containing soil and acclimatized for the next 4 weeks in a greenhouse. After 8 weeks of plantlets acclimatization, their survival percentage was recorded and they were transferred to the field conditions.

Statistical analysis

During multiplication stage, each treatment consisted of 12 explants (for initial material) and at least 16 shoots (for the following subcultures), and during rooting stage, 24 shoots for each treatments, were used. All experiments were carried out in two replicates. Results were statistically analyzed by means of ANOVA and the mean values were evaluated by Kruskal-Wallis test for varied numbers of collections (multiplication stage) or by Tukey test for equal numbers of collections (rooting stage) at $p < 0.05$.

Results and Discussion

Shoot multiplication

In the present studies on *I. germanica*, morphogenetic responses of seedlings explants on MS medium containing 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA were analyzed. The highest percentage of shoot organogenesis (83.3%) was recorded for hypocotyl fragments with average of 6.3 ± 1.1 adventitious shoots per explant (Table 1). The shoot organogenesis frequency for shoot tips and cotyledons was 66.7 % and 50%, respectively. Whereas the average number of shoots per explant was 12.0 ± 1.2 for shoot tips and 10.1 ± 0.8 for cotyledons (Fig. 1A and B) and the differences were statistically significant as compared to the results obtained for the hypocotyl (Table 1). The root explants were not able to produce shoots by organogenesis. Similar results were recorded for *Carlina acaulis* and *C. onopordifolia* (Trejgell *et al*., 2009; Trejgell and Tretyn, 2011). The difference in shoot formation between different types of explants in response to BAP could be related to the levels of endogenous cytokinins (Yucesan *et al*., 2007) or to differences in tissue sensitivity to plant growth regulators (Lisowska and Wysokinska, 2000). In previous studies on different species of the *Asteracae* family, e.g. *Artemisia*, *Carlina*, *Echinacea* and *Saussurea* species and also *Stevia rebaudiana*, *Atractylodes lancea* or *Silphium perfoliatum*, it was found that BAP was the most effective growth regulator for *in vitro* proliferation (Amin *et al*., 2013). BAP was also necessary for the *I. racemosa* shoots regeneration and the highest rate of shoot multiplication was obtained on a medium fortified with 1 mg l^{-1} BAP (Kaur *et al*., 2010). Other authors observed that BAP at the same concentration in combination with NAA was the most effective for *Acmella calva* (Amudha and Shanthi, 2011), *Centaurea arifolia* (Yüzbaşıoğlu *et al*., 2012), *Eclipta alba* (Sharma *et al*., 2013), and *Carthamus tinctorius* (Ghasempour *et al*., 2014). In addition, in our previous study, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA were the most efficient concentration of those growth regulators for multiplication of shoots for *Carlina acaulis*, *Leontopodium*

Table 1. Effects of the source of explants and the number of subcultures on percentage of explants producing shoots, and proliferation rates developing on explants of *Inula germanica* isolated from seedlings after 4 weeks of culture on MS supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA

Type of explants	Initial culture		1st subculture		2nd subculture		3rd subculture	
	% of explants producing shoots	Number of shoots /explant	% of explants producing shoots	Number of shoots /explant	% of explants producing shoots	Number of shoots /explant	% of explants producing shoots	Number of shoots /explant
Shoot tip	66.7 ± 2.7	12.0 ± 1.2 a A	92.3 ± 4.3	4.1 ± 0.7 a B	83.3 ± 3.3	3.4 ± 0.7 a B	67.9 ± 5.0	4.9 ± 0.9 a B
Hypocotyl	83.3 ± 6.7	6.2 ± 1.1 b A	84.6 ± 11.4	2.2 ± 0.3 b B	96.0 ± 3.2	3.7 ± 0.5 a C	100.0 ± 0.0	3.0 ± 0.5 a BC
Cotyledon	50.0 ± 10.0	10.1 ± 0.8 a A	100.0 ± 0.0	6.6 ± 0.9 a B	98.9 ± 0.2	4.2 ± 0.4 a C	100.0 ± 0.0	4.2 ± 0.8 a C

*Means ± standard error, means with small different letter within columns and capital letters within the rows indicate significant differences by ANOVA followed by Kruskal-Wallis test at $p<0.05$

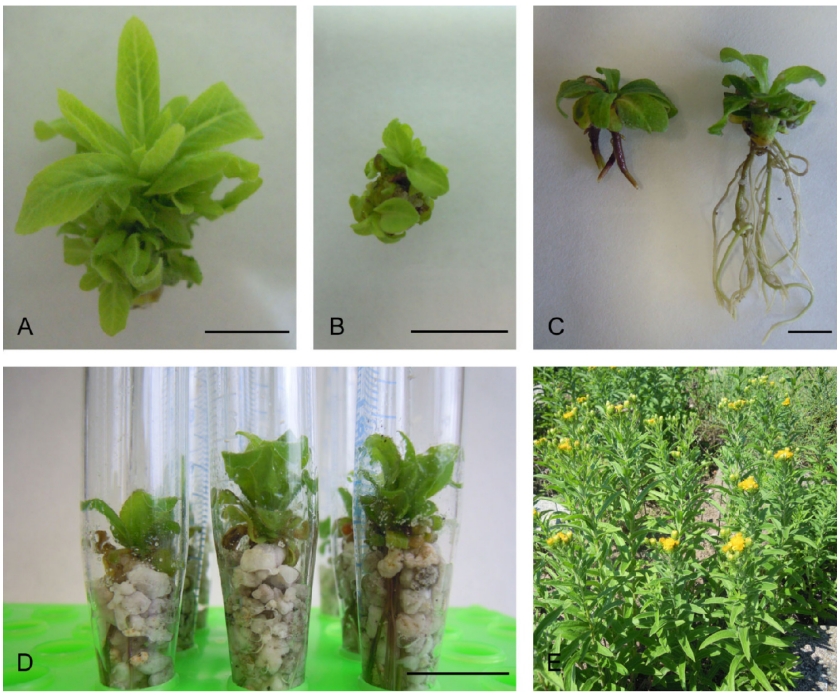

Fig. 1. Micropropagation of *Inula germanica:* (A) multiple shoots from shoot tip and (B) from fragment of cotyledon after 4 weeks of culture on MS supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, (C) comparison of root development on solid medium (left) and in liquid medium (right), (D) rooted shoots into MS liquid medium with perlite, (E) regenerated and flowered plant in the first year after acclimatization. Bar = 10 mm

alpinum, *Senecio macrophyllus* and *Cirsum pannonicum* (Trejgell and Tretyn, 2010).

The shoots of *I. germanica* obtained on proliferation medium after 4 weeks of culture were subcultured on the same medium. In the 1st passage, proliferation rate decreased independent of the origin of plant material and the differences were statistically significant. The average number of shoots per explant was 4.1 ± 0.7 and 6.6 ± 0.9 for shoots obtained from the shoot tips and cotyledons, respectively, whereas the multiplication rate for shoots obtained from hypocotyls was significantly lower (2.2 ± 0.3). In subsequent passages, the proliferation rate remained stable for shoots obtained from the shoot tips and amounted to 3.4-4.9 shoots per explant, while for those from the hypocotyl fragments it was 3.0-3.7 shoots per explant and from cotyledons 4.2 shoots per explant. The differences in multiplication rates between materials of different origins in 2nd and 3rd passages as well as between passages were not statistically significant (Table 1). The obtained shoots, independent of the source of initial explants developed normally without hyperhydratises symptoms. In previous reports on the regeneration of *Launaea sarmentosa*, Mahesh *et al*., (2012) also observed healthy shoots of good conditions on a high BAP concentration combined with a low NAA,

Rhizogenesis was not observed in any type of explants used for regeneration of *I. germanica* in both the initial material and subsequent subcultures. BAP is known as a very effective growth regulator for shoot multiplication among members of *Asteraceae* family. However, many reports describe the inhibitory effect of cytokinins on lateral and adventitious roots formation (De Klerk and Ter Brugge, 1992; Laplaze *et al*., 2007).

Rooting and acclimatization

The obtained shoots were rooted on solid MS medium without growth regulator or with 0.1 mg·l^{-1} IBA (Fig. 2). The shoots were able to root on solid medium without auxin, but less than 19% of shoots rooted on this medium (Fig. 2A). Shoots have been rooted without auxin in many *Asteraceae* species. High rooting response was achieved for species such as *Echinacea purpurea* (Korach *et al*., 2002), *Cirsium pannonicum* (Trejgell *et al*., 2012), *Arnica montana* (Surmacz-Magdziak and Sugier, 2012), *Artemisia*

amygdalina (Rasool *et al*., 2013), and *Taraxacum pieninicum* (Trejgell *et al*., 2013). Addition of IBA to medium significantly increased the percentage of rooted shoots of *I. germanica* (Fig. 2A), but had no effect on the number of roots (Fig. 2B). The average number of roots per shoot was 2.2 ± 0.4 and 1.6 ± 0.2 on medium without and with IBA, respectively. Jabeen *et al*. (2007) reported that the presence of auxin was necessary for roots induction on *I. racemosa* shoots and IBA was the most effective for this species. Stojakowska and Malarz (2004) used 0.02 mg l^{-1} IBA, which accelerated the rooting process, but had no effect on percentage of *I. royleana* rooted shoots. Moreover, the shoots of *I. verbascifolia* were rooted on MS medium supplied with 0.2 mg l^{-1} IBA (Thiem *et al*., 2003). The effective role of IBA in *in vitro* root induction has been described not only for the species of the genus *Inula* but also for other species, including *Asteraceae*, e.g. *Artemisia absinthium* (Shekhawat and Manokari, 2015), *Centaurea arifolia* (Yüzbaşıoğlu *et al*., 2012), or *Carlina onopordifolia* (Trejgell and Tretyn, 2010). However, in the case of many species, negative correlation between the root number and their length has been observed. Growth of roots of *I. germanica* on medium supplemented with 0.1 mg l^{-1} IBA was inhibited as compared to roots on medium without IBA, and root lengths were 9.9 ± 1.4 and 18.2 ± 3.1 mm, respectively, and the differences were statistically significant (Fig. 2C).

The rooting of shoots into liquid medium was comparable to solid medium with IBA. The effectiveness of rooted shoots was 62.2% and number of roots per shoot was 2.4 ± 0.2 (Fig. 1D, Fig. 3A and B). The average length of roots was nearly 54 mm, and they were 3-fold longer than in solid medium, the difference was statistically significant (Fig. 3C). Moreover, they were 5-fold longer than roots obtained on solid medium with IBA (Fig. 1C). The liquid medium for the *in vitro* rooting was used with success for *Rosa* sp, *Iresine* sp, *Rubus* sp, and *Ribes nigrum* (Clapa and Fira, 2008). Also a positive effect of using medium with perlite during *in vitro* rooting on the increase of the length and weight was obtained for the *Chrysanthemum* root system (Tymoszuk *et al*., 2009). Suthar *et al.* (2011) observed that in the medium without agar, rooting of shoots of *Boswellia serrata* was more effective, not only for a greater shoot rooting percentage,, but also for the number and the roots length. Rooting percentage and root growth of *Musa sapientum* were also superior in the liquid medium as compared to solid medium (Akbar and Roy, 2006), while in the case of *Spilanthes acmella*, induction of roots was carried out on a solid medium supplemented with IBA, and then microcuttings were transferred to Magenta containing coir fiber pith irrigated with liquid MS for better growth of roots. This clearly shows that liquid medium increased the root growth. This is possibly due to the easier uptake of components from the medium. In addition, the presence of perlite in the medium might increase the content of air, which could have a significant impact on the growth of roots.

The regenerated plants with well-developed roots were transferred to *ex vitro* conditions. Percentage of survival was approximately 87% for plantlets rooted in solid medium and 100% for plantlets from liquid medium. These plants were able to flower in first years after acclimatization (Fig. 1E). The probable reason of higher rate of plantlets survival, which were rooted in liquid medium, is due to a higher root length, the presence of larger number of lateral roots, better formed root hairs, and vascular bundles (Tymoszuk *et al*., 2009). Similar effect was reported for *Artemisia absinthium*, when shoots were rooted *ex vitro* in mixture of perlite, peat and vermiculite moistened with aqueous MS basal salt solution (Shekhawat and Manokari, 2015).

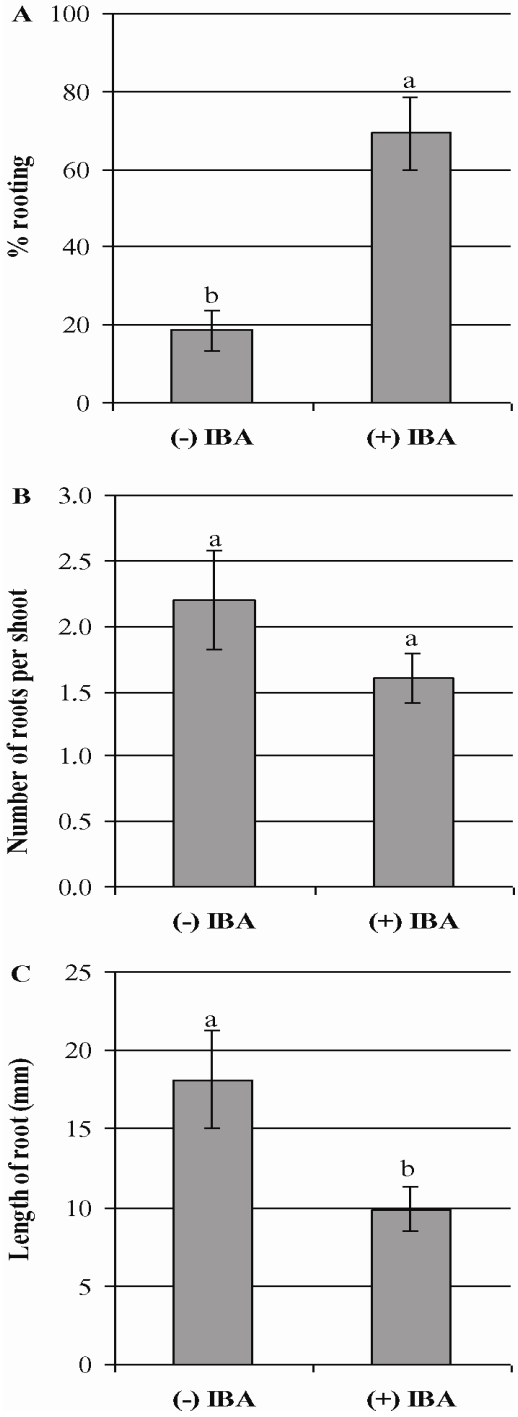

Fig. 2. The effect of 0.1 mg l^{-1} IBA into solid MS medium on rooting of *I. germanica* shoots after 4 week of culture

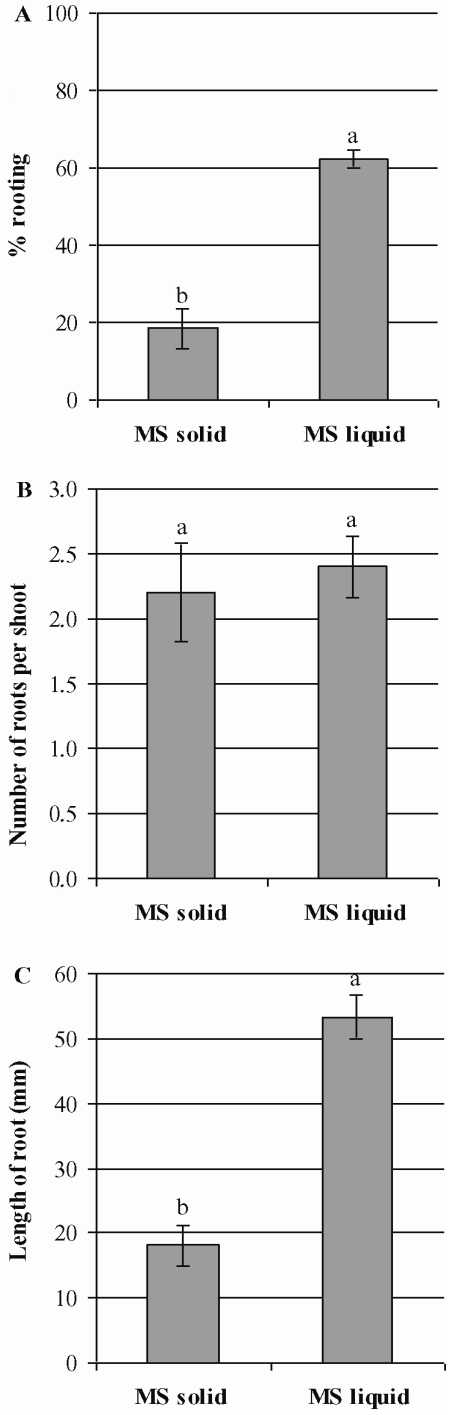

Fig. 3. The effect of liquid MS medium on rooting of *I. germanica* shoots after 4 week of culture

Conclusions

Developed micropropagation system for *I. germanica* can be a useful tool for the active protection of this species. It requires small amounts of initial material, which will not significantly affect the depletion of the natural environment. After induction of shoots all types of explants, multiplication of shoots on medium with 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (during 3 subcultures) and rooting them in liquid MS medium without growth regulators, it is possible to obtain 440 plantlets per 10 seeds used to establish *in vitro* culture.

Acknowledgements

This Project was supported by funds provided by Nicolaus Copernicus University (Toruń, Poland) for the research program of the Chair of Plant Physiology and Biotechnology.

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