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 μmol m\(^{-2}\)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 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 Bielinie on the Oder River (Kazmierczakowa 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 (Ryczyski and Mikula, 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 *Helianthus annuus* (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 uteiae* (Mallón et al., 2011), *Carlina onopordiphila* (Trejgell et al., 2011), *Arnica montana* (Surmacz-Magdzik and Sugier, 2012), or *Taraxacum pennisetum* (Trejgell et al., 2013) and others (Amin et al., 2013).

The species of *Inula* genus, *I. viscosa* (Romano, 1997), *I. royleana* (Stojakowska and Malarz, 2004), *I. verbascifolia*
(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 μmol m−2 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 NAA 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 μmol m−2 s−1) at 26 ± 1 °C in growth chambers.

Rooting and acclimatization

The shoots (1 cm and longer) obtained in the 3rd 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 Asteraceae 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 Achillea calca (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
alpinum, Senecio macrophyllus and Cirsium 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 abietinum (Shekhawat and Manokari, 2015), Centaurea arifolia (Yüzbaşoğlu 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 abietinum, 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.
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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.

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

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