

## AN EFFICIENT SYSTEM FOR REGENERATING *TARAXACUM PIENINICUM* PAWŁ. FROM SEEDLING EXPLANTS

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An efficient micropropagation system for *Taraxacum pienanicum* using seedling explants germinated in vitro is described. Shoot tips and fragments of cotyledons, hypocotyls and roots were isolated from several-day-old seedlings. The highest response, 100% frequency with 12.3 axillary shoots/explant, was from shoot tips on medium supplemented with 0.5 mg L<sup>-1</sup> BA and 0.05 mg L<sup>-1</sup> NAA. In subsequent subcultures the number of shoots was significantly higher on all explants cultured on medium containing 0.25 and 0.5 mg L<sup>-1</sup> BA, and the multiplication rate was highest (20 shoots/explant) in the 4th passage. Shoots rooted on MS and 1/2 MS medium; the highest rooting frequency was 90% and the highest number of roots 2.7/shoot. Rooted plants showed 96.2% survival in sterile soil:sand, and 100% survival in hydroponic culture. Regenerated plants flowered in the second year after acclimatization and yielded viable seeds. This protocol for obtaining complete plants through micropropagation may prove useful for conservation of the genetic resources of this and other endangered species.

**Key words:** *Taraxacum pienanicum*, endangered species, in vitro culture, multiple shoots, benzylaminopurine, hydroponic culture, fertile plants, conservation.

### INTRODUCTION

*Taraxacum pienanicum* Pawł. (syn. *T. hoppeanum* Gris. subsp. *pienanicum* Pawł.) (Asteraceae), is a hemicryptophyte perennial growing up to 15 cm high. Its thickened root neck is surrounded by remnants of old leaves forming a so-called tunic. The species flowers in May and its bright yellow flowers are pollinated by insects (Wróbel and Zarzycki, 2008). *T. pienanicum* probably is the oldest endemite of the Pieniny Mountains (Western Carpathians) (Zarzycki, 1976) and the only diploid (2n = 16) *Taraxacum* species in the Polish flora (Małecka, 1962). It occurs in the upper parts of the Trzy Korony massif, on Okraglica Mt. (above 960 m a.s.l.). It is an element of rocky grassland *Dendranthemo-Seslerietum* growing on initial rendzinas (Wróbel and Zarzycki, 2008). The *locus classicus* of this species has been destroyed by a landslide (Zarzycki et al., 2001). The present Polish

population of *T. pienanicum* consists of two clusters of a small number of individuals (Wróbel, 2004). This extremely threatened species is listed as critically endangered (CR) on two Polish red lists (Zarzycki et al., 2001; Wróbel and Zarzycki, 2008). The "Red List of the Vascular Plants in Poland" classified it as "declining – critically endangered" (E) (Zarzycki and Szelać, 2006). Protection of *T. pienanicum* involves constant monitoring of its natural habitat (Wróbel and Zarzycki, 2008). As part of measures to protect the gene pool of this species, its seeds are deposited in the Seed Bank of the Polish Academy of Sciences (PAS) Botanical Garden – Center for Biological Diversity Conservation (CBDC) in Powsin, Warsaw (Muranyi and Wróbel, 2006), and since 2007 *T. pienanicum* has been kept in the collections of the Polish flora of this botanical garden. Such measures are not sufficient for such a small population of an endangered species. Propagation in vitro seems needed. Plant propaga-

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TABLE 1. Effects of different concentrations of BA in combination with NAA on shoot organogenesis on seedling explants of *Taraxacum pieninicum*

| Concentration<br>BA<br>[mg·L <sup>-1</sup> ] | Shoot tip |                              |                         | Hypocotyl |                              |                         | Cotyledon |                              |                         | Root |                              |                         |
|--|-----------|------------------------------|-------------------------|-----------|------------------------------|-------------------------|-----------|------------------------------|-------------------------|------|------------------------------|-------------------------|
|  | %         | No. of<br>shoots/<br>explant | Shoot<br>length<br>[mm] | %         | No. of<br>shoots/<br>explant | Shoot<br>length<br>[mm] | %         | No. of<br>shoots/<br>explant | Shoot<br>length<br>[mm] | %    | No. of<br>shoots/<br>explant | Shoot<br>length<br>[mm] |
| 0.25   | 100       | 9.4±1.9a                     | 16.9±1.3a               | 87.5      | 5.8±1.4a                     | 10.5±0.8a               | 100       | 10.2±1.3a                    | 12.8±1.0a               | 77.8 | 10.3±0.8a                    | 14.7±1.1a               |
| 0.5  | 100       | 12.3±1.6a                    | 16.7±1.3a               | 100       | 7.1±1.0a                     | 11.6±1.2a               | 100       | 6.4±0.7b                     | 6.4±1.1b                | 57.1 | 4.3±1.1b                     | 5.8±0.3b                |
| 1.0  | 100       | 3.6±0.9b                     | 7.4 ± 0.7b              | 57.5      | 8.5±2.8a                     | 9.1±0.6a                | 43.8      | 4.0±1.4b                     | 8.1± 0.8b               | 0    | -                            | -                       |

\*means ± standard error (n = 30); means with the same letter within column do not differ significantly by ANOVA followed by Tukey's test at p < 0.05.

tion through tissue culture consists of the following stages: establishment of aseptic cultures, induction of meristematic centers and their development into adventitious buds or development of axillary buds, elongation of buds to shoots, rooting the shoots, and acclimatization of microcuttings to ex vivo conditions (Debergh and Maene, 1981). Shoot multiplication depends on the conditions of culture and primarily on the type and relative proportions of the growth regulators. The presence of cytokinins in the culture medium at a concentration exceeding that of auxin leads to formation of callus and adventitious bud differentiation within it (Skoog and Miller, 1957). Cytokinin triggers induction of ectopic expression of the *WUS* gene within the callus, and it is sufficient to induce shoot regeneration (Gordon et al., 2009; Cheng, 2010), whereas adding cytokinins to the growth medium in lateral bud culture reduces apical shoot dominance, resulting in axillary bud development (Gaspar et al., 2003; Shani et al., 2006). The shoots obtained require rooting. This process is stimulated by endogenous auxin and by exogenous application of this hormone, which stimulates expression of the *RUC* gene (Sugiyama, 1999; Tyburski and Tretyn, 2004).

The multiplication procedure has been commonly used for ornamental plants such as *Gerbera* (Kanwar and Kumar, 2008) and *Chrysanthemum* (Bhattacharya et al., 1990), but also offers a useful tool for conservation of protected species. In vitro vegetative propagation of declining (critically endangered, E) and vulnerable (V) species of the Polish flora has been attempted in some dicotyledonous species: for example, *Arnica montana* (Surmacz-Magdziak and Sugier, 2012), *Carlina onopordifolia* (Trejgell and Tretyn, 2011), *Drosera anglica* (Kawiak and Łojkowska, 2004), *D. intermedia* (Kromer et al., 2000), *D. rotundifolia* (Kawiak et al., 2003), *Gentiana pneumonanthe* (Bach and Pawłowska, 2003), *Leontopodium alpinum* (Trejgell et al., 2010a), and *Senecio macrophyllus* (Trejgell et al., 2010b). The monocotyledons selected for such studies include *Fritillaria meleagris* (Liliaceae),

*Gladiolus palustris* (Iridaceae) and a few Orchidaceae species (Rybczyński and Mikula, 2006). Here we present a protocol for in vitro micropropagation and acclimatization of *Taraxacum pieninicum*. To our knowledge this is the first protocol for in vitro propagation of this species.

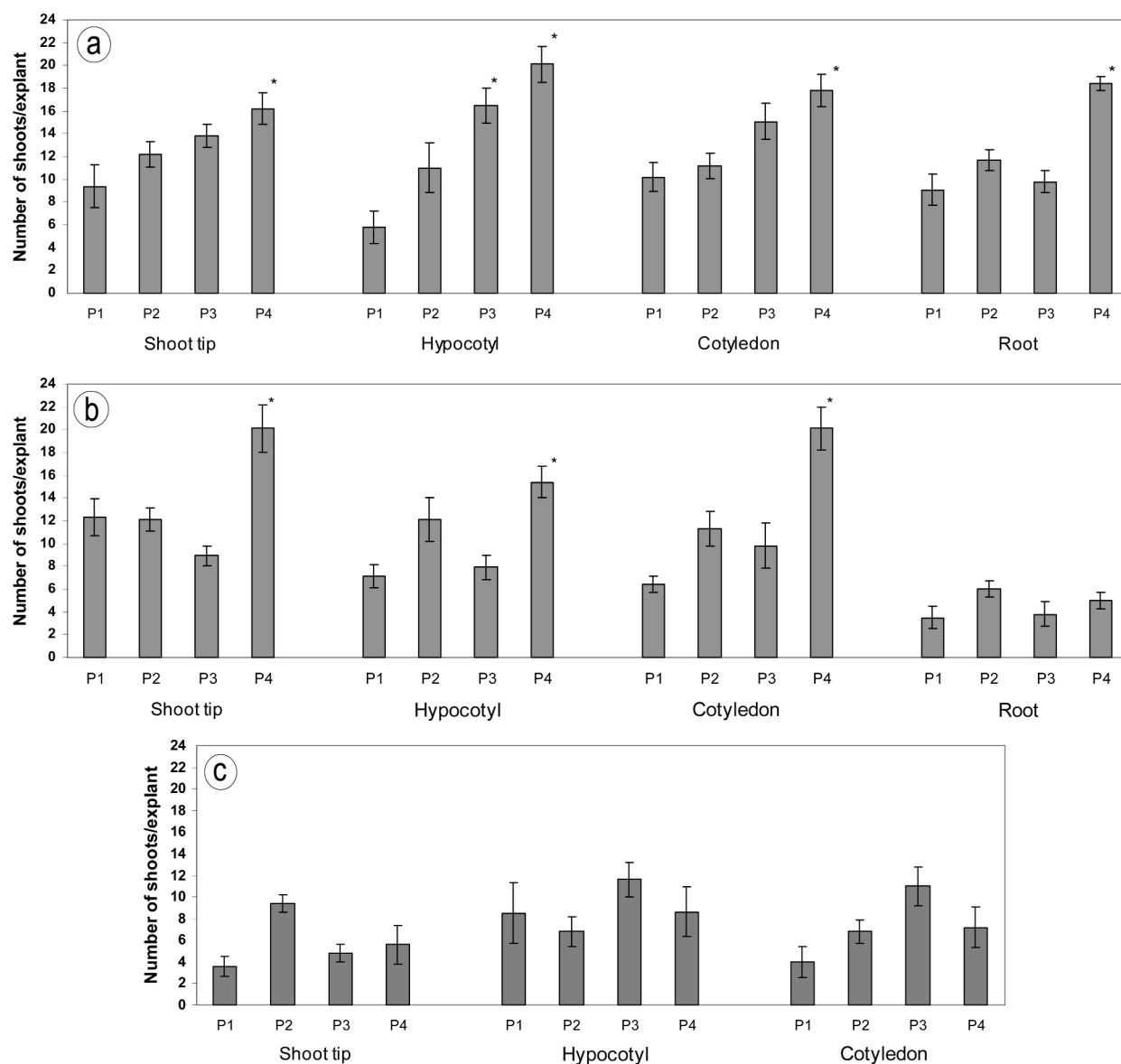
## MATERIAL AND METHODS

### PLANT MATERIAL AND DISINFECTION

For this work we used several-day old sterile seedlings of *Taraxacum pieninicum*. The seeds came from the collection of the PAS Botanical Garden – CBDC in Powsin, Warsaw (Poland). They were surface-disinfected with 70% (v/v) ethanol for 30 s and then 20% (v/v) commercial bleach (Domestos) for 20 min, then washed 4 times with sterile distilled water and transferred to Petri dishes (6 cm diam.) containing 10 ml MS (Murashige and Skoog, 1962) supplemented with 1 mg L<sup>-1</sup> GA<sub>3</sub> and solidified with 0.7% (w/v) agar (Sigma-Aldrich, Germany).

### INITIAL CULTURE AND MULTIPLICATION

Shoot tips cut under the cotyledonary node (2–3 mm long) and fragments of cotyledons (2–3 mm long), hypocotyls (1 mm long, cut out under the node), and roots (5 mm long) were isolated from seedlings. The explants were placed on MS (Murashige and Skoog, 1962) medium containing BA at concentrations of 0.25, 0.5 or 1 mg L<sup>-1</sup> in combination with NAA at 1/10 the concentration of BA. Medium pH was adjusted to 5.8 before autoclaving. The explants were cultivated in 100 ml Erlenmeyer flasks containing 30 ml medium for 4 weeks under continuous white fluorescent light (45 μmol m<sup>-2</sup>s<sup>-1</sup>) at 26 ± 1°C. After this period the percentage of explants producing shoots, number of shoots per explant, and shoot length were recorded. Individual shoots obtained on the initial material were excised from the shoot clusters and



**Fig. 1.** Effect of different concentrations of BA in combination with NAA in the medium on proliferation of axillary shoots developing on various explants of *Taraxacum pienenicum* in successive subcultures (P1, P2, P3, P4). (a) 0.25 mg L<sup>-1</sup> BA, (b) 0.5 mg L<sup>-1</sup> BA, (c) 1.0 mg L<sup>-1</sup> BA. Statistical analysis was done separately for each explant type (n = 30); \* statistically significant difference.

transferred to glass jars with 50 ml fresh proliferative medium containing the same combination of growth regulators for shoot multiplication. The shoots were subcultured 4 times at 4-week intervals. After this period the multiplication rate and percentage of explants producing shoots were analyzed.

#### ROOTING AND ACCLIMATIZATION

Shoots measuring 1 cm and longer from the 3<sup>rd</sup> and 4<sup>th</sup> subcultures were excised and transferred to root-

ing medium. Some of the shoots were rooted in glass jars with 50 ml solidified medium with full-strength (MS) or half-strength MS salts (1/2 MS). The rest of the shoots were transferred to hydroponic culture in 10 ml tubes with quarter-strength MS salt solution. The plantlets obtained after 4 weeks of culture were removed from the in vitro cultures, washed gently in sterile water and transferred to plastic pots filled with a sterile mixture of vermiculite and sand (1:1), a sterile mixture soil and sand (1:2), a nonsterile mixture of soil and sand (1:2), or hydroponic culture with quarter-strength MS salt solution. The

TABLE 2. Effect of MS salt strength on rooting of *Taraxacum pieniticum* shoots developed on different seedling explants after 4 weeks of cultivation on rooting medium

| Shoots developed on | MS           |                     |                   | ½ MS         |                     |                   |
|---------------------|--------------|---------------------|-------------------|--------------|---------------------|-------------------|
|                     | % of rooting | No. of roots/shoot* | Root length (mm)* | % of rooting | No. of roots/shoot* | Root length (mm)* |
| Shoot tips          | 47.8±8.7     | 1.7±0.2a            | 60.3±5.4bc        | 68.0±11.8    | 2.3±0.2ab           | 83.1±6.2a         |
| Hypocotyls          | 72.6±10.6    | 1.7±0.2a            | 73.4±3.9ab        | 65.2±11.6    | 1.7±0.2b            | 77.6±6.3a         |
| Cotyledons          | 54.4±8.6     | 1.8±0.2a            | 56.1±3.1c         | 59.8±13.5    | 1.6±0.2b            | 72.2±5.9a         |
| Roots               | 85.1±7.3     | 2.1±0.2a            | 80.6±3.4a         | 90.0±5.8     | 2.7±0.3a            | 88.1±3.1a         |

\*means ± standard error (n=30); means with the same letter within column do not differ significantly by ANOVA followed by Tukey's test at  $p < 0.05$

pots and tubes were covered with transparent boxes to maintain humidity. After 4 weeks of hardening in hydroponic culture the microcuttings were transferred to pots containing soil and acclimatized for another 4 weeks in a greenhouse. After 8 weeks of acclimatization all the plantlets were transferred to field conditions. Regenerated plants were replanted in the Polish Academy of Sciences Botanical Garden – CBDC in Powsin, Warsaw (100 plants), in the Maria Curie-Skłodowska University (MCSU) Botanical Garden in Lublin (15 plants), and in an experimental plot at Nicolaus Copernicus University (NCU) in Toruń (15 plants). After 4 months and in May during the next vegetation cycle, the survival rate, morphological traits, flowering ability and vitality of the seeds of regenerated plants were analyzed. Seed vitality was analyzed as germination percentages for seeds planted in pots filled with soil (greenhouses in both botanical gardens) and on MS medium supplemented with  $1 \text{ mg L}^{-1}$   $\text{GA}_3$ , and was recorded when the radicle was at least 2 mm long. Germination was monitored for 3 weeks.

#### STATISTICAL ANALYSIS

The in vitro experiments were done in three replicates. Each treatment consisted of 30 experimental units – explants or individual shoots (6 per Erlenmeyer flask/glass jar). The data given are means ± standard error (SE). The significance of differences between treatments was analyzed by ANOVA followed by Tukey's multiple range test at  $p < 0.05$ .

#### RESULTS AND DISCUSSION

Previous studies on micropropagation of selected Asteraceae species found that cytokinin BA supplementation, either alone or in combination with an auxin, is almost universally effective for in vitro proliferation (Banerjee et al., 2010; Trejgell et al., 2009,

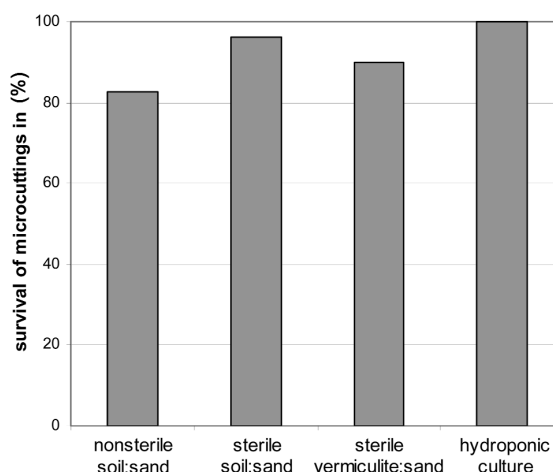
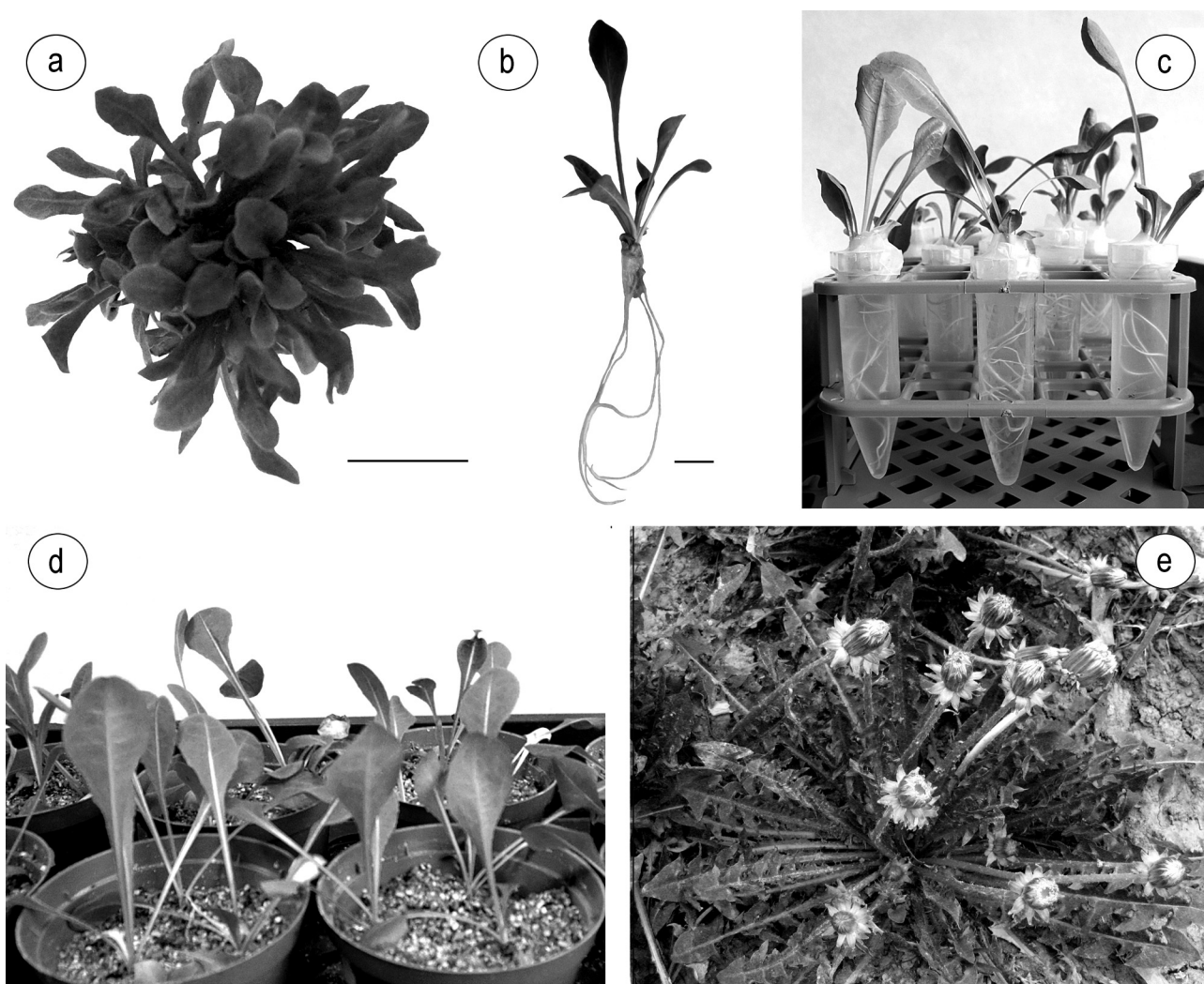


Fig. 2. Effect of substrate type on survival of *Taraxacum pieniticum* microcuttings during acclimatization ex vitro (n = 30).

2010b, and 2012), so in our experiments the medium was supplemented with only BA in combination with NAA at various concentrations. All the tested types of explants showed shoot induction on all the proliferation media used, except for roots on medium with  $1 \text{ mg L}^{-1}$  BA (Tab. 1). Lower concentrations of BA increased the explant organogenesis percentage, shoot number and shoot length (except for shoots from hypocotyls). Shoot tips cultured on medium with  $0.5 \text{ mg L}^{-1}$  BA gave the highest shooting frequency (100%) and number of shoots (12.3 axillary shoots per explant); cotyledons cultured on medium with  $0.25 \text{ mg L}^{-1}$  BA produced 10.2 adventitious shoots per cotyledon (Tab. 1). Further multiplication of well-grown shoots was achieved by sub-culturing on fresh culture medium of the same composition. In subsequent passages the number of shoots increased significantly on all the explants cultured on medium containing  $0.25 \text{ mg L}^{-1}$  BA; shoots from hypocotyls in the 4<sup>th</sup> passage gave the highest rate of multiplication, 20.1 shoots/explant (Fig. 1a,



**Fig. 3.** Micropropagation of *Taraxacum pieninicum*. (a) Axillary shoots developing on medium with  $0.25 \text{ mg L}^{-1}$  BA (4th subculture). Bar = 1 cm, (b) Rooted shoots on 1/2 MS medium. Bar = 1 cm, (c) Acclimatization of microcuttings in hydroponic culture, (d) Acclimatization of microcuttings in sterile soil:sand, (e) Regenerated plants planted in the MCSU Botanical Garden in Lublin.

Fig. 3a). During successive subcultures the shoots developed normally (no vitrification) and were similar in size from one subculture to the next. On medium with  $0.5 \text{ mg L}^{-1}$  BA the shoot multiplication rates were 20.0 per cotyledon and 20.8 per shoot tip (Fig. 1b). Similar findings have been reported for several initial passages during regeneration on BA-supplemented medium in Asteraceae species including *Centaurea rupestris* (Ćurković, 2003) and *Carlina acaulis* (Trejgell et al., 2009), and in species from other families, for example *Musa* spp. (Sadiq et al., 2012), *Camellia sinensis* (Mondal et al., 1998) and *Citrus grandis* (Paudyal and Haq, 2000). For some treatment groups there were no significant differences in shoot multiplication rates between successive subcultures: for all explant types at BA

concentration of  $1.0 \text{ mg L}^{-1}$ , and for root explants on medium with 0.25 and  $0.5 \text{ mg L}^{-1}$  BA (Fig. 1c). Higher concentrations of BA in the culture medium ( $0.5$  and  $1.0 \text{ mg L}^{-1}$ ) inhibited shoot elongation; shoot elongation differed significantly between shoots from shoot tips and shoots from cotyledon and root fragments (Tab. 1). The inhibitory effect of BA on shoot growth has also been noted in reports on regeneration of different Asteraceae species such as *Saussurea obvallata* (Joshi and Dhar, 2003), *Eclipta alba* (Baskaran and Jayabalan, 2005), *Carlina acaulis* (Trejgell et al., 2009) and *Cirsium pannonicum* (Trejgell et al., 2012).

Shoots were rooted on full- or half-strength MS growth-regulator-free solid medium and 1/4 MS solution in hydroponic cultures. Shoots have rooted

without the presence of auxin in many Asteraceae species such as *Echinacea purpurea* (Korach et al., 2002), *Senecio macrophyllus* (Trejgell et al., 2010b) and *Cirsium pannonicum* (Trejgell et al., 2012). In our study, isolated shoots transferred directly to hydroponic culture were not capable of rooting. Rooting of shoots on solid medium was effective: more than 85% of the shoots rooted on MS medium, with an average 2.1 roots per shoot. Reducing the mineral content in the rooting medium (1/2 MS) slightly stimulated growth but did not significantly affect the percentage of rooted shoots (90%) or the number of roots (2.7) (Tab. 2, Fig. 3b). Similar effects were observed in *Ensete ventricosum* (Birmeta and Welander, 2004) and *Senecio macrophyllus* (Trejgell et al., 2010b). Reducing the MS salts concentration has been shown to promote in vitro rooting of shoots in several other species (Joshi and Dhar, 2003; Baskaran et al., 2006).

During acclimatization it is particularly important to minimize water stress. Good root development is a key factor affecting the success of acclimatization, but poor vascular connections between the root and shoot are usually observed when plantlets are removed from culture; this restricts water uptake (Hazarika, 2006). Roots growing in agar often lack root hairs and may die shortly after transplanting to soil, affecting plantlet survival ex vitro (Debergh and Maene, 1981). The microcuttings showed high survival after 8 weeks of acclimatization: over 82% in non-sterile soil:sand, and higher in sterile soil:sand (96.2%) or sterile vermiculite:sand (90%). This suggests a negative effect of pathogenic organisms when microcuttings are transferred from aseptic cultures to field conditions (Hazarika, 2006). All the rooted shoots survived after two-step acclimatization, that is, 4 weeks in hydroponic culture and transfer to non-sterile soil (Fig. 2, Fig. c, d).

In the first year, the plantlets grown in both botanical gardens and in the experimental plot at NCU in Toruń produced only rosette leaves and did not enter the generative stage. In the PAS Botanical Garden – CBDC in Powsin, Warsaw, snails of the genus *Limax* and *Arion rufus* grazed the plantlet leaves, drastically reducing survival to 10% after 6 months of acclimatization. In the next year, 27% of the plants developed rosette leaves. In contrast, 100% of the plants survived in the MCSU Botanical Garden in Lublin and in the experimental plot at NCU in Toruń (Fig. 3e). In the second year after acclimatization the plants flowered: 100% of them in the MCSU Botanical Garden in Lublin, with an average  $7.7 \pm 0.6$  inflorescence stems per plant, and 93.3% with  $3.9 \pm 0.8$  inflorescence stems per plant at NCU in Toruń. In the PAS Botanical Garden – CBDC in Powsin, Warsaw, only 25.9% of the plants flowered, with only one inflorescence stem each. Adding cytokinins to the culture medium may lead

to cytokinin accumulation in plant tissues and stimulate lateral bud growth (Kotov and Kotova, 2000; Shani et al., 2006). Flowering occurred in May at all the sites, the same as in natural localities. The flowers of the regenerated plants were fertile;  $23.4 \pm 0.7\%$  of the seeds germinated in soil, and  $24.5 \pm 4.7\%$  germinated in medium supplemented with the cytokinin GA<sub>3</sub>.

The method of plant regeneration described yields huge numbers of regenerants and can be used for propagation of this species. It should be part of a strategy for reintroduction and restitution of this declining, critically endangered species.

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