INTRODUCTION

Senecio macrophyllus M. Bieb. is a member of the Astreaceae family. This species is extremely rare and has a scattered distribution in Central Europe. In Poland it occurs at only a few locations in the Lublin Uplands (SE Poland), and is listed in the Polish Red Data Book. S. macrophyllus is a rhizomatous plant with an erect flowering stem up to 190 cm high. Its inflorescence occurs as subcorymbs consisting of numerous yellow capitula. It has very slow growth and a long life cycle – flowering occurs 7–13 years after germination (Tutin et al., 1976; Czarnecka, 2006).

Previous papers describe only morphological, phytocoenological and ecological studies of Senecio macrophyllus (Czarnecka, 1995; Czarnecka, 2006). To our knowledge this is the first protocol for in vitro micropropagation of this species via multiple shoot formation. The only efficient micropropagation procedures previously reported for the genus Senecio is for Senecio × hybridus (Gertsson, 1988) and S. hadrosomus (Bramwell, 1990). Successful clonal micropropagation of most species of the family Asteraceae has been achieved using different types of explants (Cuenca et al., 1999; Korach et al., 2002; Vanegas et al., 2002; Evenor and Reuveni, 2004; Dhaka and Kothari, 2005), among them explants of seedlings (Joshi and Dhar, 2003; Baskaran and Jayabalan, 2005; Sujatha and Ranjitha Kumari, 2007; Trejgell et al., 2009). Vegetative propagation of Senecio could help in its conservation, because its seeds completely lose their ability to sprout after one year, making it unfeasible to set up a seed bank. In this work we aimed to develop methods for micropropagation of Senecio macrophyllus.

MATERIAL AND METHODS

PROPAGATION CULTURE

Ten-day-old Senecio macrophyllus M. Bieb. seedlings were the experimental material. The seeds were obtained from the Botanical Garden of Maria Curie-Skłodowska University (Lublin, Poland). They were surface-sterilized with 70% (v/v) ethanol for 30 s and then in 20% (v/v) solution of NaOCl (Domestos *e-mail: trejgell@biol.uni.torun.pl

 abbreviations: BA – 6-benzylaminopurine; GA3 – gibberellic acid; IAA – indole-3-acetic acid, IBA – indole-3-butyric acid; KN – kinetin; NAA – naphthaleneacetic acid; MS – Murashige and Skoog medium; ZEA – zeatin.
commercial bleach) for 30 min. The materials were then rinsed 4 times in sterilized distilled water and placed on MS growth medium (Murashige and Skoog, 1962) supplemented with 2.9 \( \mu \text{M} \) GA\(_3\) and solidified with 0.7% (w/v) agar. Shoot tips with a cotyledonary node (3–4 mm length), fragments of cotyledons (0.25 sq. cm), hypocotyls (1 mm length, cut under the node) and basal parts of roots (2–3 mm length) were isolated from the seedlings. 

Explants were transferred to MS medium supplemented with different types of cytokinins: BA (2.2 \( \mu \text{M}, 4.4 \mu \text{M} \) or 13.3 \( \mu \text{M})\), KN (4.7 \( \mu \text{M} \) or 13.9 \( \mu \text{M} \)) and ZEA (4.6 \( \mu \text{M} \) or 13.7 \( \mu \text{M} \)) in combination with NAA (0.54 \( \mu \text{M} \)); in the case of BA 2.2 \( \mu \text{M} \) we used NAA at 0.27 \( \mu \text{M} \) concentration. Medium pH was adjusted to 5.8 before autoclaving. The cultures were incubated in a growth chamber at 26 ± 1°C under continuous white fluorescent light (45 \( \mu \text{mol m}^-2 \text{s}^{-1} \)).

Individual shoots were excised from the shoot clusters and transferred to media containing the same growth regulator combination for shoot multiplication. The cultures were subcultured 4 times, at 4-week intervals. After this period, shoot organogenesis frequency, number of shoots/roots per explant, and axillary shoot length were analyzed.

### ROOTING AND ACCLIMATIZATION

The shoots obtained in the last subculture were rooted in vitro. Individual shoots were excised from shoot clusters and transferred to full- or half-strength MS medium without auxin. The plantlets obtained after four weeks of cultivation on the rooting medium were removed from in vitro cultures, washed gently in sterile water and transferred to plastic pots containing a sterile mixture of vermiculite and sand (1:1, v/v) or nonsterile garden soil. Rates of survival after 4-week acclimatization were noted, and the plantlets were transferred to field conditions (in June). Flowering ability, inflorescence stem morphology and seed germinability on GA\(_3\)-supplemented medium were analyzed.

### STATISTICAL ANALYSIS

Twenty explants or individual shoots were used for each treatment in three replicates; in the variants with kinetin and zeatin, however, the number of shoots in subsequent subcultures depended on their organogenesis capability. Data were analyzed by ANOVA, and the mean values of treatments were compared with Tukey’s test (P=0.05).

### RESULTS AND DISCUSSION

#### SHOOT MULTIPLICATION

Callus induction was observed on all types of primary explants, but only shoot tips were capable of shoot organogenesis. The results indicate that BA-supplemented medium is the best one for shoot organogenesis in *Senecio macrophyllus* (Table 1). The frequency of shoot organogenesis was highest (100%) for explants cultured on medium supplemented with 4.4 \( \mu \text{M} \) and 13.3 \( \mu \text{M} \) BA in combination with 0.54 \( \mu \text{M} \) NAA; it was 80% when the BA concentration was decreased to 2.2 \( \mu \text{M} \) and NAA to 0.27 \( \mu \text{M} \). The multiplication rate was highest (8.3 ± 1.0) for explants treated with 4.4 \( \mu \text{M} \) BA. These results are in conformity with earlier reports for *Senecio × hybridus* (Gertsson, 1988) and other plants of the Asteraceae family, such as *Tagetes erecta* (Misra and Dutta, 1999), *Wedelia calendulacea* (Emmanuel et al., 2000), *Echinacea purpurea* (Korach et al., 2002), *Eclipta alba* (Dhaka and Kothari, 2005) and *Carlina acaulis* (Grubisić et al., 2004; Trejgell et al., 2009), where the highest multiplication rate was achieved with BA-supplemented

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**TABLE 1. Effect of different cytokinins in combination with NAA on organogenesis of Senecio macrophyllus shoot tips**

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>NAA</th>
<th>Percentage of explants producing roots</th>
<th>Number of roots per explant</th>
<th>Percentage of explants producing shoots</th>
<th>Number of shoots per explant</th>
<th>Shoot length [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA 2.2</td>
<td>0.27</td>
<td>20.0</td>
<td>0.4±0.2 a</td>
<td>80</td>
<td>3.1±0.6 b</td>
<td>31.9±1.6 c</td>
</tr>
<tr>
<td>4.4</td>
<td>0.54</td>
<td>0</td>
<td>–</td>
<td>100</td>
<td>8.3±1.0 a</td>
<td>24.2±1.4 de</td>
</tr>
<tr>
<td>13.3</td>
<td>0.54</td>
<td>0</td>
<td>–</td>
<td>100</td>
<td>6.7±0.7 a</td>
<td>19.5±1.5 e</td>
</tr>
<tr>
<td>KN 4.7</td>
<td>0.54</td>
<td>48.3</td>
<td>0.9±0.3 a</td>
<td>30.8</td>
<td>0.6±0.2 c</td>
<td>32.6±1.4 cd</td>
</tr>
<tr>
<td>13.9</td>
<td>0.54</td>
<td>56.6</td>
<td>2.0±0.6 a</td>
<td>50.0</td>
<td>0.8±0.2 c</td>
<td>42.9±1.8 b</td>
</tr>
<tr>
<td>ZEA 4.7</td>
<td>0.54</td>
<td>54.5</td>
<td>1.1±0.3 a</td>
<td>46.5</td>
<td>1.2±0.4 bc</td>
<td>55.8±5.7 a</td>
</tr>
<tr>
<td>13.9</td>
<td>0.54</td>
<td>63.6</td>
<td>1.4±0.4 a</td>
<td>79.2</td>
<td>1.9±0.5 bc</td>
<td>36.4±1.9 bc</td>
</tr>
<tr>
<td>F</td>
<td>1.28</td>
<td>13.65*</td>
<td></td>
<td>23.97*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means ± standard error, n=20 (in triplicate); means with different letters differ significantly by ANOVA followed by Tukey’s test; * significant at p≤0.05.
MS medium. In subsequent subcultures of *S. macrophyllus* the number of shoots per explant decreased (Fig. 2b). A higher concentration of BA (13.3 μM) supported callus induction and reduction of shoot bud differentiation (Fig. 2c). The same phenomenon has been observed in other species (Tiwari et al., 2001).

The presence of BA in the medium strongly inhibited the elongation growth of axillary shoots; mean shoot length was 24.2 ± 1.4 mm (4.4 μM BA) and 19.5 ± 1.5 mm (13.3 μM BA). The leaf lamina surface was much reduced on medium with a higher concentration of BA (Fig. 1a) as compared to shoots induced on medium with ZEA and KN (Fig. 1b). Similar results have been described for other species of the *Asteraceae* family (Joshi and Dhar, 2003; Baskaran and Jayabal, 2005; Trejgell et al., 2009). BA at a concentration of 2.2 μM in combination with NAA at 0.27 μM caused limited inhibition of shoot elongation; average shoot length was 31.9 ± 1.6 mm (Tab. 1). It also decreased the proliferation rate, both from donor material (3.1 ± 0.6) and in subsequent subcultures (Fig. 2a). Moreover, the presence of BA in regeneration media inhibited root organogenesis as compared with other cytokinins (Tab. 1). This result suggests that high concentrations of BA have an inhibitory effect on root induction. A similar effect has been observed in a few species belonging to the *Poaceae* family (Poonawala et al., 1999) and *Asclepiadaceae* family (Chaudhuri et al., 2004).

The use of zeatin and particularly kinetin resulted in a lower shoot organogenesis frequency and proliferation rate (Tab. 1). The proliferation rate for donor material treated with KN was 0.6 ± 0.2 and 0.8 ± 0.2 on average, depending on the cytokinin concentration. In subsequent subcultures the proliferation rate was the same as for the donor material on medium with 13.9 μM KN (Fig. 2d); on medium with 4.7 μM KN the ability to form shoot buds in subsequent subcultures was lost. The presence of ZEA in the medium gave a 1.2 ± 0.4 shoot/explant ratio on medium with 4.7 μM ZEA, and 1.9 ± 0.5 at the higher ZEA concentration (Tab. 1). These values remained stable in successive subcultures (Fig. 2e, f). The differential response to various cytokinins may be due to the differences in their uptake, recognition by the cells, and/or the mechanism of action of the cytokinins (Sujatha and Reddy, 1998; Kim et al., 2001).

### ROOTING AND SURVIVAL

Shoots were excised from multiple shoot cultures and were rooted on full- or half-strength MS growth regulator-free medium. Full-strength MS slightly boosted rooting of shoots developed on medium with BA and KN (Tab. 2). However, neither the number of roots per shoot nor root length were greater than the values obtained with half-strength MS (Tab. 2). Earlier studies have examined the rooting process on MS medium without auxin in many members of the *Asteraceae* family: *Guizotia abyssinica* (Sujatha, 1997), *Centaurea paut* (Cuenca et al., 1999), *Echinacea purpurea* (Korach et al., 2002) and *Carlina acaulis* (Trejgell et al., 2009), as well as many other species. The incidence of root formation on auxin-free medium may be due to the presence of endogenous auxin in regenerated shoots (Minocha, 1987). Decreasing the amount of salts in the rooting medium has been shown to cause various effects depending on the species. In *Saussurea obvallata* it increased the number of rooted shoots as well as the number of roots per
Halving the MS salts boosted the rooting frequency in *Eclipta alba* (Baskaran and Jayabalan, 2005) and gave the best root growth from shoots in *Ensete ventricosum* (Birmeta and Welander, 2004). Root induction on shoots formed on BA-supplemented medium occurred a week later than for shoots differentiating on medium with ZEA and KN, and on full-strength MS medium supplemented with BA the number of roots per shoot was significantly lower (1.8 ± 0.2) than the corresponding shoots in the ZEA and KN treatments (Tab. 2). Shoots formed on BA-supplemented medium needed up to 6 more weeks of culture to increase rooting to 100%. Root formation is often inhibited by cytokinins, especially BA (Poonawala et al., 1999; Chaudhuri et al., 2004). Rooting of microshoots plays
TABLE 2. Effect of MS salt strength on shoot rooting of Senecio macrophyllus induced on medium supplemented with different types of cytokinins after 4 weeks of culture on rooting medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Shoots developed on medium with</th>
<th>Rooting percentage</th>
<th>Number of roots per shoot</th>
<th>Root length [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>BA</td>
<td>87.5</td>
<td>1.8±0.2 b</td>
<td>37.2±5.6 d</td>
</tr>
<tr>
<td></td>
<td>ZEA</td>
<td>100</td>
<td>3.4±0.3 a</td>
<td>85.0±5.1 a</td>
</tr>
<tr>
<td></td>
<td>KN</td>
<td>96.5</td>
<td>3.6±0.4 a</td>
<td>73.5±4.7bc</td>
</tr>
<tr>
<td>1/2 MS</td>
<td>BA</td>
<td>75.6</td>
<td>2.7±0.4ab</td>
<td>28.0±3.1 d</td>
</tr>
<tr>
<td></td>
<td>ZEA</td>
<td>100</td>
<td>4.7±0.8 a</td>
<td>82.6±6.0ab</td>
</tr>
<tr>
<td></td>
<td>KN</td>
<td>85.7</td>
<td>3.4±0.6 a</td>
<td>60.6±6.2 c</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>8.8*</td>
<td></td>
<td>20.1*</td>
</tr>
</tbody>
</table>

Means ± standard error, n=20 (in triplicate); means with different letters differ significantly by ANOVA followed by Tukey's test; * significant at p≤0.05.

an important role in acclimatization (Hazarika, 2003).

Plantlets with well developed roots were transferred to a sterile mixture of vermiculite and sand or to nonsterile garden soil. The plantlets grew intensively; their survival rate was 100% on vermiculite and 92% on soil after 4 weeks of acclimatization. No variation of morphological characteristics between the plants growing under field conditions was detected. In the first year after acclimatization they produced only a leaf rosette but were not able to flower. In the second year the survival rate was 82%, and 53.6% of the plants flowered (Fig. 1c) in the typical season (June and July). The obtained achenes contained viable seeds, but after 6 months only 8% of the seeds were able to germinate on GA3-supplemented medium. Plants that grow in nature are able to flower 7–13 years after germination, and the seeds are able to germinate for only a few months thereafter (Czarnecka, 1995).

This study showed that multiplication of shoots from shoot tips offers a useful method for active protection of Senecio macrophyllus, a species for which it is not possible to establish a seed bank.

REFERENCES


