ACTA BIOLOGICA CRACOVIENSIA Series Botanica 52/1: 67–72, 2010

MICROPROPAGATION OF *SENECIO MACROPHYLLUS* M. BIEB.

ALINA TREJGELL*, MAGDALENA MICHALSKA, AND ANDRZEJ TRETYN

Department of Biotechnology, Institute of General and Molecular Biology, Nicolaus Copernicus University ul. Gagarina 9, 87-100 Toruń, Poland

Received July 30, 2009; revision accepted September 20, 2009

This is the first protocol for *in vitro* micropropagation of *Senecio macrophyllus*. Shoot tips and fragments of the cotyledon, hypocotyls and roots were isolated from 10-day-old sterile seedlings. The morphological response was tested on MS medium supplemented with different types of cytokinins: BA (2.2 μM, 4.4 μM or 13.3 μM), KN (4.7 μM or 13.9 μM) and ZEA (4.6 μM or 13,7 μM) in combination with 0.54 μM NAA or 0.27 μM NAA (with 2.2 μM BA only), but only shoot tips were capable of shoot organogenesis. Shoot proliferation was highest for explants cultured on MS medium supplemented with 4.4 μM BA in combination with 0.54 μM NAA. The shoots formed were then multiplied on the same medium. Rooting was achieved on full- and half-strength MS medium without auxin, but shoots cultured on medium BA-supplemented began inducing roots a week later than shoots obtained on media with other types of cytokinins. Well-rooted plantlets were transferred to ex vitro conditions. The survival rate of rooted plants was 100% for plants cultured in a mixture of vermiculite and sand, and 92% for those planted in soil after 4 weeks of acclimatization. In the first year the plants grew intensively under field conditions and were able to develop a leaf rosette. In the second year the plants were able to flower and produce viable seeds.

Key words: *Senecio macrophyllus*, micropropagation, shoot tip, benzylaminopurine, flowering plantlets.

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 propagation 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; GA_3 – gibberellic acid; IAA – indole-3- acetic acid, IBA – indole-3-butyric acid; KN – kinetin; NAA – naphthaleneacetic acid; MS – Murashige and Skoog medium; ZEA – zeatin.

PL ISSN 0001-5296

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

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

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.

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 μM 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 μM, 4.4 μM or 13.3 μM), KN (4.7 μM or 13.9 μM) and ZEA (4.6 μM or 13.7 μM) in combination with NAA (0.54 μM); in the case of BA 2.2 μM we used NAA at 0.27 μM 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 μmol m^{-2} 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 germinabilty 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 μM and 13.3 μM BA in combination with 0.54 μM NAA; it was 80% when the BA concentration was decreased to 2.2 μM and NAA to 0.27 μM. The multiplication rate was highest (8.3 ± 1.0) for explants treated with 4.4 μM 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

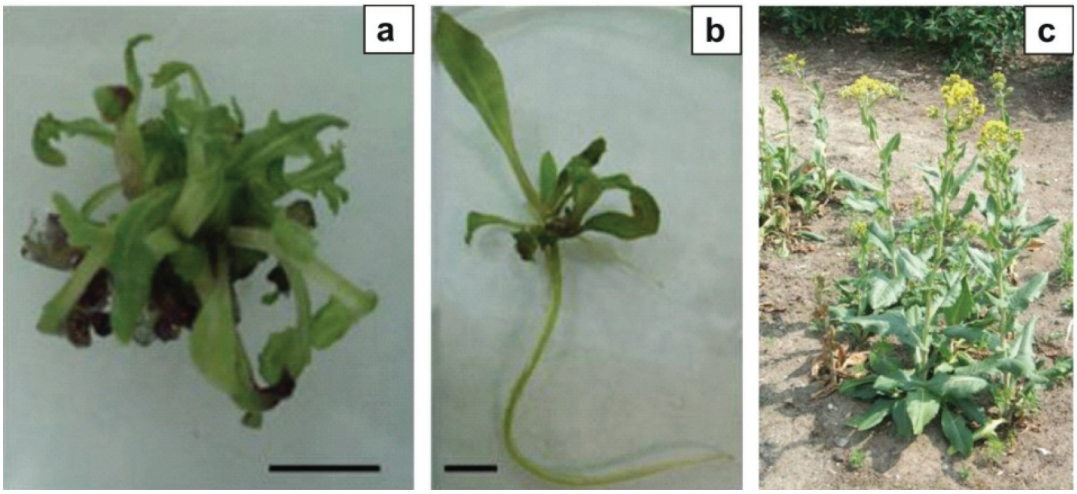

Fig. 1. Micropropagation of *Senecio macrophyllus*. **(a–b)** Multiple shoot formation on medium with 13.3 μM BA **(a)**, 13.9 μM KN **(b)** after 4 weeks. Bar = 10 mm. **(c)** Hardened flowering plants in field conditions after 2 years.

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 Jayabalan, 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 multiplication 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 *Asteracae* family: *Guizotia abyssinica* (Sujatha, 1997), *Centaurea paui* (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

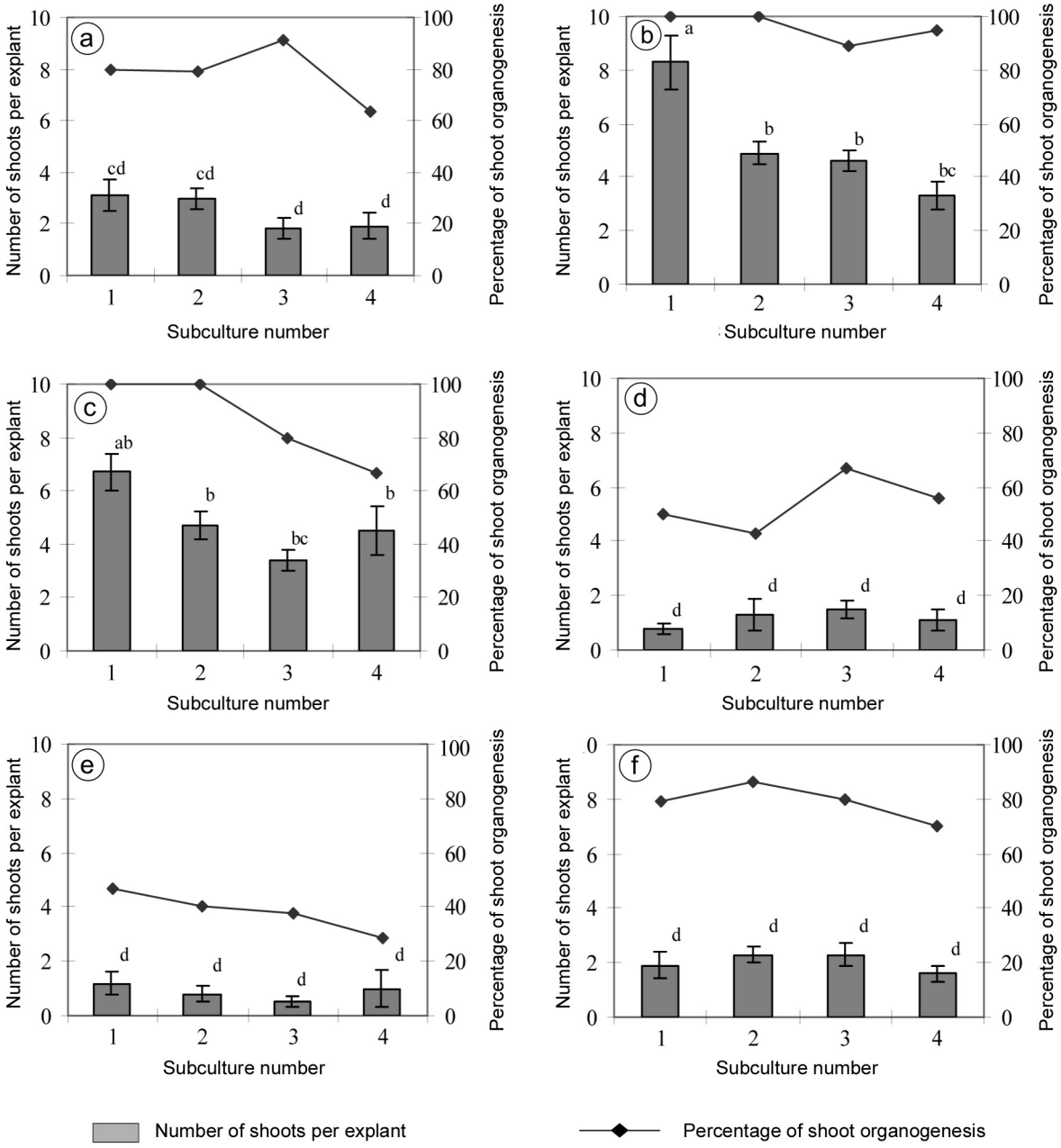

Fig. 2. Effect of different cytokinins in combination with NAA on multiplication rate and shoot organogenesis frequency in 4 subcultures of *S. macrophyllus*. **(a)** 2.2 μM BA and 0.27 μM NAA, **(b)** 4.4 μM BA and 0.54 μM NAA, **(c)** 13.3 μM BA and 0.54 μM NAA, **(d)** 4.7 μM KN and 0.54 μM NAA, **(e)** 4.6 μM ZEA and 0.54 μM NAA, **(f)** 13.7 μM ZEA and 0.54 μM NAA.

shoot (Joshi and Dhar, 2003). 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

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

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 GA_3-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

Baskaran P, and Jayabalan N. 2005. An efficient micropropagation system for *Eclipa alba* – a valuable medicinal herb. *In Vitro Cellular and Developmental Biology – Plant* 41: 532–539.

Birmeta G, and Welander M. 2004. Efficient micropropagation of Ensete ventricosum applying meristem wounding: a three-step protocol. *Plant Cell Reports* 23: 277–283.

Bramwell B. 1990. The role of in vitro cultivation in the conservation of endangered species. In: Hernandez Bermejo JE, Clemente M, Heywood V [eds.], *Conservation Techniques in Botanic Garden*, 3–15. Koeltz Scientific Books, Koenigstein.

Chaudhuri KN, Ghosh B, and Jha S. 2004. The root: a potential new source of competent cells for high-frequency regeneration in *Tylophora indica*. *Plant Cell Reports* 22: 731–740.

Cuenca S, Amo-Marco JB, and Parra R. 1999. Micropropagation from inflorescence stems of the Spanish endemic plant *Centaurea paui* Loscos ex Willk. (Compositae). *Plant Cell Reports* 18: 674–679.

Czarnecka B. 1995. Biology and ecology of the isolated population of *Senecio rivularis* (Waldst. et Kit.) DC. and *Senecio umbrosus* Waldst. et Kit., 27–39. Maria Curie-Skłodowska University Press, Lublin.

Czarnecka B. 2006. Large-scale vs. small-scale factors affecting flowering patterns in *Senecio macrophyllus* M. Bieb., a long-lived perennial, *Acta Agrobotanica* 59: 233–239.

Dhaka N, and Kothari SL. 2005. Micropropagation of *Eclipta alba* (L.) Hassk – an important medicinal plant. *In Vitro Cellular and Developmental Biology – Plant* 41: 658–661.

Emmanuel S, Ignacimuthu S, and Kathiravan K. 2000. Micropropagation of *Wedelia calendulacea* Less., a medicinal plant. *Phytomorphology* 50: 195–200

Evenor D, and Reuveni M. 2004. Micropropagation of *Achillea filipendulina* cv. 'Parker'. *Plant Cell, Tissue and Organ Culture* 79: 91–93.

Gertsson UE. 1988. Large-scale in vitro propagation of *Senecio x hybridus* Hyl. *Journal of Horticultural Science* 63: 131–136.

Grubisić D, Savikin-Fodulovic K, Misic D, Giba Z, and Konjevic R. 2004. *In vitro* stem elongation of stemless carline thistle. *Plant Growth Regulation* 44: 65–69.

Hazarika BN. 2003. Acclimatization of tissue-cultured plants. *Current Science* 85: 1704–1712.

Joshi M, and Dhar U. 2003. In vitro propagation of *Saussurea obvallata* (DC.) Edgew. – an endangered ethnoreligious medicinal herb of Himalaya. *Plant Cell Reports* 21: 933–939.

Kim KH, Park HK, Park MS, and Yea UD. 2001. Effects of auxin and cytokinin on organogenesis of soybean *Glycine max* L. *Journal of Plant Biotechnology* 3: 95–100.

Korach A, Juliani HR, Kapteyn J, and Simon JE. 2002. In vitro regeneration of *Echinacea purpurea* from leaf explants. *Plant Cell, Tissue and Organ Culture* 69: 79–83.

Minocha SC. 1987. Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. In: Bonga JM, Durjan DJ [eds.], *Cell and Tissue Culture in Forestry*, vol. 1, 50–66. Martinus Nijhoff Publishers, Dordrecht.

Misra P, and Dutta SK. 1999 *In vitro* propagation of white marigold (*Tagetes erecta* L.) through root tip proliferation. *Current Science* 77: 1138–1140.

Murashige T, and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 437–497.

Poonawala IS, Jana MM, and Nadgauda RS. 1999. Factors influencing bud break and rooting and mass-scale micropropagation of three *Phragmites* species: *P. karka, P. communis* and *P. australis*. *Plant Cell Reports* 18: 696–700.

Sujatha M. 1997. *In vitro* adventitious shoot regeneration for effective maintenance of male sterile niger [*Guizotia abyssinica* (L.f.) Cass.]. *Euphytica* 93: 89–95.

Sujatha M, and Reddy TP. 1998. Differential cytokinin effects on the stimulation of in vitro shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). *Plant Cell Reports*17: 561–566.

Sujatha G, and Ranjitha Kumari BD. 2007. Effect of phytohormones on micropropagation of *Artemisia vulgaris* L. *Acta Physiologica Plantarum* 29: 189–195.

Tutin FG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, and Webb DA. 1976. *Flora Europea*, vol. 4. Plantaginaceae to Compositae (and Rubiaceae), 196. Cambridge University Press, Cambridge – New York-Melbourne.

Tiwari V, Tiwari KN, and Singh BD. 2001. Comparative of cytokinins on *in vitro* propagation of *Bacopa monniera*. *Plant Cell, Tissue and Organ Culture* 66: 9–16.

Trejgell A, Dąbrowska G, and Tretyn A. 2009. In vitro regeneration of *Carlina acaulis* subsp. simplex from seedling explants. *Acta Physiologica Plantarum* 31: 445–453.

Vanegas PE, Cruz-Hernandez A, Valverde ME, and Peredes-López O. 2002. Plant regeneration via organogenesis in marigold. *Plant Cell, Tissue and Organ Culture* 69: 279–283.