http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 8 (5): 691-695, 2005
ISSN 1028-8880
© 2005 Asian Network for Scientific Information

In vitro Micropropagation of *Anthemis xylopoda* O. Schwarz, a Critically Endangered Species from Turkey

Bengi Erdağ and Yelda Emek
Department of Biology, Faculty of Science and Arts, Adnan Menderes University, 09010 Aydin, Turkey

Abstract: A micropropagation method was developed for *Anthemis xylopoda* O. Schwarz (Asteraceae), a critically endangered endemic species of Turkey to provide an efficient alternative propagation method. Seeds of *Anthemis xylopoda* which were collected from natural habitat were germinated on Murashige and Skoog medium (MS) supplemented with different concentrations of Gibberellic Acid (GA_3). The highest percentage germination was observed on MS medium with 1 mg L^{-1} GA_3. Shoots obtained from *in vitro* germinated seeds used as initial explant for shoot multiplication experiments. Shoot multiplication was achieved on MS medium with different cytokinins. The highest number of shoots per explant was obtained on MS medium containing 0.005 mg L^{-1} Thidiazuron (7.83 per explant). The highest mean of maximum shoot length was found MS without growth regulators and Kinetin (0.1 mg L^{-1}) at low concentration. Elongated shoots (~3 cm), excised from multiple shoot cultures were transferred to rooting media. Indole-3-butyric acid (IBA) was more effective than Indole-3-acetic acid (IAA) for the root induction. The best rooting result (60%) was obtained on the MS medium with 0.5 mg L^{-1} IBA. The plantlets with well developed root were transferred to *ex vitro* conditions. About 70% of the rooted shoots were successfully established in pots after hardening.

Key words: *Anthemis xylopoda*, cytokinins, endemic plant, micropropagation, *ex situ* conservation, TDZ

INTRODUCTION

Conservation of germ-plasm requires co-operation among a spectrum of different technologies. Falk[1] uses the term integrated strategies and discusses use of reserve, land restoration, botanic gardens and seed banks in this manner.

In the last two decades, micropropagation and other *in vitro* techniques have been widely used in conservation and propagation of wild plant species[2]. Many endangered species can be quickly propagated and preserved from a minimum of plant material, rendering low impact on wild populations by using this technology[3].

Anthemis xylopoda O. Schwarz, a stenoendemic of Turkey is in CR category in Red Data Book of Turkey[4]. There are only two populations including less than 400 individuals on two mountains of Western Turkey. Seçmen *et al*.[5] reported that number of individuals in these populations can be easily decreased under natural conditions if an useful conservation method is not applied for the species.

The main objective of present study was to develop an efficient rapid micropropagation protocol for *Anthemis xylopoda* O. Schwarz using seedlings from *in vitro* germinated seeds as an aid for the ongoing local *ex situ* conservation programs for this critically endangered species.

MATERIALS AND METHODS

Seeds of *Anthemis xylopoda* were collected during summer period (July-August) in 2003 from Mahmut mountain of province Izmir-Kemalpaşa, an altitude of 1200 m. Seeds were washed thoroughly under running tap water for 30 min. Subsequently, seeds were surface-sterilized for 10 min with 70% (w/v) ethanol followed by 4.5% (w/v) sodiumhypochloride for 15 min in laminar air flow cabinet and then the materials were washed with sterile distilled water (3-4 times) for 10 min. Shoots originated from 30-40 day old seedlings obtained in *in vitro* conditions from seeds of *A. xylopoda* collected from natural habitat were used as initial explants.

All the experiments were maintained on solidified basal medium. Basal medium contained Murashige and Skoog[6] mineral salts, 100 mg L^{-1} myo-inositol, 2 mg L^{-1} glisin, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} thiamine HCl, 30 g L^{-1} sucrose, 8 g L^{-1} agar (Agar-agar) and various concentrations of plant

Corresponding Author: Dr. Bengi Erdağ, Department of Biology, Faculty of Science and Arts, Adnan Menderes University, 09010 Aydin, Turkey
Tel: +90 256 2128498/2119 Fax: +90 256 2135379 E-mail: berdag@adu.edu.tr

growth regulators (GA_3, BA, KIN, TDZ, IAA and IBA) depending on experimental objectives.

The pH of media were adjusted to 5.8 before autoclaving at 121°C for 15 min. Filter-sterilized GA_3 was added to media after autoclaving and used for germination experiments.

In germination experiments, seeds were individually cultured on solidified MS basal medium in addition with 1, 2.5, 5 and 10 mg L^{-1} GA_3. A set containing MS basal medium without growth regulators was used as the control.

After 30 to 40 days of incubation, when the length of seedlings reached 1-2 cm, they were separated from primary roots and transferred to the multiplication medium (MS basal medium) supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar with addition of three different BA (0.05, 0.1 and 0.2 mg L^{-1}), KIN (0.05, 0.1 and 0.2 mg L^{-1}) or TDZ (0.005, 0.01 and 0.05 mg L^{-1}) concentrations or without growth regulators. In shoot multiplication experiments, cultures were subcultured three times and subculturing was periodically carried out at 4 weeks intervals.

Elongated shoots (~ 3 cm), excised from multiple shoot cultures were transferred to MS medium containing 30 g L^{-1} sucrose, 8 g L^{-1} agar supplemented with different concentrations of IBA (0.5, 1 and 1.5 mg L^{-1}) or IAA (0.5, 1 and 1.5 mg L^{-1}) or without growth regulators for rooting. The percentages of rooted shoots were recorded after 6 weeks.

After 9 weeks of rooting *in vitro*, the plantlets were removed from culture flasks, washed thoroughly with sterile distilled water and transplanted in 10 cm diameter plastic pots containing autoclaved Sand:Perlite (3:1 v/v) mixture. To maintain initially the plantlets at high humidity, pots were covered with clear plastic bags. Meanwhile, the plantlets were kept in culture room and watered with MS salts for 2 weeks. Plantlets were acclimatized to a reduced relative humidity by gradually opening the plastic cover. After two weeks the plantlets were completely uncovered and transferred in plastic pots containing soil:sand (1:1 v/v) mixture.

The culture environment for all experiments was maintained at 24±2°C with illumination provided by cool white fluorescent lamps at 40 $\mu E\ m^{-2}\ s^{-1}$ with a 16 h light period.

Each experiment was applied to 30 explants (shoot multiplication experiments) or 20 explants (germination and rooting experiments) following a completely randomized design. All experiments were repeated two times. The data were subjected to analysis of variance and means were compared using Duncan's Multiple-Range Test at $p < 0.05$.

RESULTS AND DISCUSSION

The sterilization procedure was successful. Sterile cultures were obtained in high proportion (98%) after three weeks of incubation. Radicle emergences were evaluated as primer indicator of seed germination. At 5th week of incubation, the highest percentage germination was observed in the presence of 1 mg L^{-1} GA_3 (Table 1). The percentage germination decreased with the increase of the GA_3 concentrations. The lowest germination value was observed in media without GA_3.

Plant hormones such as gibberellins (GA_s) are known to have an important role in the germination process[7] and gibberellins are generally synthesized by seeds. External application of gibberellins may break dormancy and promote development of seedling[8-12].

In all shoot multiplication experiments, an increase in number of shoots per explant have been observed at the end of 1st and 2nd subculture but it was decreased in 3rd subculture (Table 2). Thus, evaluations were based on shoot number at the end of 2nd subculture. A decrease in number of shoots was also reported in *Gerbera* cultivars by Vardja[13].

Although BA was evaluated as an effective cytokinin for shoot multiplication in many species of Asteraceae[14,15], 0.005 mg L^{-1} of concentration of TDZ (7.83±0.26 shoot/explant) gave the highest number of shoot per explant in this study (Fig. 1). The cytokinin-like compound TDZ has been used for micropropagation of many woody species, but has not been widely tested for members of the Asteraceae.

Fig. 1: Shoot multiplication on MS medium containing 0.005 mg L^{-1} TDZ. Scale Bar: 1 cm

Table 1: The effect of different concentrations of GA_3 on seed germination of *Anthemis xylopoda* after 40 days incubation. The means±Standard Deviation (SD) of two replicates

Medium	Germination (%)
MS	20±0.41
MS + 1 mg L^{-1} GA_3	65±0.49
MS + 2.5 mg L^{-1} GA_3	40±0.50
MS + 5.0 mg L^{-1} GA_3	30±0.47
MS + 10 mg L^{-1} GA_3	25±0.44

Table 2: Effect of different cytokinins and concentrations on shoot multiplication of *Anthemis xylopoda*. The means±Standard Error (SE) of two replicates

		Number of shoots per explant			
Cytokinins	mg L^{-1}	1st subculture	2nd subculture	3rd subculture	Maximum shoot length (cm)
Control	0	1.10±0.11d	1.45±0.20e	1.63±0.30de	4.40±0.36a
BA	0.05	1.70±0.12c	3.30±0.44cd	3.20±0.53bc	2.46±0.30bc
	0.1	2.13±0.15bc	3.53±0.39bcd	2.67±0.38cd	2.30±0.25cd
	0.2	2.80±0.23a	3.73±0.38bcd	2.63±0.42cd	2.13±0.18cd
KIN	0.05	2.27±0.15b	2.87±0.27d	2.70±0.40cd	2.13±0.23cd
	0.1	2.07±0.15bc	4.47±0.45b	4.03±0.53b	3.06±0.20b
	0.2	2.27±0.17b	3.92±0.30bcd	2.40±0.30cde	2.73±0.26bc
TDZ	0.005	2.90±0.14a	7.83±0.26a	6.40±0.34a	2.66±0.15bc
	0.01	2.13±0.16bc	4.33±0.48bc	3.07±0.38bc	2.03±0.23cd
	0.05	2.13±0.12bc	2.90±0.18d	1.37±0.14e	1.70±0.10d

In each column, the means with different letter(s) are significantly different (Duncan's Multiple-Range Test, $p<0.05$)

Table 3: Effect of IAA and IBA on *in vitro* rooting, number of roots per explant, longest root and maximum shoot length of *Anthemis xylopoda* on Murashige and Skoog (MS) medium. Data (mean±Standard Error, SE) were recorded as two replicate after 6 weeks of culture

Auxins	mg L^{-1}	Rooting (%)	Number of roots per explant	Longest root (cm)	Maximum shoot length (cm)
Control	0	15	0.45±0.31b	0.70±0.39b	2.77±0.15b
IAA	0.5	30	1.15±0.48ab	2.60±0.97ab	1.85±0.41bc
	1.0	20	0.75±0.43b	1.10±0.52b	1.40±0.34c
	1.5	30	1.35±0.56ab	1.50±0.56b	1.75±0.41bc
IBA	0.5	60	2.50±0.63a	4.15±1.03a	4.10±0.42a
	1.0	55	1.60±0.50ab	1.95±0.52b	4.60±0.49a
	1.5	50	1.80±0.52ab	1.82±0.50b	2.75±0.25b

In each column, the means with different letter(s) are significantly different (Duncan's Multiple-Range Test, $p<0.05$)

Fig. 2: Root development on MS medium containing 0.5 mg L^{-1} IBA. Scale Bar: 0.5 cm

Fig. 3: A well developed root system on MS medium containing 0.5 mg L^{-1} IBA. Scale Bar: 0.5 cm

Little callus formation at bases of multiple shoots were observed in all experimented medium. Giusti *et al.*[16] reported that TDZ stimulates shoot proliferation but it cause hyperhydrioty and callus induction in higher concentrations. In this study, relatively higher concentration of TDZ (0.05 mg L^{-1}) decreased number of shoots per explant. Additionally, vigorous callus formation at bases of shoots were observed but no hyperhydrioty problem was occurred in that concentration. No significant differences were found among other concentrations of BA, KIN and TDZ. A very low increase was also observed in media without growth regulators.

The highest mean of maximum shoot length was found MS without growth regulators and with KIN (0.1 mg L^{-1}) at low concentration (respectively, 4.40±0.36 and 3.06±0.20). It is accordant with a report Cuenca and Amo-Marco[17] for *Centaurea spachii* Bip ex Wilk. and Lange. Elongated shoots (~3 cm), excised from multiple shoot cultures were transferred to rooting media.

Rhizogenesis was occurred in all media that used in present study. The highest value of rooting was obtained in the medium containing 0.5 mg L^{-1} IBA (Table 3 and Fig. 2). Pevalek-Kozlina[18] reported similar result with same auxin concentrations in half-strength MS medium for *Centaurea ragusina* L. This auxin concentration was useful also both root system development and shoot quality (Fig. 3). Additionally, flower bud formation was also observed in the same media (Fig. 4). Rooting percentages were lower in all media containing IAA. IBA is more resistant than IAA to chemical degradation in tissue culture media, both during autoclaving and at room temperature[19,20].

Fig. 4: Flower buds on MS medium supplemented with 0.5 mg L^{-1} IBA. Scale Bar: 0.5 cm

Fig. 5: An acclimatized plant, one month after hardening

Robust yellow-green calli formations were observed at shoot bases in medium with IAA. The robust calli probably inhibited further shoot development and shoots died as a result of this inhibition. Shoots in rooting media also showed multiplication in low rate (Data not shown).

The plantlets with well developed root were transferred to *ex vitro* conditions described in materials and methods (Fig. 5). Percentage of survival of shoots was approximately 70%. Small shoots showed a low rate of survive after transplanting or died during acclimatization.

To date, there is no report on micropropagation of *Anthemis xylopoda* O. Schwarz. The present study is the first report on micropropagation of this rare and critically endangered species using seedlings from *in vitro* germinated seeds and aims to contribute ongoing *ex situ* conservation programs.

REFERENCES

1. Falk, D.A., 1990. Integrated strategies for conserving plant genetic diversity. Ann. Missouri Bot. Gard., 77: 38-47.
2. Fay, M.F., 1992. Conservation of rare and endangered plants using *in vitro* methods. *In vitro* Cell Dev. Biol., 28: 1-4.
3. Cuenca, S., J.B. Amo-Marco and R. Para, 1999. Micropropagation from inflorescense stems of the Spanish endemic plant *Centaurea paui* Loscos ex Wilk. (Compositae). Plant Cell Reports, 18: 674-679.
4. Ekim, T., M. Koyuncu, M. Vural, H. Duman, Z. Aytaç and N. Adigüzel, 2000. Red Data Book of Turkish Plants (Pteridophyta and Spermatophyta).Turkish Association for the Conservation of Nature. Ankara, pp: 64.
5. Seçmen, Ö., A. Güvensen and E. Çetin, 2004. Conservation studies of *Anthemis xylopoda* O. Schwarz population on Mahmut mountain (Kemalpaşa-Izmir). Proc. XVII. Natl. Biol. Congr. 21-24 June, Adana, Turkey, pp: 40.
6. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.
7. Ritchie, S. and S. Gilroy, 1988. Gibberellins: Regulating genes and germination. New Phytologist, 140: 363-383.
8. Metzger, J.D., 1983. Promotion of germination of dormant weed by substituted phtalimides and gibberellic acid. Weed Sci., 31: 285-289.
9. Pitel, J.A. and B.S.P. Wang, 1988. Improving germination of basswood (*Tilia americana* L.) seeds with gibberellic acid. Seed Sci. Technol., 16: 273-280.
10. Karssen, C.M., S. Zagorski, J. Kepczyski and S.P.C. Groot, 1989. Key role for endogenous gibberellins in the control of seed germination. Ann. Bot., 63: 71-80.
11. Dunand, R.T., 1992. Enhancement of Seedling Vigor in Rice (*Oryza sativa* L.) by Seed Treatment With Gibberellic Acid. In: Karssen, C.M., L.C. Van Loon and D. Vreugdenhil (Eds.), Kluwer Acad. Pub., London, pp: 835-841.
12. Lecat, S., F. Corbineau and D. Come, 1992. Effects of gibberellic acid on the germination of dormant oat (*Avena sativa* L.) seeds as related to temperature, oxygen and energy metabolism. Seed Sci. Technol., 20: 421-433.
13. Vardja, R. and T. Vardja, 2001. The effect of cytokinin type and concentration and the number of subcultures on the multiplication rate of some decorative plants. Proc. Estonian Acad. Sci. Biol. Ecol., 50: 22-32.
14. Ruffoni, B. and F. Massabo, 1991. Tissue culture in *Gerbera jamesonii* hybrida. Acta Horti., 289: 147-148.
15. Hammatt, N. and P.K. Evans, 1985. The *in vitro* propagation of an endangered species: *Centaurea junoniana* Svent. (Compositae). J. Hortic. Sci., 60: 93-97.

16. Giusti, P., D. Vitti, F. Fiocchetti, G. Colla, F. Saccardo and M. Tucci, 2002. *In vitro* propagation of three endangered cactus species. Sci. Hortic., 95: 319-332.
17. Cuenca, S. and J.B. Amo-Marco, 2000. *In vitro* propagation of *Centaurea spachii* from inflorescence stems. Plant Growth Regulation, 30: 99-103.
18. Pevalek-Kozlina, B., 1998. *In vitro* propagation of *Centaurea ragusina* L: A Crotian endemic species. Acta Biologica Cracoviensia series Botanica, 40: 21-24.
19. Dunlap, J.R. and K.M. Robacker, 1988. Nutrient salts promote ligh-induced degradation of indole-3-acetic acid in tissue culture media. Plant Physiol., 88: 379-382.
20. Nissen, S.J. and E.G. Sutter, 1990. Stability of IAA and IBA in nutrient medium to several tissue culture procedures. Hortscience, 25: 800-802.