Asia Pacific Journal of Molecular Biology and Biotechnology, 2004
Vol. 12 (1&2) : 21-25

In Vitro Micropropagation of the aquarium plant *Ludwigia repens*

Meryem Öztürk[1], Khalid Mahmood Khawar[2], Hasan Hüseyin Atar[1],
Cengiz Sancak[2] and Sebahattin Özcan[2*]

[1]*Department of Fisheries, Faculty of Agriculture, University of Ankara, 06110 Diskapi, Ankara, Turkey*
[2]*Department of Field Crops, Faculty of Agriculture, University of Ankara, 06110 Diskapi, Ankara, Turkey*

Received 25 November 2003 / Accepted 20 May 2004

Abstract. Apical meristems, first, second and third-fourth axillary buds of *Ludwigia repens* were cultured on Murashige and Skoog (MS) medium containing various concentrations of 6-benzylaminopurine (BAP), thidiazuron (TDZ) and α-naphthaleneacetic acid (NAA) for micropropagation. Micropropagation was best achieved from apical meristem on MS medium containing 0.05 mg dm^{-3} TDZ and 0.1 mg dm^{-3} NAA. It was noted that TDZ or BAP had inhibitory effect on shoot elongation, which was overcome by subculturing shoots on half-strength MS media without growth regulators after 4 weeks of culture. This also served as rooting media. Rooted plantlets were finally transferred to aquariums containing fresh water with 100% adaptation.

Keywords. ***Ludwigia repens*, micropropagation, thidiazuron (TDZ), 6-benzylaminopurine, and carry over effect**

Abbreviations: MS : Murashige and Skoog; TDZ : thidiazuron [1 Phenyl 3-(1,2,3-thiadiazol -5YL) urea]; BAP : 6-benzylaminopurine; NAA : α-naphthaleneacetic acid

INTRODUCTION

Ludwigia repens, belonging to the family *Onargaceae,* is an evergreen amphibian herbaceous plant which is largely found in Southern parts of North America. *Ludwigia* sp. is widely distributed in America, Africa, Asia and Australia (Rataj and Horeman, 1977) and is mainly used for filtration and cleaning of water in canals and lakes. Some of the species belonging to *Ludwigia* genus are used as vegetables, ornamental aquarium plants, pollen source for honey bees, fish feed and medicinal purposes (Brunson, 1988; Chen et al., 1989; Greenway and Wooley, 1999; Mooi et al., 1999; Kuo et al., 1999; Brundu et al., 2001). *L. repens* with small yellow flowers (Cirik et al., 2001) and pinkish red to bright green leaves is widely used as an aquarium plant. It grows rapidly in slightly acidic waters at 19-28 °C (Rataj and Horeman, 1977).

L. repens is propagated from cuttings without ensuring genetic uniformity by amateur workers, which results in production of undesired phenotypes and subsequently influence the quality and regeneration potential of the plants; as they select the plants randomly without taking necessary care. This results in negative economic implications for mass production of this important aquarium plant. In spite of a considerable progress in developing *in vitro* micropropagation protocols in a variety of land plants (Khawar and Özcan, 2002; Özcan et al., 1996; Özcan et al., 1993), aquatic plants in general have lagged behind to a considerable extent. *In vitro* micropropagation of *Ludwigia repens* has not been reported previously. The present study describes a rapid, simple and efficient micropropagation system from several explants of *L. repens.* Culture conditions described here may also be applicable for *in vitro* micropropagation of other plants belonging to *Ludwigia* species.

MATERIALS AND METHODS

Plants of *Ludwigia repens* were obtained from local dealers of commercial aquarium plants by ensuring that they were visibly healthy and free from any signs of stress or surface blemishes. The selection was further carried out by growing the plants under environmentally controlled fresh water aquariums at 28 ± 2°C with 12-h photoperiod.

For surface-sterilization, plants were first scrubbed gently under running tap water for 0.5 h to remove coating layer of microorganisms ubiquitously found on them. Upper portions of the shoot twigs containing apical meristem through fourth axillary bud were isolated and submerged for 20 min in 30% commercial bleach (Axion) with 1-2 drops

*Author for Correspondence.
Mailing address: Department of Field Crops, Faculty of Agriculture, University of Ankara, 06110 Dýþkapý, Ankara, Turkey. Tel: +90-312-3179815; Fax: +90-312-3179815; E-mail: ozcan@agri.ankara.edu.tr

Table 1. Shoot regeneration from different explants of *Ludwigia repens* after 8 weeks in culture on MS media supplemented with various concentrations of 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA) and thidiazuron (TDZ).

Growth reg. (mg dm^{-3})			Frequency (%) of shoot regeneration				Number of shoots per explant[2]			
BAP	NAA	TDZ	Apic. mer.	1st bud	2nd bud	3rd-4th bud	Apic. mer.	1st bud	2nd bud	3rd-4th bud
0	0	0	87.50^{a1}	100.00^{a}	16.75^{bc}	4.75^{b}	2.25^{c}	1.94^{e}	4.19^{d}	1.19^{c}
0.1	0.1	0	52.50^{bc}	100.00^{a}	21.50^{bc}	8.75^{ab}	2.94^{c}	4.31^{cd}	5.36^{cd}	2.19^{bc}
0.2	0.1	0	37.50^{c}	56.25^{d}	17.50^{bc}	19.00^{a}	1.81^{c}	3.06^{de}	4.38^{d}	4.75^{a}
0.3	0.1	0	68.75^{b}	81.25^{bc}	14.00^{c}	18.25^{ab}	3.69^{c}	4.75^{cd}	3.50^{d}	4.56^{a}
0	0.1	0.05	100.00^{a}	87.50^{abc}	30.25^{ab}	20.50^{a}	12.31^{a}	10.00^{a}	7.56^{a}	5.12^{a}
0	0.1	0.1	100.00^{a}	68.75^{cd}	38.50^{a}	1.75^{b}	10.69^{a}	9.63^{a}	5.94^{bc}	0.44^{c}
0	0.1	0.15	100.00^{a}	93.75^{ab}	27.00^{abc}	14.50^{b}	7.31^{b}	6.81^{b}	6.75^{bc}	3.63^{ab}

Each value is the mean of 4 replicates each with 4 explants.
[1]Values with in a column followed by different letters are significantly different at 0.01 level using Duncan's Multiple Range Test.
[2]From explants which regenerated shoots

Table 2. *In vitro* rooting of regenerated shoots in *Ludwigia repens* after 4 weeks in half-strength MS medium

Reg. medium Growth reg. (mg dm^{-3})			Root no./ shoot				Root length (cm) /shoot			
BAP	TDZ	NAA	Apic. mer.	1st bud	2nd bud	3rd-4th bud	Apic. mer.	1st bud	2nd bud	3rd-4th bud
0	0	0	8.89^{aA1}	6.56^{aAB}	4.22^{aB}	8.56^{aAB}	5.11^{aA}	4.28^{aA}	3.83^{aA}	4.89^{aA}
0.3	0	0.1	7.67^{aA}	2.11^{bB}	4.67^{aAB}	4.11^{bAB}	4.39^{aAB}	2.56^{aB}	4.89^{aA}	3.44^{abAB}
0	0.05	0.1	6.00^{aA}	7.00^{aA}	4.44^{aA}	2.67^{bB}	3.78^{aA}	3.83^{aA}	4.28^{aA}	2.67^{bB}

Each value is the mean of 4 replicates each with 4 explants.
[1]Values with in a column followed by different lowercase letters and values within a row followed by different uppercase letters are significantly different at 0.05 level
using Duncan's Multiple Range Test.

of Tween 20 and continuous stirring. Stirring dispersed the air bubbles adhering to explants and facilitated even distribution of the disinfectant. After discarding bleach, the explants were washed 3 times with sterile water. Apical meristems, first, second and third-fourth axillary buds from shoot tips were excised and cultured on to shoot regeneration media in Magenta GA7 vessels for 4 weeks. Explants were then cultured to subculture medium for 4 weeks. The number of explants producing shoots and the number of shoots per explant were scored after 8 weeks of culture. Well developed shoots were isolated and rooted in rooting medium. Rooted plantlets were transferred to fine sand in aquariums (100 x 30 x 40 cm) with 4 cm spacing at 28 ± 2°C water (pH 6.0) temperature with 12 h photoperiod for 3 months.

The shoot regeneration media consisted of MS mineral salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.7% agar (Sigma agar type A), 0.1-0.3 mg dm^{-3} 6-benzylaminopurine (BAP), 0.1 mg dm^{-3} α-naphthaleneacetic acid (NAA) or 0.05-0.15 mg dm^{-3} thidiazuron (TDZ) and 0.1 mg dm^{-3} NAA (Table 1). Subculture and rooting medium consisted of half-strength MS medium, 3% sucrose and 0.7% agar. The pH of the medium was adjusted to 5.6 with 1N NaOH or 1N HCl before autoclaving at 1.4 kg/cm^{2} and 121 °C for 20 min. All cultures were incubated at 24 ± 1°C under cool white fluorescent light (35 μmol photons m^{-2} s^{-1}) with 16 h photoperiod.

Each treatment had 4 replicates containing 4 explants for both micropropagation and rooting experiments and was repeated twice. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range test using MSTAT-C computer program (Michigan State University). Data given in percentages were subjected to arcsine ($\sqrt{X}$) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS AND DISCUSSION

Shoot regeneration. Any micropropagation system must produce large number of genotypically uniform plants similar to the original plant from which they were propagated. This aim could be easily achieved using meristematic regions. After surface sterilization, apical meristem, first, second and third-fourth axillary buds from shoot tips of the *L. repens* were subjected to different concentrations of BAP, NAA or TDZ, NAA. Shoot initials were clearly visible on all explants within 6-8 d, which subsequently developed into

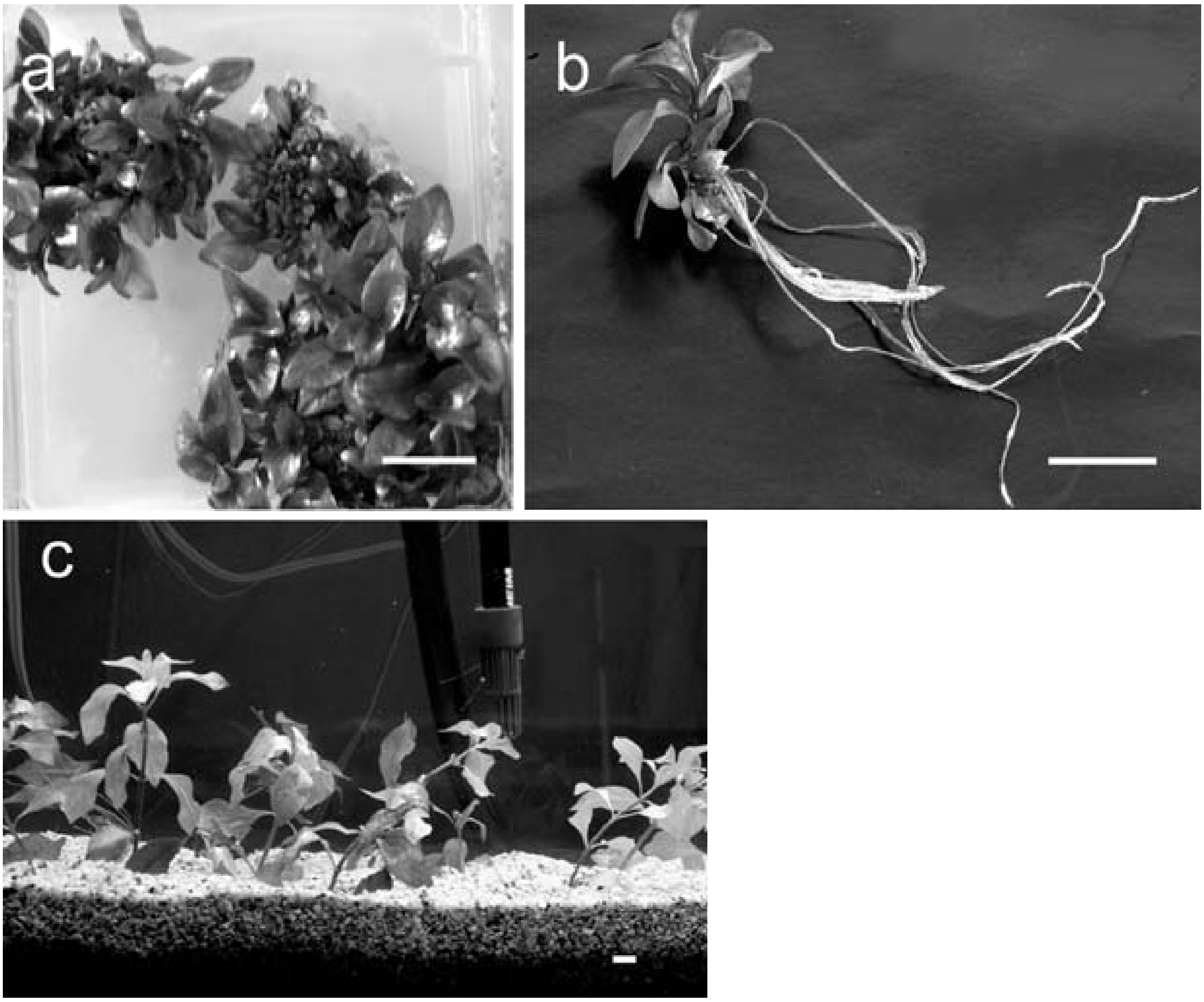

Figure 1. *In vitro* micropropagation of *Ludwigia repens* (a) Four weeks old regenerated shoots on MS medium containing 0.05 mg dm^{-3} Thidiazuron and 0.1 mg dm^{-3} α-naphthaleneacetic acid (b) rooting on ½ strength MS medium (c) successful establishment of rooted plants in aquariums. Bar= 1 cm.

normal shoots after 7-8 weeks of culture initiation. This shoot regeneration was accompanied with a minute callus formation at the cut ends of explants touching the media containing TDZ and NAA.

Analysis of variance test revealed that explants and growth regulators interacted significantly with respect to the percentage of explants producing shoots and mean number of shoots per explant ($p<0.01$). All apical meristems produced shoots on media containing any concentration of TDZ with 0.1 mg dm^{-3} NAA; whereas, shoot regeneration was not consistent on media supplemented with BAP and NAA (Table 1). Moreover, shoot regeneration frequency was higher in apical meristems and first node on all media compared to second and third-fourth bud. This is reduced in descending order from apical meristem to the third-fourth axillary buds (Table 1).

The pattern of shoot development was dissimilar between the media containing BAP and NAA or TDZ and NAA. TDZ resulted in production of meristematic areas adjacent to the cut end of meristematic regions and produced numerous shoots. Extremely low TDZ concentrations were more effective for shoot proliferation and even a slight increase resulted in reduction of shoot proliferation. In general, TDZ and NAA combinations were more effective in all explants compared to BAP and NAA for shoot initiation. The results also revealed that apical meristems were the best and the third-fourth axillary buds are the poorest explants in terms of shoot regeneration. In case of all explants, the highest number of shoots per explant was achieved from apical meristems on MS medium containing 0.05 mg dm^{-3} TDZ and 0.1 mg dm^{-3} NAA.

It was found that shoots were longer and fewer with green and wider leaves on growth regulator-free MS media than media containing any concentration of BAP or TDZ. Especially, presence of TDZ in the media resulted in formation of clustered short shoots with very small leaves. Very similar observation was also described in *Cercis canadensis* var *alba* (Yusnita et al., 1990) and *Hibiscus rosa- sinensis* (Preece et al., 1987). Similarly Pijut et al. (1991) found that number of shoots in *Pinus strobus* L increased but their elongation ceased with higher levels of TDZ. The reason for high activity of low concentration of TDZ has not been investigated in amphibian plants. We assume that TDZ is persistent in the plant tissue and presumably metabolize in a manner similar to that reported for *Phaseolus* (Mok and Mok, 1985). They found that even when bean callus was cultured on medium with [^{14}C]-thidiazuron for 33 d, most of the label remained in TDZ molecule. A portion of TDZ was glycolsylated by the bean tissue, possibly to inactivate the compound for storage.

Since TDZ and BAP stimulate shoot proliferation tremendously, they have been used for micropropagation of many plant species. We observed pronounced inhibition or suppression in elongation and growth of regenerated shoots after 2-3 weeks on all explants on media containing any concentration of TDZ or BAP. This suppressive activity was more severe on media containing TDZ and NAA with numerous shoots bearing small green red-shaded leaves than BAP and NAA containing media or growth regulators-free media which had fewer shoots with large leaves. This negative effect on shoot elongation was reduced and minimized by transferring regenerated shoots on secondary medium containing half-strength MS medium lacking growth regulators after 4 weeks of culture, where growth accelerated. This suggests that presence of TDZ or BAP may facilitate shoot proliferation but is not essential during the growth and elongation of shoots. Similar results were also obtained in apple (Fasolo et al., 1989), pear (Singha and Bhatia, 1988), populus (Russel and McCown, 1986) and rhododendron (Preece and Imel, 1991).

Rooting development in regenerated shoots. Regenerated shoots (10-20 mm in length) were excised from the explants previously cultured on growth regulator-free MS, MS medium containing 0.05 mg dm^{-3} TDZ and 0.1 mg dm^{-3} NAA or 0.3 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA and transferred to half-strength MS medium without growth regulators. All regenerated shoots rooted on this medium within four weeks (Figure 1b) and it is believed that rooting of regenerated shoots is difficult because of a "carry over effect" from cytokinins in the shoot proliferation medium (Huetteman and Preece, 1993). We found that TDZ or BAP in regeneration medium did not inhibit the frequency of rooting as reported by Fasolo et al. (1989), Yunsita et al. (1990) and Preece et al. (1991). However, shoots regenerated on media containing BAP or TDZ had significantly reduced number of roots, compared to the control. The phenomenon was not consistent for root length. In general, root length was shorter on shoots regenerated on media containing TDZ compared to those regenerated on media containing BAP in all explants except first bud where the reverse was true (Table 2). This result may be attributed to "carry over effect" from cytokinins in the regeneration medium. Rooted plantlets were transferred to aquariums and later established with 100% success (Figure. 1c). After 4-5 weeks, shade of redness reduced or diminished completely with uniform green leaves and roots.

In conclusion, to our knowledge the present study is the first report for *in vitro* shoot regeneration of *Ludwigia repens*. The procedure described here provides a rapid and prolific micropropagation system that may also be applicable to other species belonging to *Ludwigia* genus with small modifications.

ACKNOWLEDGEMENT

The authors are thankful to the University of Ankara and State Planning Commission of Turkey (DPT) for financial support (Project No. 2001 K 120240).

REFERENCES

Brundu, G., Brock, J., Camarda, I., Child, L. and Wade, M., 2001. Plant Invasions-Species Ecology and Ecosystem Management. Leiden, The Netherlands: Backhuys Publishers.

Brunson, M.W., 1988. Forage and feeding systems for commercial crawfish culture. *J. Shellfish Res. 7: 210.*

Chen, C.P., Lin, C.C. and Namba, T., 1989. Screening of Taiwanese crude drugs for antibacterial activity against Streptococus mutants. *J. Ethnopharmacol. 27: 285-295.*

Cirik, S., Cirik, S. and Dalay, M.C., 2001. Içsu Bitkilerinin Biyolojisi, Ekolojisi, yetiþtirme Teknikleri. Su Bitkileri II. Ege Üniv., Su Ürünleri Fak., Yayin No. 61, Izmir, Turkiye (Biology Ecology and Growing Techniques of fresh water plants, Water Plants II. Publication No. 61 of Faculty of Fishries, Aegean Univ. Izmir, Turkey) (In Turkish).

Fasolo, F., Zimmerman, R.H. and Fordham, I., 1989. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. *Plant Cell Tiss. Org. Cult. 16: 75-87.*

Greenway, M. and Wooley, A., 1999. Constructed wetlands in Queensland: Performance efficiency and nutrient bioaccumulation. *Ecological Eng. 12: 39-55.*

Huetteman, C.A. and Preece, J.E., 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tiss. Org. Cult. 33: 105-119.*

Khawar, K.M. and Özcan, S., 2002. High frequency shoot induction from cotyledonary node explants of different lentil (*Lens culinaris* Medik) genotypes and *in vitro* micrografting. *Biotechnol. & Biotechnol. Equipment. 16: 12-17.*

Kuo, Y.C., Sun, C.M., Tsai, W.J., Ou, T.C., Chen, W.P. and Lin, C.Y., 1999. Blocking of cell proliferation, cytokines production and gene expression following administration of Chinese herbs in the human mesangial cells. *Life Sci. 64: 2089-2099.*

Mok, M.C. and Mok, D.W.S., 1985. The metabolism of [^{14}C]-thidiazuron in callus tissue of *Phaseolus lunatus. Physiol. Plant. 65: 427-432.*

Mooi, L.Y., Ali, A.M., Norhanom, A.B., Salleh, K.M., Murakami, A. and Koshimizu, K., 1999. Anti tumor promoting activity of some Malaysian traditional vegetables (Ulam). *Nat. Prod. Sci. 5: 32-37.*

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant. 15: 473-497.*

Özcan, S., Barghchi, M., Firek, S. and Draper, J., 1993. Efficient adventitious shoot regeneration and somatic embryogenesis in pea. *Plant Cell, Tissue and Organ Culture.* 34: 271-277.

Özcan, S., Sevimay, C.S., Yildiz, M., Sancak, C. and Özgen, M., 1996. Prolific shoot regeneration from immature embryo explants of sainfoin (*Onobrychis viciifolia* Scop.). *Plant Cell Reports. 16: 200-203.*

Pijut, P.M., Michler, C.H. and Voelker, T.M., 1991. Effects of embryo explant orientation, Thidiazuron and agar on eastern white pine (*Pinus strobus* L.) adventitious shoot initiation. *In* Proc. Intl. Symposium on Applications of Biotechnology to tree culture, protection and Utilisation. , p 126. Columbus, OH.

Preece, J.E., Huetteman, C.A., Ashby, W.C. and Roth, P.L., 1991. Micro and cutting propagation of silver maple. I. Results with adult and juvenile propagules. *J. Amer. Soc. Hort. Sci. 116: 142-148.*

Preece, J.E., Huetteman, C.A., Puello, C.H. and Neuman, M.C., 1987. The influence of Thidiazuron on *in vitro* culture of woody plants. *Hort. Science. 22: 1071.*

Preece, J.E. and Imel, M.R., 1991. Plant regeneration from leaf explants of *Rhododenderon,* P.J.M. Hybrids. *Scientia Hort. 48: 159-170.*

Rataj, K. and Horeman, T.J., 1977. Aquarium plants, their identification, cultivation and ecology. New Jersey: T. F.H. publications, Inc.

Russel, J.A. and McCown, B.H., 1986. Thidiazuron stimulated shoot differentiation from protoplast- derived calli of *Populus.* VI Intl. Congr. Plant Tissue Cell Culture Abstracts. p 49.

Singha, S. and Bhatia, S.K., 1988. Shoot proliferation of pear cultures on medium containing thidiazuron and Benzylaminopurine. *Hort. Sci. 23: 802.*

Snedecor, G.W., Cochran, W.G., 1967. Statistical Methods. Iowa, USA: The Iowa State University Press.

Yusnita, S., Geneve, R.L. and Kester, S.T., 1990. Micropropagation of white flowering eastern redbud (*Cercis canadensis* var. *alba* L.). *J. Environ. Hort. 8: 177-179.*