BIOLOGIA PLANTARUM 53 (4): 750-754, 2009

BRIEF COMMUNICATION

An efficient *in vitro* regeneration system for *Lythrum salicaria*

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Abstract

This report describes an efficient plant regeneration system for the medicinal plant *Lythrum salicaria via* direct adventitious shoot development from leaf and stem explants. Leaf explants were much more responsive to regeneration than stem segments. Of the hormonal combinations tested, those involving thidiazuron (TDZ; 0.1, 0.3 or 0.5 mg dm^{-3}) were more effective than the combinations of other hormones and 0.1 mg dm^{-3} TDZ combined with either indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was the most productive. Rooting was readily achieved when multiple shoots were singled out and cultured on medium containing different auxins. IAA was the most effective on root development in terms of both the number of roots per shoot and the frequency of rooted shoots. More than 90 % of the regenerants survived after hardening for four weeks at gradually decreased air humidity.

Additional key words: auxins, direct adventitious shoot development, leaf explants, purple loosestrife, stem explants, thidiazuron.

Lythrum salicaria L. (purple loosestrife) is a perennial wetland herb belonging to the family *Lythraceae*. It has a widespread circumpolar distribution throughout the northern hemisphere with the exception of extremely cold and arctic regions. Micropropagation of many medicinal plant species has been achieved through various tissue culture techniques. In many cases, actively growing shoot-tips or axillary buds, both of which already contain *de nova* primordia, were used as a starting material (Heuser 1982, Heuser 1983, Rout *et al*. 2000, Kalia *et al*. 2007, Loureiro *et al*. 2007, Mallikarjuna and Rajendrudu 2007, Vasudevan *et al*. 2007). The present study describes, to our knowledge for the first time, an efficient *in vitro* plant regeneration protocol for *L. salicaria via* direct adventitious shoot development from leaf explants cultured on medium containing different concentrations and combinations of various plant growth regulators.

Seeds of *L. salicaria* were collected from the vicinity of Lake Abant, Bolu, Turkey in 2006. Seeds were washed with an anti-bacterial soap, rinsed with distilled water and surface sterilized by shaking in 70 % ethanol for 20 min, rinsed well with sterilized distilled water and then dipped into 20 % *Domestos*® (5 % sodium hypochloride) for 25 min, finally followed by washing with sterile distilled water three times. Sterilized seeds were germinated in plastic Petri dishes containing Murashige and Skoog (1962) medium with minimum organics (MSMO) (*Sigma*, St. Louis, MO, USA) supplemented with 30 g dm^{-3} sucrose, 8 g dm^{-3} *Difco-Bacto* agar (pH 5.7, autoclaved for 20 min at 121 °C and 105 kPa). After a one-week incubation on this medium, seedlings were transferred to Magenta containers (*GA-7*, *Sigma*) containing the same medium for an additional three weeks. For shoot regeneration, leaf lamina pieces (5 × 5 mm) and stem internode segments (5 mm) explants were excised from 4-week-old sterile seedlings and placed in plastic Petri dishes containing 20 cm^3 MSMO medium supplemented with different combinations and concentrations of plant growth regulators; thidiazuron (TDZ; 0.01, 0.05, 0.1, 0.3, 0.5 or 1.0 mg dm^{-3}) and indole-3-acetic acid

Received 3 January 2008, *accepted* 5 May 2008.

Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellin; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MSMO - Murashige and Skoog medium with minimum organics; NAA - naphthaleneacetic acid; TDZ - thidiazuron.

Acknowledgements: The authors are grateful to The Scientific and Technological Research Council of Turkey (TUBITAK) for financial support [TBAG-2278(103T024) and TBAG-HD/27(105T040)]. Special thanks are expressed to Dr. Hakan Turker for his technical support and Dr. Kemal Kazan (CSIRO Plant Industry, Australia) for the critical reading of the manuscript.

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(IAA; 0.1, 0.5, 1.0 or 2.0 mg dm^{-3}) and TDZ (0.1, 0.5, 1.0 or 3.0 mg dm^{-3}) and indole-3-butyric acid (IBA; 0.1, 0.5, 1.0 or 3.0 mg dm^{-3}). After three weeks of incubation on these media, regenerated shoots were transferred to Magenta boxes containing the same MSMO media for shoot elongation for two additional weeks. Then, well-developed shoots (3 - 4 cm in length) were singled out and placed on a rooting medium containing MSMO medium supplemented with varying concentrations of different auxins: IAA (0.5, 1.0 or 3.0 mg dm^{-3}), IBA (0.5, 1.0 or 3.0 mg dm^{-3}), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1, 0.3, 0.5 or 1.0 mg dm^{-3}) or naphthaleneacetic acid (NAA; 0.1, 0.5, 1.0, 2.0 or 4.0 mg dm^{-3}) for three additional weeks. Well-rooted regenerants (plantlets) were finally transferred to foam cups containing potting soil and kept under growth room conditions for four weeks before finally moving to room conditions at low humidity. All cultures were incubated at 23 °C under a 16-h photoperiod (cool-white fluorescent tubes, irradiance of 22 - 28 µmol m^{-2} s^{-1}). For shoot regeneration, the mean number of shoots per leaf and stem explants, and percentage of explants producing shoots were determined. For rooting, the number of developing roots per shoot and the percentage of shoots that produced roots were determined. Eight replicates for shoot regeneration and ten replicates for rooting experiments were used for each treatment and all experiments were repeated three times. Results were expressed as means ± standard error of the mean. All data were analyzed by analysis of variance (*ANOVA*) and mean values were compared with Duncan's Multiple Range Tests using *SPSS* vers. *15* (*SPSS Inc*., Chicago, IL, USA).

An efficient plant regeneration system was established for *Lythrum salicaria* by culturing leaf lamina explants on MSMO medium containing several combinations of different plant growth regulators. To our knowledge, this is the first report on *in vitro* regeneration of this species *via* direct adventitious shoot development. The method described here is relatively quick enabling to regenerate *L. salicaria* plants within a period of around 12 weeks.

Leaf and stem explants excised from 4-week-old *in vitro* germinated seedlings were used to examine the effects of combinations of TDZ with IAA or IBA at varying concentrations. Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation and these processes are influenced both by exogenous plant growth regulators and endogenous hormones (Schwarz and Beaty 1996). Different tissues may have different hormone levels and, therefore, the explant source would have a critical impact on the regeneration success. In our study, when leaf and stem explants were compared, leaf explants were more productive than stem explants in terms of both the mean number of shoots per explant and percentage of explants producing shoots in all combinations and concentrations of plant growth regulators tested (data not shown). Similarly, leaf explants were also reported to be more responsive on shoot organogenesis in some other species

Table 1. Shoot regeneration from leaf explants cultured on MSMO medium containing different concentrations and combinations of TDZ and IAA or IBA. Means ± SE. Means with the same letters within columns are not significantly different at $P > 0.05$.

Treatments [mg dm^{-3}]	Number of shoots [$explant^{-1}$]		Explants forming shoots [%]
0.01 TDZ + 0.1 IAA	1.9 ± 1.3	hij	29.2
0.01 TDZ + 0.5 IAA	3.9 ± 2.1	efghij	37.5
0.01 TDZ + 1.0 IAA	7.3 ± 2.8	defghij	50.0
0.01 TDZ + 2.0 IAA	0		0
0.05 TDZ + 0.1 IAA	0		0
0.05 TDZ + 0.5 IAA	5.1 ± 2.6	defghij	41.7
0.05 TDZ + 1.0 IAA	6.6 ± 1.8	defghij	75.0
0.05 TDZ + 2.0 IAA	4.6 ± 2.4	defghij	37.5
0.1 TDZ + 0.1 IAA	0		0
0.1 TDZ + 0.5 IAA	10.3 ± 2.5	cdef	75.0
0.1 TDZ + 1.0 IAA	17.0 ± 5.0	ab	66.7
0.1 TDZ + 2.0 IAA	11.4 ± 1.7	bcd	87.5
0.3 TDZ + 0.1 IAA	0		0
0.3 TDZ + 0.5 IAA	1.0 ± 0.1	ij	16.6
0.3 TDZ + 1.0 IAA	14.5 ± 2.2	abc	91.7
0.3 TDZ + 2.0 IAA	4.4 ± 1.7	defghij	50.0
0.5 TDZ + 0.1 IAA	1.4 ± 0.8	ij	33.3
0.5 TDZ + 0.5 IAA	4.4 ± 1.1	defghij	75.0
0.5 TDZ + 1.0 IAA	17.3 ± 2.7	ab	91.7
0.5 TDZ + 2.0 IAA	1.4 ± 0.9	ij	20.8
1.0 TDZ + 0.1 IAA	1.4 ± 0.8	ij	41.7
1.0 TDZ + 0.5 IAA	3.5 ± 1.4	efghij	50.0
1.0 TDZ + 1.0 IAA	5.4 ± 2.6	defghij	41.7
1.0 TDZ + 2.0 IAA	9.8 ± 2.4	cdefg	75.0
0.1 TDZ + 0.1 IBA	11.5 ± 4.2	bcd	79.2
0.1 TDZ + 0.5 IBA	5.8 ± 2.6	defghij	50.0
0.1 TDZ + 1.0 IBA	9.1 ± 2.4	cdefgh	75.0
0.1 TDZ + 3.0 IBA	2.6 ± 1.0	ghij	45.8
0.5 TDZ + 0.1 IBA	1.1 ± 0.7	ij	20.8
0.5 TDZ + 0.5 IBA	19.3 ± 5.7	a	66.7
0.5 TDZ + 1.0 IBA	3.3 ± 0.9	fghij	79.2
0.5 TDZ + 3.0 IBA	0		0
1.0 TDZ + 0.1 IBA	1.6 ± 1.2	ij	25.0
1.0 TDZ + 0.5 IBA	3.0 ± 1.5	fghij	54.2
1.0 TDZ + 1.0 IBA	10.6 ± 1.6	bcde	91.7
1.0 TDZ + 3.0 IBA	3.5 ± 1.4	efghij	50.0
3.0 TDZ + 0.1 IBA	1.5 ± 0.5	ij	16.6
3.0 TDZ + 0.5 IBA	1.6 ± 1.1	ij	25.0
3.0 TDZ + 1.0 IBA	7.6 ± 2.3	defghi	62.5
3.0 TDZ + 3.0 IBA	4.1 ± 1.9	efghij	45.8

such as *Carthamus tinctorius* (Sujatha and Kumar 2007), *Nicotiana tabacum* (Lucyszyn *et al*. 2007), and *Cichorium intybus* (Yucesan *et al*. 2007). Leaf explants gave the best shoot proliferation on media containing 0.5 mg dm^{-3} TDZ + 0.5 mg dm^{-3} IBA (19.3 ± 5.7 shoots per explant with a frequency of 66.7 %), 0.5 mg dm^{-3} TDZ + 1.0 mg dm^{-3} IAA (17.3 ± 2.7 shoots per explant with a frequency of 91.7 %), and 0.1 mg dm^{-3} TDZ +

Table 2. Effects of the tested auxins on root formation from regenerated shoots. Means ± SE. Means with the same letter within columns are not significantly different at $P > 0.05$.

Auxins [mg dm^{-3}]	Number of shoots [explant^{-1}]	Explants forming roots [%]
IAA 0.5	15.0 ± 0.5 ^{a}	100
IAA 1.0	8.3 ± 0.4 ^{bc}	100
IAA 3.0	7.2 ± 0.7 ^{cde}	100
IBA 0.5	9.1 ± 1.6 ^{bc}	83.3
IBA 1.0	6.2 ± 1.1 ^{de}	80.0
IBA 3.0	6.0 ± 1.3 ^{e}	66.6
2.4-D 0.1	2.3 ± 0.7 ^{fg}	60.0
2.4-D 0.3	0	0
2.4-D 0.5	1.3 ± 0.5 ^{fg}	53.3
2.4-D 1.0	0	0
NAA 0.1	5.4 ± 0.2 ^{e}	100
NAA 0.5	8.1 ± 0.3 ^{bcd}	100
NAA 1.0	9.3 ± 0.3 ^{b}	100
NAA 2.0	2.7 ± 0.3 ^{f}	100
NAA 4.0	1.3 ± 0.3 ^{fg}	73.3

1.0 mg dm^{-3} IAA (17.0 ± 5.0 shoots per explant with a frequency of 66.7 %) (Table 1). In all set of experiments, treatments without growth regulators (control treatments) did not produce any shoots at all.

With regard to the effects of the combinations of TDZ with IAA on shoot development, significantly higher numbers of shoots per explant were obtained than the rest of the treatments when moderate concentrations of TDZ (0.1, 0.3 or 0.5 mg dm^{-3}) were combined with 1.0 mg dm^{-3} IAA, with a mean number of 17.0, 14.5 and 17.3 shoots per leaf explant, respectively (Table 1). For the combinations of TDZ and IBA, it was clear that the 0.5 mg dm^{-3} TDZ and 0.5 mg dm^{-3} IBA was the most effective, producing 19.3 shoots per leaf explant (Table 1, Fig. 1*A,B*).

The promoting effect of TDZ on shoot and somatic embryo development has been lately reported for many species (Siddique and Anis 2007, Sujatha and Kumar 2007, Jones *et al*. 2007). However, despite its wide use in recent years, not much is known about the mechanisms underlying the TDZ-promoted regeneration. Recently, Jones *et al*. (2007) have suggested that TDZ-induced regeneration is linked to accumulation and transport of certain endogenous signals such as auxins or the related compounds like melatonin and serotonin. Of the hormonal combinations tested in our study, combinations involving TDZ were always found significantly more effective than those of other plant growth regulators. It was previously shown in several species that low concentrations (0.01 - 0.02 mg dm^{-3}) of TDZ promote axillary shoot formation while high concentrations (2.2 - 4.4 mg dm^{-3}) triggering adventitious shoot development (Yusnita *et al*. 1990, Huetteman and Preece 1993, Qu *et al*. 2002, Ledbetter and Preece 2003). Also, Singh *et al*. (2003) reported that low concentrations of TDZ promoted organogenesis from the callus of pigeon pea whereas high concentrations were more effective for somatic embryogenesis. These results may suggest that the TDZ concentration is critical for the induction of different developmental events.

We also tested the combinations of several other plant growth regulators for shoot regeneration including TDZ (0.01, 0.05 or 0.1 mg dm^{-3}) and 2,4-D (0.01, 0.1, 0.5 or 1.0 mg dm^{-3}); KIN (0.1, 0.5, 1.0 or 3.0 mg dm^{-3}) and IAA (0.05, 0.1, 0.5 or 1.0 mg dm^{-3}); KIN (1.0, 3.0, 5.0 or 7.0 mg dm^{-3}) and NAA (0.5 or 1.0 mg dm^{-3}); BA (1.0, 3.0 or 5.0 mg dm^{-3}) and IBA (0.5, 1.0, 3.0 or 5.0 mg dm^{-3}); BA (0.5, 1.0, 3.0, 5.0, 7.0 or 10.0 mg dm^{-3}) and NAA (0.5, 1.0 or 3.0 mg dm^{-3}); zeatin (0.1, 0.5, 1.0 or 2.0 mg dm^{-3}) and IBA (0.5, 1.0, 3.0 or 5.0 mg dm^{-3}); TDZ (0.5, 1.0 or 3.0 mg dm^{-3}), IAA (0.1, 0.5 or 1.0 mg dm^{-3}) and GA_3 (1.0 mg dm^{-3}). However, in many cases, no shoot regeneration was observed (data not shown).

Rooting was readily achieved when multiple shoots were singled out and cultured on MSMO medium containing different concentrations of IAA, IBA, 2,4-D or NAA (Table 2). IAA was the most effective auxin in root induction and development in terms of both the number of roots per shoot and the frequency of shoot explants that rooted. Of the different concentrations of IAA tested, 0.5 mg dm^{-3} was the most effective, producing a mean number of 15.0 roots per shoot at 100 % frequency (Table 2, Fig. 1*C*). The other auxins IBA and NAA were moderately effective, while 2,4-D being the least effective in terms of both the mean number of roots per shoot and the mean frequency of shoot explants that rooted. 2,4-D appeared to have promoted callus development at the basal part of the shoots which were in contact with the medium. It was also observed that the number of roots produced per shoot explant decreased with increasing concentrations in all types of auxins, except for NAA in which there was a visible increase up to 1.0 mg dm^{-3}, followed by a decrease at higher concentrations. Auxin is generally considered necessarry for the acquisition of the meristematic competence of the responsive cells. Once this competence is established, excessive auxin concentrations can be often inhibitory for further embryonic (Charriére *et al*. 1999) or adventitious root (Gurel and Wren 1995, Mingozzi and Morini 2009) development.

More than 90 % of the shoots survived through the hardening process when the rooted shoots (regenerated plantlets) were transferred to foam cups and kept under growth room conditions for four weeks. These plants were subsequently moved to low humidity conditions (Fig. 1*D*).

Despite being both an important medicinal species and an ornamental plant, no study has been carried out on the *in vitro* propagation of *L. salicaria* through direct or indirect adventitious shoot organogenesis or somatic embryogenesis. Therefore, the protocol described in this report is expected to contribute to the future studies in *L. salicaria*, including genetic transformation and large-scale production of certain biologically active plant metabolites from *in vitro* propagated elite materials.

Further studies are, however, recommended for the possible improvement of the plant regeneration efficiency by refining the optimal concentration and combination of TDZ with other hormones, and for the establishment of suspension cultures for metabolite production.

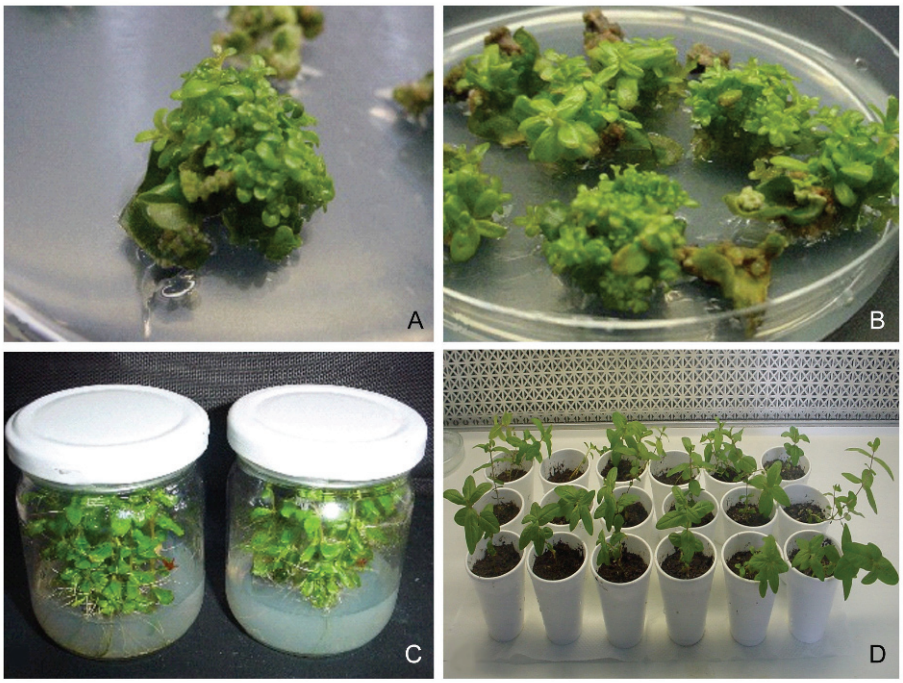

Fig. 1. Shoot regeneration from leaf explants cultured on MSMO medium containing 0.5 mg dm^{-3} TDZ and 0.5 mg dm^{-3} IBA for three weeks (*A* and *B*). Well-rooted shoots on rooting medium containing 0.5 mg dm^{-3} IAA after three weeks of incubation (*C*). Regenerated plants transferred from rooting medium to foam cups and kept under growth room conditions for four weeks (*D*).

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