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RESEARCH ARTICLE

MICROPROPAGATION OF *LYSIMACHIA LAXA* BAUDO

***Sanjoy Gupta, S. Kaliamoorthy, [1]Jayashankar Das, A. A. Mao and [2]Soneswar Sarma**

*Botanical Survey of India, Eastern Regional Center, Lower New Colony, Laitumkhrah, Shillong – 793003, Meghalaya, India
[1]Plant Bioresources Division, Regional Centre of IBSD, Ganktok-737102, Sikkim, India
[2] Department of Biotechnology, Gauhati University, Guwahati-781014, Assam, India

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ABSTRACT

An in vitro regeneration protocol was developed for *Lysimachia laxa* Baudo. Maximum number of shoot (9.10±0.32) was induced by culturing nodal explants in Murashige and Skoog (MS) medium supplemented with 17.76 µM benzyladenine (BA) and 5.37 µM α – naphthalene acetic acid (NAA). Elongated shoots were best rooted, with 100% rooting efficiency, in MS medium containing 2.85 µM indole-3-acetic acid (IAA). Among the two explants (nodal and shoot tips) used, nodal explants were found to be the best option for in vitro regeneration of *Lysimachia laxa*. Rooted plantlets thus developed were hardened in 2 months time by growing in root trainers containing potting mixture of a 1:1 mix of garden soil and leaf mould. The established plants were then transferred to earthen pots and subsequently transferred to field condition.

Key words: Apical shoot tips, *Lysimachia laxa* Baudo., micropropagation, Murashige and Skoog medium, nodal segments

INTRODUCTION

The genus *Lysimachia* L. consists of approximately 180 species of perennial, biennial or annual herbs, or sometimes shrubs, with cosmopolitan distribution (Stahl and Anderberg 2004; Oh *et al*. 2008). *Lysimachia* is traditionally placed in the family Primulaceae (Cronquist 1981; Takhtajan 1997) however, recent phylogenetic studies based on molecular and morphological data, transferred *Lysimachia* genus to the family Myrsinaceae (Källersjö 2000; APGII 2003; Oh *et al*., 2008). The genus mainly occurs in temperate and subtropical parts of northern hemisphere but a few species occurs in Africa, Australia and South America (Zhang *et al*., 2006). More than 75% of the species are reportedly occurs in China (Fang 2003). In China, the genus *Lysimachia* plays an important role in folk medicine. *Lysimachia davurica* grows in northeast China is being used in folk medicine for treating hypertension and also has anti-tumor activity (Liang *et al*., 2006). *Lysimachia clethroides* Duby, has been used widely by the Chinese for the treatment of throat ache, edema, and menoschesis, etc (Jiang *et al*., 2007). In India, the species *Lysimachia laxa* Baudo (Syn; *Lysimachia ramosa* Wall ex Duby), a perennial herb, reported from the states of Meghalaya, Arunachal Pradesh, Manipur and Nagaland at an altitude between 1800 and 2000 m. The fresh leaves of *L. laxa* are widely used for de-worming by the tribal people of Meghalaya (Mao *et al*., 2009; Challam *et al*., 2010). In view of its pharmacological value, the plant has been overexploited in nature and as a result, its survival in nature is highly challenged. Further, the seedling recruitment of *L. laxa* in nature is poor due to low seed germination percentage. The restoration of this plant is, therefore, possible with the intervention of plant tissue culture technique. Except for a single report on the in vitro regeneration of three species of *Lysimachia* viz., *Lysimachia christinae, L. rubinervis and L. nummularia* (Zheng *et al*., 2009), no further work on this field of research has been undertaken. In view of the above, the present study has been taken up with an objective to develop a reproducible protocol for in vitro regeneration of *Lysimachia laxa* using nodal and shoot tip explants.

Corresponding author: sanjoy12biotech@yahoo.com

MATERIALS AND METHODS

Seeds and live plant materials of *Lysimachia laxa* Baudo. were collected from Talle valley of lower Subansiri district, Arunachal Pradesh, India at an altitude of 1800-2000 m. Voucher specimen of *Lysimachia laxa* (Mao and Gupta, field number 19205), was deposited in the 'ARUN' Herbarium, Botanical Survey of India, Arunachal Pradesh Regional Center, Itanagar, Arunachal Pradesh, India. The seeds were washed thoroughly in running tap water followed by treatment with 2-3 drops of Tween-20 (v/v) per 100 ml distilled water and finally rinsed five times with distilled water. These seeds were then disinfected with 10% (v/v) Sodium- hypochlorite solution for 30 min and washed five times with sterile distilled water. Finally the seeds were treated with 0.1% $HgCl_2$ (w/v) for 1 min followed by several rinses with sterile distilled water. The sterilized seeds were inoculated on half-strength MS (Murashige and Skoog 1962) medium containing 3% sucrose (w/v) and 0.8% Bacto agar (w/v) (Sd Fine Chem, Mumbai, India), pH was adjusted to 5.8. Eight-week old in vitro germinated seedlings were used as the source of explants. Apical shoot tips (0.5-1 cm) and nodal

segments (1 cm) were excised from these seedlings and inoculated onto shoot bud induction medium consisting of full strength MS salts supplemented with 3% sucrose (w/v) and various concentrations of either benzyladenine, BA (2.22 - 26.64 µM) or kinetin, Kn (2.32 -27.90 µM). In addition to this, combination of BA (17.76 µM) and different concentrations of either α-naphthalene acetic acid, NAA (1.34-8.05 µM) or indole butyric acid, IBA (1.22-7.35 µM) were also tried for multiple shoot induction. All media were autoclaved at 121 ºC, 1.05 kg cm^{-2} pressure for 15 min. Cultures were inoculated and maintained at 25 ± 2 ºC, 16-h photoperiod with an irradiance of 60 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Percentage of response, number of shoot per explant and shoot length were recorded after 8 weeks.

Microshoots (1.2 -1.5 cm) were then excised from the cluster and transferred to root induction medium consisting of full strength MS salts, 3% sucrose and various concentrations of either indole-3-acetic acid, IAA (2.85-8.55 µM) or indole-butyric acid, IBA (2.45-7.35 µM). Percentage of response, number and length of roots were recorded after six weeks of inoculation. The well rooted shoots were removed and washed thoroughly with distilled water. The plantlets were then treated with 0.1% Bavistin (w/v) (BASF India Ltd) for 5 min before transplanted in root trainers (Agro Vision Biotech, Jaipur, India) containing garden soil and leaf mould (1:1). Plants were watered daily and maintained in a green house at 25 ± 2 ºC with 80 ± 5 % relative humidity. Finally, the hardened plants were transferred to earthen pots and subsequently to field condition. All experiments were carried out with 30 replicates each and repeated thrice. The data were analyzed using one way analysis of variance (ANOVA) and the difference between the mean of sample was analyzed by the least significant difference (LSD) test at a probability level of 0.05, using SPSS software version-10.

RESULTS AND DISCUSSION

In the present study, effects of two cytokinins, BA and Kn either alone or in combination with two auxins, NAA and IBA on in vitro plantlet regeneration from shoot tips and nodal explants of *Lysimachia laxa* were presented. A varied response was observed with two cytokinins, treated either alone or in combination with two auxins at different concentration, as summarized in Table1. MS medium supplemented with different concentration of BA (2.22-26.64 µM) stimulated axillary shoot sprouting after 7-9 days of culture, and 17.76 µM BA was found to be most effective for maximum (3.2±0.12 shoots per nodal explant) shoot induction after 8 weeks of incubation (Fig. 1A). Shoot tip explants on the other hand, recorded a maximum mean shoot number of 2.20±0.12 shoots per explant. However, at higher concentration of BA 26.64 µM, the cultures showed decline in shoot number and length; and subsequently the cultures became fragile and eventually died. A similar observation was reported in *Salix tetrasperma* by Khan *et al*., (2011). This negative impact of BA on regeneration potential was believed to be due to its detrimental effect at high concentration on the cells predetermined to form vegetative buds (Khan *et al*., 2011). Further the lethal effect of BA at higher concentration might be attributed to the induction of apoptosis (Carimi *et al*., 2004; Mlejnek and Dolezel 2005). When Kn was used as the sole source of cytokinin, the regeneration frequency was found to be low (Table 1) and there was a decrease in shoot number as well. A maximum of 2.03±0.10 shoots per nodal explant with the highest percentage (56%) of responding explants has produced at 18.60 µM Kn after 8 weeks of inoculation. While shoot tip explants showed a maximum of 46% regeneration frequency and 1.90 ± 0.11 shoots per explant at this concentration of Kn. From these observations, it is evident that Kn was inferior to BA in this species, which is in accordance with earlier reports on many plant species (Ahmad *et al*., 2008; Anis *et al*., 2010; Khan *et al*., 2011)

A beneficial effect from the use of BA in combination with two auxins, α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) was also recorded in the present study (Table 1). Of the various combinations tested, 17.76µM BA and 5.37µM NAA found to be very effective with a maximum mean shoot number and length respectively of 9.10 ± 0.32 and 5.95 ± 0.16cm per nodal explant (Fig. 1B). Shoot tip explants, on the other hand responded with a maximum mean shoot number of 4.06 ± 0.30 shoots per explant with 17.76µM BA + 8.05µM NAA. Maximum shoot regeneration frequency of 100% was achieved with nodal explants at 17.76µM BA either in combination with NAA (5.37µM and 8.05µM) or IBA (2.45, 4.90 and 7.35 µM) however, the percentage shoot regeneration frequency using shoot tip explants varies from a minimum of 56.66% to maximum of 76.66%. A similar finding was also reported in three ornamental *Lysimachia* species viz *L. christinae, L. rubinervis*, and *L. nummularia* (Zheng *et al*., 2009). The synergistic effect of cytokinin and auxin on enhanced shoot regeneration potential was reported by many workers in other medicinal plants viz., *Clitoria ternatea* (Shahzad *et al*. 2007), *Ruta graveolens* (Ahmad *et al*., 2010), *Balanites aegytiaca* (Anis *et al*. 2010), *Justicia gendarussa* (Thomas and Yoichiro 2010) and *Salix tetrasperma* (Khan *et al*., 2011). The elongated shoots were carefully excised individually from the cluster and cultured in full strength MS medium containing either IAA (2.85-8.55 µM) or IBA (2.45-7.35 µM) for root induction. The regenerated shoots rooted within 3 weeks of treatment but with different rooting frequency among the different concentrations of these two auxins tested.

In the present study, the regenerated shoot of *Lysimachia laxa* were best rooted in a medium containing 2.85µM IAA, with the maximum root number and length respectively of 10.15±0.37 and 6.22±0.19 cm. (Table 2; Fig. 1C). In addition, IAA treatment resulted in 100% rooting frequency in all the concentrations tested. While in IBA treatment, the rooting frequency varied from a minimum of 63% to a maximum of 81%. The minimum and maximum root number and length, between all concentrations of IBA varied respectively from 2.44 to 3.68 and from 3.88 to 4.74 cm. In the present study, both the auxins, at higher concentrations, developed mild callus at the shoot base. However lower concentrations of IAA (2.85 µM), and IBA (2.45 µM) responded well in rooting without the intervention of callus formation. Application of IAA and IBA in root induction has been reported in many plant systems (Rout *et al*., 2000; Senthilkumar and Rao 2007; Sujana and Naidu 2011). However, higher concentrations of these two hormones proved to be either inhibitory or produce callus at the base of microshoots (Hossain *et al*., 1993; Senthilkumer and Rao

Table 1. Effect of two different cytokinins (BA, Kn) either alone or in combination with two auxins (NAA, IBA) on in vitro regeneration from apical shoot tip and nodal explants of *L. laxa* in MS medium, after 8 weeks of culture. Means followed by common letters within a column are not significantly different (LSD, P<0.05)

Treatments (µM)				Nodal explant			Apical shoot tip		
BA	Kn	NAA	IBA	% of response	No of shoot	Shoot length (cm)	% of response	No of shoot	Shoot length (cm)
Control				0.00	1.00 ± 0.00 p q r s	3.40 ± 0.05 p q	0.00	1.0 ± 0.00 o p q r s	3.45 ± 0.05 o p
2.22	0	0	0	33.00	1.36 ± 0.05 o p q	3.47 ± 0.14 p	20.00	1.20 ± 0.4 o p q	3.63 ± 0.13 o
4.44	0	0	0	63.30	2.13 ± 0.11 k l	3.90 ± 0.13 m n o	46.60	1.80 ± 0.10 i j k l m	3.63 ± 0.13 o
8.88	0	0	0	70.00	2.63 ± 0.13 j	4.29 ± 0.14 l m	50.00	2.03 ± 0.12 h i j	4.29 ± 0.14 g h i j k l
17.76	0	0	0	86.66	3.20 ± 012 h i	5.02 ± 0.17 d e f g	56.60	2.20 ± 0.12 h i	5.36 ± 0.14 a b
26.64	0	0	0	63.33	2.40 ± 0.12 j k	4.74 ± 0.14 f g h i j	43.33	1.83 ± 011 i j k l	5.16 ± 0.12 a b c d
0	2.32	0	0	16.00	1.16 ± 0.03 p q r	3.29 ± 0.11 p q r	13.00	1.13 ± 0.03 o p q r	3.28 ± 0.08 o p q
0	4.65	0	0	36.00	1.40 ± 0.05 o p	3.90 ± 0.13 m n o	33.00	1.33 ± 0.04 n o p	4.01 ± 0.12 k l m n
0	9.30	0	0	46.00	1.96 ± 0.12 l m n	4.18 ± 0.11 l m n	43.00	1.76 ± 0.10 i j k l m n	4.31 ± 0.14 g h i j k
0	18.60	0	0	56.00	2.03 ± 0.10 k l m	4.73 ± 0.13 f g h i j k	46.66	1.90 ± 0.11 i j k	5.03 ± 0.12 b c d e
0	27.90	0	0	36.00	1.60 ± 0.09 m n o	4.49 ± 0.12 i j k l	33.33	1.50 ± 0.8 k l m n o	5.09 ± 0.12 b c d
17.76	0	1.34	0	90	3.70 ± 0.16 e f g	5.20 ± 0.17 c d e	60	2.46 ± 0.14 f g h	5.23 ± 0.14 a b c
17.76	0	2.68	0	96.66	7.36 ± 0.32 b	5.58 ± 0.19 a b c	63.33	3.30 ± 0.26 c d e	4.65 ± 0.14 e f g
17.76	0	5.37	0	100	9.10 ± 0.32 a	5.95 ± 0.16 a	71	3.90 ± 0.27 a b	4.54 ± 0.14 f g h
17.76	0	8.05	0	100	6.03 ± 0.15 c	5.65 ± 0.15 a b	66.60	4.06 ± 0.30 a	4.49 ± 0.19 f g h i
17.76	0	0	1.22	94.3	3.55 ± 0.15 f g h	5.06 ± 0.16 d e f	68.88	2.90 ± 0.16 e f	5.46 ± 0.13 a
17.76	0	0	2.45	100	4.22 ± 0.19 d	5.34 ± 0.18 b c d	76.66	3.70 ± 0.25 a b c	4.81 ± 0.14 d e f
17.76	0	0	4.90	100	4.08 ± 0.18 d e	4.91± 0.14 e f g h	66.60	3.46 ± 0.23 b c d	4.40 ± 0.15 g h i j
17.76	0	0	7.35	100	3.91 ± 0.17 d e f	4.79 ± 0.16 f g h i	56.66	2.70 ± 0.20 f g	4.10 ± 0.13 j k l m

Table 2. Effect of auxins on root induction from in vitro raised micro shoots of *L. laxa* after 6 weeks of incubation. Means followed by common letters within a column are not significantly different (LSD, P<0.05). Callus: Present (+) absent (-)

Hormone Treatment (µM)		% of Response	Callus	No of roots	Root length (cm)
IAA	IBA				
Control		42	-	2.75 ± 0.44 d e f	3.07 ± 0.48 f
2.85	0	100	-	10.15 ± 0.37 a	6.22 ± 0.19 a
5.70	0	100	+	7.23 ± 0.33 b	5.43 ± 0.17 a b
8.55	0	100	+	4.16 ± 0.57 c	5.24 ± 0.23 b c
0	2.45	63	-	3.68 ± 0.47 c d	4.20 ± 0.39 c d e
0	4.90	81	+	3.09 ± 0.39 c d e	4.74 ± 0.35 b c d
0	7.35	75	+	2.44 ± 0.31 e f	3.88 ± 0.33 d e f

2007; Sujana and Naidu 2011). The rooted plantlets after 4-5 weeks of inoculation were transferred to potting mixture of a 1:1 mix of garden soil and leaf mould and kept in green house for hardening and acclimatization (Fig.1D). Around 75% of the in vitro raised plants of *Lysimachia laxa* transferred from lab to green house condition were successfully established ex vitro after 2 months. The established plants were then transferred to earthen pots and subsequently transferred to field condition (Fig. 1E). In conclusion, 17.76 µM BA and 5.37µM NAA was found to be optimal in producing maximum number of shoots per explant. Highest rooting efficiency (100%) of individual shoot culture was obtained in 2.85 µM IAA treatment. Among the two explants (nodal and shoot tips) used, nodal explants were found to be the best option for invitro regeneration of *Lysimachia laxa.*

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