ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **31**(1): 35-42, 2021 (June)

PTC&B

An Improved liquid Culture System for Efficient Shoot Multiplication in *Aerva lanata* (L.) Juss. Ex Schult.

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Key words: *Aerva lanata*, Liquid medium, Plant growth regulators, Shoot multiplication

Abstract

An improved *in vitro* mass propagation protocol was developed for *Aerva lanata* using MS liquid medium. The influence of MS medium (solid and liquid) with cytokinin (TDZ and BAP, respectively) were studied for shoot proliferation and growth. The liquid medium perfomed better than solid medium in shoot multiplication. The maximum shoot multiplication rate was (29.37 ± 0.64 shoots per explant), obtained in MS liquid medium which is containing 0.6 mg/l TDZ, 0.3 mg/l NAA and 0.2 mg/l IBA. Different volumes of liquid medium have been used, 30 ml of medium flask showed the maximum number of shoots. Liquid medium is better suited for *in vitro* propagation of *A. lanata* since the enhanced multiplication rate was observed with shorter subculture intervals.

Introduction

Aerva lanata is an important medicinal plant belonging to the *Amaranthaceae* family. It is an erect, herbaceous weed plant, which grows everywhere in the plains of India. The whole plant, especially leaves are edible and used as food (Joseph and Mathew 2015). In the traditional system of medicine, *A. lanata* is used as diuretic, anti-helminthic, anti-diabetic and expectorant (Gupta and Neeraj 2004). It is also extensively used for many purposes in the form of ailments like arresting haemorrhage during pregnancy, burn healing, anti-inflammatory, skin diseases, for uterus clearance after delivery and to prevent lactation (John 1984).

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DOI: https://doi.org/10.3329/ptcb.v31i1.54109

The plant extract is also used to treat nasal bleeding, cough, scorpion sting, fractures and spermatorrhoea (Mukerjee et al. 1984, Sikarwa and Kaushik 1993, Girach et al. 1994). The flowers are used for dysentery, diarrhoea and bronchitis. The seeds are find to use in rheumatism and bronchitis. The plant leaves are also used for antimalarial, in fever and to expel stones from kidney, scorpion sting, spermaorrhoea, urinary troubles and antirheumatic. Further, the root is used for headache, scabies, cough, as demulcent, diuretic, to cure diarrhea, jaundice, cholera, dysentery and snake bite (Rajesh et al. 2011).

A. lanata is reported to be very effective in curing urinary risk factors associated with calcium oxalate urolithiasis and no side effects (Selvam et al. 2001, Surya et al. 2012). The plant contains bioactive compounds are responsible for the above pharmacological activities such as β-carboline, β-sitosterol, palmitic acid, alphaamyrin, aervin, methyl aervine, and aervoside (Varutharaju et al. 2014). The liquid culture system for *in vitro* mass propagation helps in the substantial reduction of plantlet production costs (Sandal et al. 2001) and it is an important step towards automation (Aitken-Christie et al. 1995).

Further, liquid medium provides other advantages in the form of uniform culturing conditions, renewal of media without changing the container, sterilization by microfilteration and ease of cleaning containers. The liquid culture system is highly efficient for multiple shoot proliferation, cell suspension culture, adventitious root formation and adventitious shoot proliferation in various plantspecies (Sivanandhan et al. 2013, Soundar Raju et al. 2015, Thilip et al. 2015, Arigundam et al. 2020). However, several reports indicate that a liquid culture system promotes hyperhydricity (Deterz et al. 1994) and is limited by low oxygen content (Smith and Spomer 1995). Although there are a few reports for *in vitro* propagation of *A. lanata* (Varutharaju et al. 2014, Shekhawat et al. 2016), there has been no study conducted on the comprehensive assessment of the potential of a liquid culture system for its mass propagation of *A. lanata*. The present work focus on the study of various parameters associated with the development of an efficient liquid culture system for *in vitro* mass propagation of *A. lanata*.

Materials and Methods

Three months old *in vitro* raised healthy node and shoot bud segments of *A. lanata* were used in this experiment. To optimize the concentration of cytokinins for shoot induction and proliferation, the MS solid medium with different concentration of cytokinins TDZ (0.3, 0.6 and 0.9 mg/l) and BAP (0.3, 0.6 and 0.9 mg/l) was used. Both solid and liquid medium was adjusted to pH 5.6-5.8 before autoclaving for 20 min at 121°C. The cultures were incubated at 24 ± 2°C under 16/8 (light and dark cycle) photoperiod (40 µmol m^{-2} s^{-1}) and irradiance provided by cool-white fluorescent (Philips, India). Further, after 2 weeks shoot induction cultures were recorded.

To determine the shoot multiplication rates in both solid and liquid MS medium supplemented with concentration of 0.6 mg/l TDZ and 0.6 mg/l BAP in combination of

0.3 mg/lNAA, 0.2 mg/l IBA were observed. Different growth parameters such as shoot length, root length, number of shoots/explants, fresh weight and dry weight (60°C) and clusters of plantlets were recorded after 4 weeks of culture.

To optimize the liquid medium conditions for growth and shoot proliferation of *A. lanata*. Different volumes of MS liquid medium (10, 20, 30, 40 and 50 ml) were supplemented with 0.6 mg/l TDZ, 0.3 mg/l NAA, 0.2 mg/l IBA and sucrose (3.0%) were tested in a conical flask. The mean number of shoots produced per explants was recorded at regular intervals of 14, 21, 28 and 35 days. After 14 days, various parameters like root induction period, root length and branching of roots were recorded.

All experiments were repeated thrice and each with three replicates. Data were recorded after 4 weeks of culture for multiple shoot formation in liquid culture. Data were statistically analyzed using of variance (ANOVA). Data were presented as mean±standard error (SE). The mean separations were carried out using Duncan's multiple range test using the SPSS and significance was determined at $p < 0.05$ was performed.

Results and Discussion

One month old *in vitro* raised shoot buds and nodal segments were used for multiple shoot induction. They were cultured on MS solid medium containing different concentration (0.3 - 0.9 mg/l) of TDZ and BAP. Among the various treatments, 0.6 mg/l TDZ and 0.6 mg/l BAP significantly showed a higher ratio of shoot induction (5.69 and 2.60 shoots for explants) compared to control and other treatments (Table 1). Optimum percentage (83%) of multiple shoot induction was obtained on MS medium supplemented with 0.6mg/l TDZ alone (Fig. 1A,B). High concentration of TDZ (above 0.6 mg/l) or BAP showed inhibited the multiple shoot induction (Table 1). Shekhawat et al. (2016) reported that the maximum number of shoot induction obtained with MS solid medium which is containing 1.0 mg/l BAP and 0.2 mg/l NAA in *A. lanata*.

Table 1. Effect of PGRs on multiple shoot induction from shoot bud and nodal explants.

PGRs (mg/l)		Shoot bud explants		Nodal explants	
TDZ	BAP	% of shoot response	No. of shoots/explants	% of shoot response	No. of shoots / explants
0.3	-	66	1.56 ± 0.18^{c}	70	1.60 ± 0.77^{e}
0.6	-	**76**	**2.60 ± 0.24^{a}**	**83**	**5.69 ± 0.20^{a}**
0.9	-	68	1.50 ± 0.24^{d}	73	2.22 ± 0.18^{c}
-	0.3	63	1.26 ± 0.20^{e}	67	1.66 ± 0.17^{d}
-	0.6	70	1.57 ± 0.66^{b}	75	2.60 ± 0.11^{b}
-	0.9	58	1.20 ± 0.57^{f}	60	1.50 ± 0.17^{f}

Data represented mean ± SE was carried out using Duncan's multiple range (DMRT) test at $p < 0.05$ level. All the experiments were carried out three times with at least 20 explants.

A comparative analysis of multiple shoot formation in solid (Agar 0.8%) and liquid medium was also studied (Fig. 1A-F). After 5 weeks, liquid medium containing 0.6 mg/l TDZ in combination with 0.3 mg/l NAA and 0.2 mg/l IBA showed a higher percentage (29.37±0.64) of shoot multiplication (Fig. 1F) compared to solid medium (12.56 ± 1.24; Fig. 1C). Further, subculture of these shootlets in liquid medium showed a higher ratio of response in terms of shoot length, root length, number of shoots, fresh weight and dry weight (Table 2). Gradually decrease in the number of shoots per explants (13.37 ± 2.67) was observed when liquid MS medium containing lower concentrations of 0.3 mg/l TDZ in combination with 0.3 mg/l NAA and 0.2 mg/l IBA, after 5 weeks of culture (Fig. 1E). Further, cultures on liquid MS medium containing 0.6 mg/l TDZ in combination with 0.3 mg/l NAA, also showed poor respondents (7.34 ± 1.12) of multiple shoot formation (Data not shown) (Fig. 1D).

Table 2. Effect of solid and liquid MS.

Parameters	Solid	Liquid
Mean number of shoots per explants	12.56 ± 1.24	29.37 ± 0.64
Shoot length (cm)	4.36 ± 0.14	5.76 ± 1.44
Root length (cm)	3.56 ± 1.04	4.66 ± 0.32
Fresh weight (% w/w)	0.54 ± 0.03	0.86 ± 0.63
Dry weight (% w/w)	0.13 ± 0.02	0.28 ± 1.03

Medium: MS medium containing 0.6 mg/l TDZ, 0.3 mg/l NAA and 0.2 mg/lIBA. Data represented mean ± SE was carried out using Duncan's multiple range (DMRT) test at $p < 0.05$ level. All the experiments were carried out three times with at least 20 explants.

The liquid culture system is the best attractive alternative compared to solid medium. The complete removal of agar, an expensive ingredient, in the multiplication medium has helped in a substantial cost reduction. It has been calculated and reported in several earlier studies (Pati et al. 2011). In the present study, *in vitro* raised nodal segments of *A. lanata* produced and enhanced shoot proliferation in the MS liquid medium compare than MS solid medium. Higher shoot proliferation and better response of other growth parameters such as shoot length, root length, fresh weight and dry weight in liquid medium. From this result, the liquid medium is an efficient system choice for micropropagation of *A. lanata*. The positive effect of liquid culture is the shoots are totally submerged in the medium, facilitating the uptake of nutrients and plant growth regulators in over the whole shoot surface (Hung et al. 2006). Further, this system is promoted better supply of oxygen and sucrose under the rotary culture. All shoots initiated in the liquid culture media showed uniform and vigorous shoots. Therefore, the present study concludes that regeneration of *A. lanata* in liquid culture was superior to solid culture.

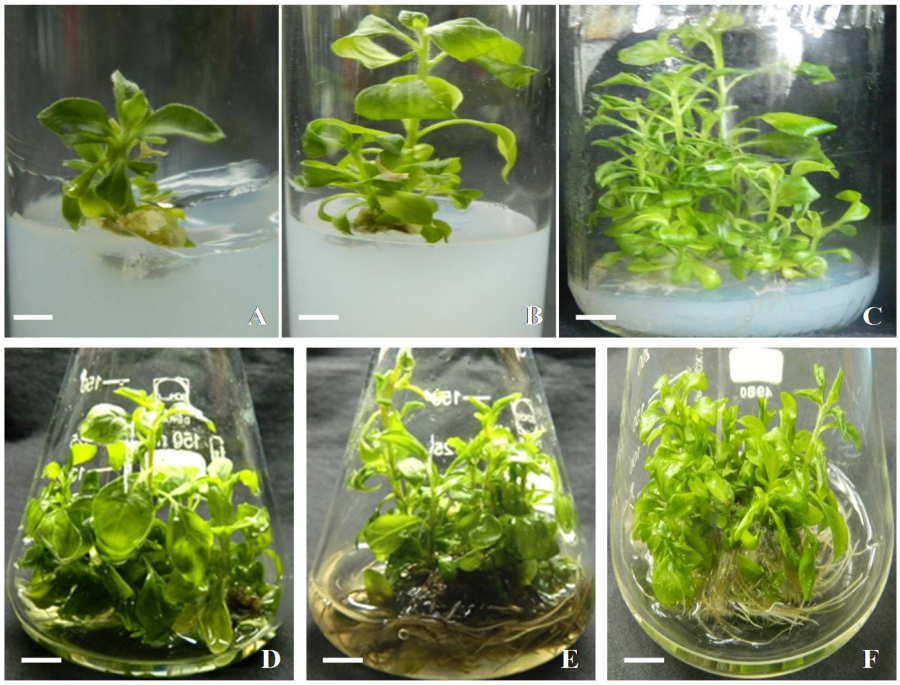

Fig. 1. Shoot multiplication in solid and liquid medium. A and B. Multiple shoot induction on MS solid medium containing 0.6 mg/l TDZ after 3 weeks. C. Multiple shoot formation on MS solid medium containing 0.6 mg/l TDZ, 0.3 mg/l NAA and 0.2 mg/l IBA. D. Multiple shoot formation on MS liquid medium containing 0.6 mg/l TDZ, 0.3 mg/l NAA. E. Multiple shoots with spontaneous root proliferation in MS liquid medium containing 0.3 mg/l TDZ in combination with 0.3 mg/l NAA and 0.2 mg/l IBA. F. MS liquid medium containing 0.6 mg/l TDZ, 0.3 mg/l NAA and 0.2 mg/l IBA. *Scale bars*: A and B = 0.5 cm; C to F = 1.5 cm.

Different volumes of liquid medium used in 150 ml conical flask, 30 ml medium showed the maximum number of shoot proliferation (Table 3). Further increase or decrease in the volume has led a reduction of the number of shoots. The present study also optimized the suitable culture time resulting in the number of shoots and short period of time leading to lower number of shoots. When shoots attained maximum level, no significant number of shoot multiplications had extended duration. 35 days of culture time were suitable for the maximum shoot proliferation (29.37 ± 0.64 shoots/explant) in 30 ml liquid medium. This hypothesis, in agreement with the earlier report of *Scutellaria alpine* (Grzegorczyk-Karolak et al. 2017).

It was also observed that, increasing liquid medium to 40 ml promoted hyperhydricity while reducing it to 10 ml led to desiccation of shoots within 2 weeks of subculture. Medium volume is one of the key factors for rate of shoot multiplication. In *A. lanata*, within 35 days of culture period and 150 ml vessel containing 30 ml of medium optimum for higher number of shoot multiplication. The 50 days of culture period were induced chlorosis. Chu et al. (1993) reported that the growth of miniature rose shoots cultured on 30 ml liquid medium had a higher multiplication ratio than those grown in

10 or 20 ml. Increasing the volume caused a least number of shoots; it could be due to hyperhydricity as a result of the lower availability of oxygen (Sandal et al. 2001, Pati et al. 2011).

Table 3. Effect of medium volume on multiple shoot formation.

Volume of medium (ml)	Mean number of shoots per explants during different days of subculture			
	14 days	21 days	28 days	35 days
10	2.34 ± 0.50^{c}	0.00^{e}	0.00^{e}	0.00^{e}
20	3.25 ± 0.60^{b}	5.87 ± 0.73^{b}	9.24 ± 0.56^{b}	14.23 ± 0.59^{b}
30	**4.54 ± 0.24^{a}**	**7.46 ± 0.56^{a}**	**16.55 ± 0.34^{a}**	**29.37 ± 0.64^{a}**
40	2.14 ± 0.14^{d}	3.39 ± 0.36^{c}	5.66 ± 0.54^{c}	9.59 ± 0.40^{c}
50	1.38 ± 0.12^{e}	2.14 ± 0.15^{d}	3.24 ± 0.36^{d}	5.34 ± 0.20^{d}

Medium-MS liquid medium containing 0.6 mg/l TDZ, 0.3 mg/l NAA and 0.2mg/l IBA

Data represented mean ± SE was carried out using Duncan's multiple range (DMRT) test at $P < 0.05$ level. All the experiments were carried out three times with at least 20 explants.

This study presents for the first time a complete protocol for *in vitro* mass propagation of *A. lanata* in a liquid culture system using cytokinnin and auxin. The protocol could also be helpful for the improvement of medicinal content by genetic engineering of this medicinally and pharmaceutically important plant of *A. lanata.*

Acknowledgments

The authors thank to DST, Government of India, for providing facilities through FIST program. They are also thank to the University Grants Commission, New Delhi, for its support through "College with Potential for Excellence" programme.

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(Manuscript received on 25/01/2020; revised on 06/04/2021)