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In vitro Shoot Multiplication of a Seasonal and Vulnerable Medicinal Plant-*Aerva lanata* L.

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Abstract: The present contribution focused on standardization of *in vitro* propagation protocols of *Aerva lanata*, an important medicinal plant used in diverse traditional system of medicine. MS and L_2 media supplemented with auxins and cytokinins were used to test the *in vitro* growth response of explants. L_2 media showed positive growth response and complete plant regeneration upon subsequent subculture. Using leaf segments and shoot tips as explants, callusing, shoot multiplication and rhizogenesis were obtained. L_2 media fortified with 2, 4-dichlorophenoxyacetic acid (2.5 mg L^{-1}) and benzylaminopurine (1.5 mg L^{-1}) was standardized as the suitable medium for highest number (17 shoots per shoot tip) of shoot production and half strength L_2 media with naphthaleneacetic acid (2 mg L^{-1}) for rooting (3.2 cm in 36 days). Hardening of rooted plants was successful on 1:1:1 ratio of Soil rite:Coco peat:Vermiculite, later plants were transferred to pots containing sterile soil and maintained in Green house then planted in the field. The survival rate was between 65 and 70%. The method established could be adopted for rapid large scale micro propagation and conservation of this important vulnerable medicinal herb.

Key words: Tissue culture, micropropagation, Murashige and Skoog media, naphthaleneacetic acid, benzylaminopurine

INTRODUCTION

Aerva lanata L. a medicinally important plant commonly called as Polpala belongs to the family Amaranthaceae which is recognized as repellants to many pests, birds and evil spirits. The plant contains palmitic acid, beta-sitosterol and alpha-amyrin; leaf is used in hepatitis, root in urinary strangury and decoction of the plant is used in catarrh of bladder; flowers and roots are used for headache relief (Khare, 2007). According to British Herbal pharmacopoeia the key applications of this herb are as diuretic and lithontriptic. *A. lanata* has a narrow germplasm base and continuously exploited for its varied advantages and medicinal properties. To combat the problem, conventional propagation methods were employed but the plant took very long time for its development with a low rate of fruit set and poor seed germination. Moreover, this plant is susceptible to fungi, bacteria and viruses which further reduces the yield. In the case herbs land degradation, transformation or clearing would wipe out the whole population.

For the past three decades *in vitro* multiplication have been proposed as an alternative tool for rapid micropropagation of useful genotypes, their improvement and conservation of endangered and vulnerable medicinal plants. A well known fact of this approach is not the rapid propagation and selection of desirable traits; it also facilitates raising plantlets in a limited space. Murgai (1959) have made an attempt on *in vitro* culture of the inflorescences, flowers and ovaries of an apomict, *Aerva tomentosa* Forsk. and reported that the results of the experiment have not been so encouraging. Pullaiah *et al*. (2000) reported that the herb *Aerva lanata* L. is seen soon after the onset of monsoon and disappears by the beginning of January. Dormancy and seasonal appearance in plants are a temporary suspension of visible growth of structures containing a meristem and also an important mechanism ensuring a seasonal synchronization of plant growth and also contributes to the control of architecture. Knowledge on impact and influence of these phenomenons on *A. lanata,* study of *in vitro* growth response and development of complete regeneration protocol will open new avenues for rapid micro propagation of this seasonally appearing herb.

In vitro shoot multiplication of *A. lanata* was not yet attempted and this study would be the first of its kind to report. Unprecedented genetic erosion, disappearance of

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species, their ecosystem and conservation of natural resources assumes paramount urgency. Krishnan *et al.* (2011) emphasised that medicinal plants continue to be an important source of lifesaving drugs for humankind. Micro propagation and eco-restoration of medicinal plants in fact support the in-situ conservation activities, facilitate population enhancement of species where natural propagation is hindered due to destructive harvesting and reproductive barriers. Therefore, the present study reports the *in vitro* response of *A. lanata* explants, their reproducibility and reliable techniques for shoot multiplication within short period of time.

MATERIALS AND METHODS

Healthy plants of *A. lanata* were collected from Botanical garden, Department of Botany, Bangalore University, Jnana Bharathi campus, Bangalore, Karnataka, India and used for experimental purpose. Stem tips, nodal segments and leaves were used as explants. The explants were thoroughly washed in running tap water for 10 min, disinfected by using tween-20 (5% v/v) for 15 min and rinsed with distilled water. The explants were then treated with 0.5% (w/v) Sodium hypochlorite solution (5 min) followed by a rinse in double distilled water. Later the explants were treated with 0.01% (w/v) Mercuric chloride (10 min) and 0.1% (w/v) Mercuric chloride solution (5 min), followed by Bavistin solution (30 min) and washed thoroughly in double distilled water. The surface sterilized explants were transferred aseptically to pre-sterilized culture tubes containing MS media (Murashige and Skoog, 1962) and L_2 media (Phillips and Collins, 1979) fortified with hormones. Then, the tubes were incubated in culture room under controlled conditions of White fluorescent light (16 h light to 8 h dark) and temperature (24±2°C). Plant growth regulators namely, benzylaminopurine (BAP) (0.5-4 mg L^{-1}), 2, 4-dichlorophenoxyacetic acid (2, 4-D), naphthaleneacetic acid (NAA), indolebutyric acid (IBA) (0.5-4 mg L^{-1}) were tried individually or in combination to obtain the multiple shoot bud induction. Observations were recorded at an interval of 2 weeks.

All the experiments were repeated three times to confirm the reproducibility. The one-way ANOVA was carried out using Microsoft Excel 2003 and mean data were compared using critical difference at 0.5% level of significance (Panse and Sukhatme, 1989).

RESULTS AND DISCUSSION

A. lanata appeared at the onset of monsoon in June and the flower buds appear during November, followed by fruit setting and disappear during January under natural condition. Seeds dispersed have not germinated till the re-appearance of monsoon marked by off-season months between January and May.

MS and L_2 media fortified with various auxins like 2, 4-D, IBA, NAA and BAP at various concentrations were screened for callus and multiple shoot formation with leaf and shoot tip as explants. In the present investigation, L_2 media gave better response compared to MS media in the thorough morphogenesis investigation of *A. lanata* (Table 1, Fig. 1). Among the hormonal combinations employed 2.5 mg L^{-1} of 2, 4-D gave friable callus growth in L_2 media with >92% response from leaf explants. The parameters that influence organogenesis under *in vitro*

Table 1: Summary of response of *A. lanata* explants under in-vitro condition to L_2 media supplemented with different concentrations of Auxins and Cytokinins

Hormones employed (mg L^{-1})			Callus formation from explants (%)		Shoot growth	
2, 4 -D	NAA	BAP	Leaf	Shoot tip	No. of shoots per shoot tip	Shoot length (cm)
0.5	-	-	0	0.0	0^{e}	0^{e}
1.0	-	-	42.5±5.00	56.1±3.41	$4±1^{d}$	$2.0±1.0^{e}$
1.5	-	-	63.6±3.88	58.7±6.03	$8±2^{c}$	$2.2±0.1^{e}$
2.0	-	-	90.4±7.32	66.0±2.65	$14±2^{a}$	$3.8±0.2^{b}$
2.5	-	-	92.9±3.96	94.7±3.51	$13±2^{b}$	$3.5±0.1^{b}$
3.0	-	-	65.7±4.62	74.7±3.21	$6±1^{d}$	$2.1±0.2^{e}$
3.5	-	-	46.4±6.52	23.3±5.51	$3±1^{e}$	$1.5±0.2^{d}$
4.0	-	-	25.0±8.67	11.7±2.31	$2±1^{e}$	$0.5±0.1^{e}$
-	1.5	-	40.5±2.65	0.0	0^{e}	0.0^{e}
-	2.0	-	50.4±4.59	0.0	0^{e}	0.0^{e}
-	2.5	-	92.7±3.84	0.0	0^{e}	0.0^{e}
-	3.0	-	85.6±2.99	0.0	0^{e}	0.0^{e}
-	3.5	-	46.5±2.00	54.0±4.00	$7±2^{c}$	$3.4±0.3^{b}$
-	4.0	-	28.3±1.11	0.0	0^{e}	0.0^{e}
1.0	-	0.5	0.0	44.0±1.73	$12±2^{b}$	$1.2±0.1^{d}$
1.5	-	0.5	25.7±3.94	65.3±3.79	$13±3^{b}$	$2.3±0.2^{c}$
2.0	-	1.0	88.0±3.50	87.2±5.01	$15±2^{a}$	$4.5±0.3^{a}$
2.5	-	1.5	26.6±2.25	88.0±1.73	$17±3^{a}$	$4.7±0.2^{a}$
CD					3.23	0.62

Values represented with the same letter were not significantly different at p = 0.05. ±SD, CD at p = 0.05

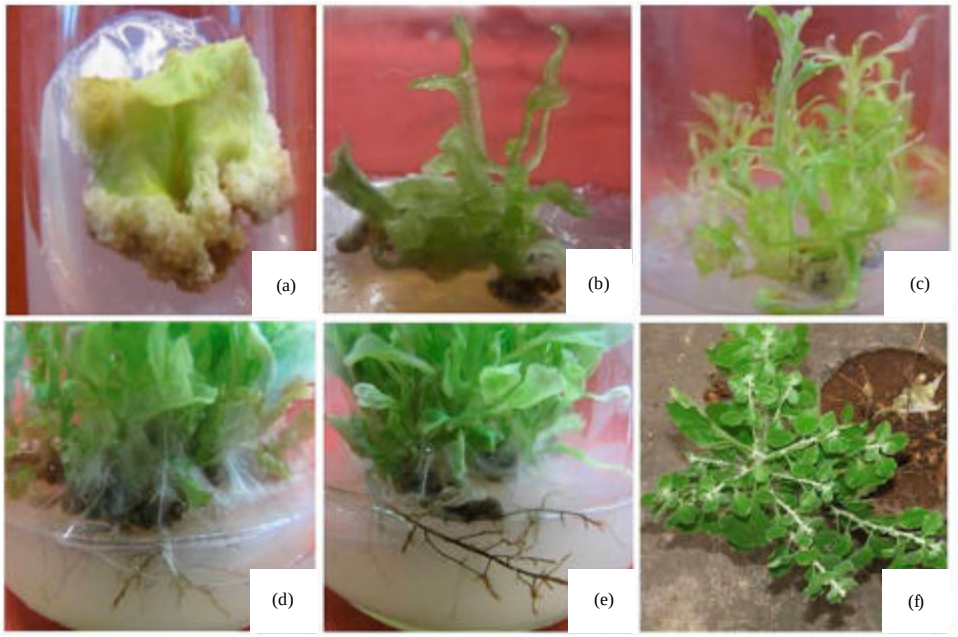

Fig. 1(a-f): (a) The callus formation in the leaf explant (L_2+2, 4-D 2.0 mg L^{-1}), (b) Multiple shoots at 21 days, (c) Multiple shoots at 54 days (L_2+ 2.5 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BAP), (c-d) Stages of root growth on half strength L_2 with NAA (2.0 mg L^{-1}), (e) Complete regenerated and (f) hardened plant

condition are the sources of carbon and nitrogen, pH of the medium and physical environment of culture including plant hormones (Rajore and Batra, 2005). Shoot tip explants on L_2 media supplemented with 2, 4-D (2.0 and 2.5 mg L^{-1}) produced three shoots after 30 days, on sub culturing 14 shoots were yielded in 74 days of incubation and the shoots were of 3 to 3.5 inch in height (Table 1; Fig. 1b, c). A combination of 2, 4-D (2.5 mg L^{-1}) and BAP (1.5 mg L^{-1}) gave multiple shoots in just 21 days which yielded 15-17 shoots within 60 days of incubation (Table 1). NAA (3.5 mg L^{-1}) addition resulted seven shoots per shoot tip in 15 days and proliferated within 43 days of incubation. Multiple shoot proliferation on L_2 with combination of 2, 4-D (2.5 mg L^{-1}) and BA (1.5 mg L^{-1}); 2, 4-D (2.5 mg L^{-1}) and BAP (1.5 mg L^{-1}) was 5% more responsive compared to media with 2, 4-D (2.0 mg L^{-1}) alone. The effect of BAP on multiple shoot bud differentiation has been demonstrated in number of plants using variety of explants (Jeong *et al*., 2001; Loc *et al*., 2005; Hiregoudar *et al*., 2003, 2006). MS media which is said to give best results with a wide range of plants did not give desirable results for the study plant although leaf explants yielded 60 and 65% callus response at 2.0 and 2.5 mg L^{-1} 2, 4-D, respectively (Table 2).

Table 2: Effect of MS media supplemented on leaf explant with different concentrations of Auxins and Cytokinins

Hormones employed (mg L^{-1})		Callus response from explant (%)	
2,4-D	NAA	Leaf	Remarks
0.5	-	0	No response
1.0	-	22±1.53	Calli only at borders
1.5	-	43±1.00	Calli at midrib and veins
2.0	-	60±3.21	White calli all over
2.5	-	65±2.08	Green Calli all over
3.0	-	47±1.53	Calli at borders
3.5	-	28±1.00	Callus induction
4.0	-	15±3.06	Minimum response
-	0.5	0	No response
-	1.0	21±4.04	Minimum response
-	1.5	42±1.00	Calli at midrib and veins
-	2.0	63±1.15	White calli all over
-	2.5	68±1.53	Green Calli all over
-	3.0	45±1.00	Calli at midrib and veins
-	3.5	29±3.79	Calli at borders
-	4.0	59±2.52	Brown calli with Rhizogenesis

Values are the mean of three replicates and ±SD

Shoots were then transferred to rooting media (half strength L_2+IBA and NAA) and rhizogenesis occurred after 36 days of incubation (Table 3, Fig 1d, e). Half strength L_2 with 0.5, 0.8, 1.0, 1.5, 2.0 mg L^{-1} of IBA and 0.5, 1.0, 1.5, 2.0, 2.5 mg L^{-1} of NAA were used separately. Out of which 2.0 mg L^{-1} of NAA gave roots within 36 days of

Table 3: Root response in the multiple shoots with half strength L_2 media supplemented with Auxins at different concentrations

Hormones employed (mg L^{-1})		Root growth		
IBA	NAA	Root response (%)	Root length (cm)	No. of days taken for root Initiation
0.5	-	0.0	0.0	50±1.53
0.8	-	85.0±2.65	2.53±0.95[a]	45±0.58
1.0	-	42.7±7.57	1.50±0.52[b]	48±1.53
1.5	-	25.2±4.20	1.03±0.47[c]	44±1.15
2.0	-	10.8±2.57	0.93±0.15[c]	50±1.53
-	0.5	10.3±2.08	0.58±0.30[f]	65±1.15
-	1.0	24.7±5.13	1.20±0.62[b]	54±1.53
-	1.5	36.0±2.65	2.20±0.40[a]	51±0.58
-	2.0	85.7±6.66	3.20±0.53[a]	36±2.08
-	2.5	21.6±3.86	1.80±0.40[b]	46±1.00
	CD		1.11	

Values represented with the same letter were not significantly different at p = 0.05. ±SD, CD at p = 0.05

incubation compared to IBA between 50 and 65 days. The success of NAA in promoting efficient root induction has been reported for *Holostemma ada-kodien* (Martin, 2002), *Ceropegia candelabrum* (Beena *et al*., 2003) and *Mucuna pruriens* (Faisal *et al*., 2006).

Hardening of regenerated plants was made on 1:1:1 ratio of Soil rite:Coco peat:Vermiculite and then transferred to pots with sterile soil in Green house and then transferred to field. In the current investigation, *A. lanata* plantlets derived through *in vitro* method have survival rate between 65 and 70%. Hardened plants were comparatively well branched and leaves were broader than donor plants and the parent plants (Fig. 1f). Success of *in vitro* response of these explants is strongly influenced by the source of the explants, media supplementation, growth regulators and controlled environment. Methodology standardised in the present study for complete plant regeneration of this *A. lanata* can be applied to produce plants throughout the year irrespective of season. Similarly, Azadi and Khosh-Khui (2007) observed a remarkable influence of the season on tissue culture response of barley (*Hordeum vulgare*) for cultivars Salome and Golden Promise.

Fungal contamination of explants and established shoots in culture was a major crisis for *in vitro* propagation despite under controlled condition plant growth has been observed and reduction of biotic stress was overcome by stringent plant sanitary measures. Maximum fungal contamination of shoots collected during October to December was observed and allied with conditions of high humidity and low temperatures. Regularly, contaminated cultures were discarded. Explant material collected during the monsoon months between June and July with optimum temperatures and high rainfall resulted in less fungal contamination and high bud break. In the present investigation, a perspective on response of explants of seasonally appearing *A. lanata* was studied to report the plant regeneration capacity under *in vitro* condition. The preferential seasonal appearance of *A. lanata* could be attributed to the active growth phase during high rainfall, optimum temperature and high humidity. Under *in vitro* conditions, however once the shoots are established, recurrent plantlet regeneration is derived throughout the year. These finding indicate that the implementation of rapid micropropagation techniques for seasonally appearing plants are not restricted.

We recommend the L_2 media supplemented with 2.5 mg L^{-1} of 2, 4-D and BAP (1.5 mg L^{-1}) for multiple shoot production and half strength L_2 with 1.5 mg L^{-1} of NAA for root growth establishment which were found to be the optimum combination for complete regeneration of *A. lanata* plant. Implications of the present investigation could be employed for *in vitro* multiplication of the medicinally important, seasonally appearing vulnerable herb *A. lanata*.

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