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RESEARCH ARTICLE

IN VITRO PROPAGATION OF *Oldenlandia umbellata L.* – A HIGHLY MEDICINAL & DYE-YIELDING PLANT OF COROMANDEL COAST

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ABSTRACT

Oldenlandia umbellata L. is an ancient Indian herb used as a source of red dye and various medicinal products. In present study successful protocol has been developed for mass propagation of *O. umbellata* in Murashige and Skoog (MS) medium. The explants, collected in the months of January-February respond well with 3.0 mgl^{-1} Benzylaminopurine (BAP) (13.9 shoots per culture). The shoots were multiplied good on MS liquid medium supplemented with 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} Indole-3 acetic acid (IAA) (63.2 shoots per culture). 96% shoots were rooted through *in vitro* rooting method and about 83% shoots were rooted using *ex vitro* methods with help of auxins (Indole-3 butyric acid and IAA). The plantlets were hardened in green house.

INTRODUCTION

The genus *Oldenlandia* (family Rubiaceae) consists of different species, many of which are used in traditional medicine (Seydel and Dornenburg 2006). *Oldenlandia umbellata* is commonly known as ''Indian madder'', known to yield a color-fast red dye from its roots, and has been used in diverse applications since ancient times. The root bark, preferably of a two-years-old plant, when used with a mordant will confer red color to calico, wool, and silk fabrics (Siva 2007). It is a low growing plant native to India and commonly found in parts of India (Coromandel coast), Burma, Sri Lanka, Cambodia and Indonesia.

The plant is well-known in Siddha Medicine for its styptic property. It is also a drug that can be administered for bronchial asthma, as a decoction of the entire plant, a decoction made from its root and liquorice in the ratio 10:4 or the powdered root is given either with water or honey. A decoction of the root is also used as a febrifuge. Both leaves and roots are also deemed good expectorants, and used for treatment of asthma, bronchitis, and bronchial catarrh (Gupta *et al.*, 2007). A decoction of leaves is used as a rinse to treat poisonous bites (Rekha *et al.*, 2006). Siva *et al.*, (2009a) reported a novel pH indicator dye from this plant. Siddha Medicine prepared from *O. umbellata* in the form of Mathirai, Vadagam or Leghyam is administered as directed by the Siddha Physician. For toxic bites and ulcers the decoction prepared from its roots and leaves are also used externally as a wash.

These varied uses have increased utilization and exploitation of *O. umbellata* for medicinal and dye extraction purposes (Cardon and du Chatenet 1990). As a result, natural stands of *O. umbellata* are fast disappearing and are threatened with extinction due to indiscriminate collection. The plant grows wild in forests, among other areas, and there is no propagation system available to replenish these stands. Therefore, an effort has been undertaken to develop a reliable protocol for mass multiplication of *O. umbellata* in present investigation.

MATERIALS AND METHODS

The mature plants of *Oldenlandia umbellata* growing in the campus of K.M. Centre for Postgraduate Studies, Pondicherry, India were selected for explants source. The nodal segments (2.0-3.0 cm in length) harvested from the field grown plants were used as explants. Explants were surface sterilized under aseptic condition in Laminar air flow hood with 0.1% $HgCl_2$ (w/v) for 3-5 min followed by 4-5 times washing with autoclaved water.

Media and culture conditions

Murashige and Skoog (1962) medium supplemented with 3% sucrose and additives (50 mgl^{-1} of ascorbic acid and 25 mgl^{-1} each of adenine sulphate, arginine and citric acid) were used for culture initiation. Culture medium was solidified by 0.8% (w/v) Agar (Hi-Media, India). The pH of the medium along with Plant Growth Regulator (PGR) was adjusted to 5.8±0.02 prior to autoclaving at 15 psi, 121°C for 15 min. The cultures were maintained at 25±2°C under 12-h photoperiod with light intensity of 40-50 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) provided by cool white fluorescent lamps and 60% Relative Humidity (RH).

Multiple shoot induction

To evaluate the influence of different culture medium composition on shoot induction, nodal segments were inoculated on various media viz. full or half-strength MS medium, Woody Plant (WP) medium (Llyod and McCown 1980) and B5 medium (Gamborg *et al.*, 1968). Since MS medium was found to be best for shoot induction, therefore, explants were cultured on MS medium supplemented with

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different concentrations (0.5-5.0 mgl^{-1}) of BAP and Kinetin (Kn) either alone or in combination of optimum concentration of BAP (3.0 mgl^{-1}) with IAA (0.1-2.0 mgl^{-1}) for shoot induction.

Multiplication of shoots in culture

The shoot cultures were further multiplied by two approaches (i) repeated transfer of mother explants and (ii) subculture of *in vitro* produced shoots. The original explants were repeatedly transferred (after harvesting shoots) to fresh MS medium supplemented with 3.0 mgl^{-1} BAP and 0.5 mgl^{-1} IAA to yield more shoots up to five passages. After 2-3 weeks regenerated shoots isolated from explants were cut into segments with 1-2 nodes (1.0-2.0 cm in length) and subculture on MS agar gelled as well as liquid medium containing different concentrations of BAP (0.1-3.0 mgl^{-1}). These experimental sets were maintained for three subcultures, with each passage of 3-4 week intervals.

Rooting of *in vitro* produced shoots

Rooting of shoots was attempted on agar-gelled medium. The healthy shoots were excised, separated and transferred individually to full, half, one-third and one-fourth strength of MS medium + 0.1% activated charcoal with 3% sucrose and various concentrations (0.5-5.0 mgl^{-1}) of IBA or IAA.

***Ex vitro* rooting of regenerated plants**

The *in vitro* produced shoots (3-4 cm) were carefully excised from shoot clumps and treated with auxins for root induction under *ex vitro* conditions and harden directly on soilrite under the green house conditions. The bases (4-5 mm) of shoots were treated with different concentrations (25-200 mgl^{-1}) of IBA and IAA for 5 min. Auxin-treated shoots were transferred to autoclaved soilrite in bottles moistened with one-fourth strength of MS basal salts. These were kept in the green house.

Hardening of plantlets in green house

Plantlets (3-4 cm in length) were hardened by two step method. In the first stage, the plantlets were kept in the green house for 4-5 days in capped glass bottles containing autoclaved soilrite moistened with 1/4 strength MS salts. The plantlets were gradually exposed to green house conditions by loosening and removing the caps. In the second stage, the plantlets were transferred to the pots containing sand, garden soil, and organic manure (2:2:1) and successful hardened plantlets of *O. umbellata* were transferred to the nursery.

Experimental design, data collection and statistical analysis

All the experiments were repeated thrice with 15 replicates for each treatment. The results are expressed as mean ± SD of three experiments.

RESULTS & DISCUSSION

Effect of season and culture medium on shoot induction

The nodal segments harvested during January-February from field grown plants were found to be the most suitable for establishment of cultures of *O. umbellata*. Similar types of season specific responses were earlier reported in *Capparis decidua* (Deora and Shekhawat 1995). *Quercus euboica* (Kartsonas and Papafotiou 2007) and *Salvadora persica* (Phulwaria *et al.,* 2011b). In the present investigation, full-strength MS medium was found to be the best for shoot induction. Regeneration of comparatively less number of shoots on half-strength MS medium, WP medium or B5 medium confirm the essentiality of sufficient amount of nitrates in the medium.

Table 1 Effects of cytokinin (BAP) on induction of shoots from explants of *Oldenlandia umbellata* on MS medium

BAP concentrations (mgl^{-1})	Number of Shoots / Explant ± SD	% of Response
Control (0.0)	0.0 ± 0.00	8
0.5	3.1 ± 0.39	17
1.0	4.3 ± 0.21	34
1.5	6.8 ± 0.86	58
2.0	8.2 ± 0.62	79
2.5	12.9 ± 0.83	93
3.0	13.9 ± 0.23	100
3.5	13.6 ± 0.16	82
4.0	9.3 ± 0.33	71
4.5	7.8 ± 0.81	49
5.0	4.2 ± 0.67	23

Note: Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with fifteen replicates per treatment

Bud breaking and initiation of cultures

Among all these explants tested, fresh shoot segments of length 2 to 3 cm with 1-2 nodes were found to be most suitable for culture initiation. These shoots when surface sterilized with 0.1% $HgCl_2$ for 4.0 min did not showed any kind of contamination in cultures. A 100% bud break occurred after 15 days of inoculation on MS semisolid medium. On MS medium supplemented with 3.0 mgl^{-1} BAP + additives, a maximum of 13.9±0.23 shoots were obtained (Fig. 1A and Table 1). This rate of shoot regeneration is very high as compared to previous report (Siva *et al.*, 2009b). Fewer shoots (4.2±1.03) were differentiated on MS medium with higher concentration of Kn along with additives. The rejuvenation of instant meristem was also achieved in *M. oblongifolia* by selection of explant type, and season for explant harvest and by treatment of cytokinins (Aitken- Christie and Connett 1992, Rathore *et al.,* 1993, Shekhawat *et al.,* 1993). Higher light conditions (25 to 30 $\mu molm^{-2}s^{-1}$) favored early bud breaking.

Table 2 Effects of Cytokinin (BAP) concentrations on multiplication of shoots from subcultured shoots on MS liquid Medium containing 0.5 mgl^{-1} IAA.

BAP Conc. (mgl^{-1})	Shoot Number ± SD	Shoot Length (cm) ± SD
0.1	33.1 ± 0.82	2.8 ± 0.57
0.5	54.6 ± 0.61	3.1 ± 0.23
1.0	63.2 ± 0.77	3.1 ± 0.83
1.5	52.3 ± 0.69	3.2 ± 0.30
2.0	43.6 ± 0.17	2.9 ± 0.58
2.5	36.7 ± 0.53	2.8 ± 0.62
3.0	**31.3 ± 0.92**	**2.7 ± 0.17**

Note: Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with fifteen replicates per treatment

***In vitro* multiplication of shoots**

Shoots initiated from activation of axillary meristem were further amplified by two ways (i) repeated transfer of mother explant (Boulay 1985, Franclet and Boulay 1989, Deora and

Shekhawat 1995) or (ii) subculturing of *in vitro* rejuvenated shoots. Repeated transfer was carried out on MS semisolid medium supplemented with 3.0 mgl^{-1} of BAP and 0.5 mgl^{-1} IAA + additives. On this medium 43-45 shoots were obtained after 15-20 days. This medium was supportive for shoot development up to fourth culture cycle. For subculturing of *in vitro* rejuvenated shoots agar gelled MS medium was also tried for further amplification of shoots but, that was not found suitable for multiplication and maintenance of cultures. Multiplication of cultures was best achieved by subculturing shoots on MS liquid medium supplemented with 1.0 mgl^{-1} of BAP + 0.5 mgl^{-1} IAA and additives for mass multiplication of shoots. About 63.2 ± 0.77 shoots were obtained after 3-4 weeks of inoculation (Fig. 1B and Table 2). Increase in IAA resulted in callus formation at the base of shoots. Among the cytokinins BAP was found superior to Kinetin (Bonga and Von-Aderkas 1992) when taken along with additives and placed in 12 h photo-period (Rathore *et al.*, 2005) (Table 3). It was also observed during the investigation that culture medium containing more than the average cytokinin, number and length of shoots was reduced (Rathore *et al.*, 1993, Shekhawat *et al.*, 2011) as shown in Table 4.

Table 3 Effects of Kn concentrations on multiplication of shoots from *in vitro* regenerated shoots on MS Medium containing 0.5 mgl^{-1} IAA

Kn Concentrations (mgl^{-1})	Shoot Number ± SD	Shoot Length (cm) ± SD
0.1	32.6 ± 0.54	2.6 ± 0.8
0.5	43.2 ± 0.83	2.7 ± 0.13
1.0	44.1 ± 0.89	2.8 ± 0.67
1.5	33.7 ± 0.14	2.8 ± 0.43
2.0	32.8 ± 0.87	2.6 ± 0.19
2.5	21.9 ± 0.66	2.2 ± 0.43
3.0	17.4 ± 0.34	1.7 ± 0.67

Note: Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with fifteen replicates per treatment

Table 4 Effects of Cytokinins (BAP + Kn) concentrations on multiplication of shoots from *in vitro* regenerated shoots on MS liquid Medium containing 0.5 mgl^{-1} IAA

BAP + Kn Concentrations (mgl^{-1})	Shoot Numbers ± SD	Shoot Length (cm) ± SD
0.1	32.3 ± 0.54	2.5 ± 0.83
0.5	43.9 ± 0.81	2.6 ± 0.87
1.0	53.6 ± 0.83	2.7 ± 0.62
1.5	42.8 ± 0.14	2.5 ± 0.31
2.0	32.3 ± 0.67	2.3 ± 0.29
2.5	21.9 ± 0.28	1.8 ± 0.47
3.0	15.2 ± 0.37	1.4 ± 0.76

Note: Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with fifteen replicates per treatment

Rooting of shoots

In vitro rooting of individual shoots was achieved with half-strength semisolid MS medium containing 2.5 mgl^{-1} of IBA + 0.1% activated charcoal. A 96% response was recorded in terms of *in vitro* rooting. About 11-12 roots of length 2-3 cm were obtained after 3-4 weeks of inoculation (Fig. 1C and Table 5). The *in vitro* root induction was low on medium supplemented with IAA (2-3 roots per shoot). Activated charcoal is said to promote *in vitro* rooting as it provides darkness and adsorbs PGRs (Thomas 2008). Diffused light (10 -20 $\mu molm^{-2}s^{-1}$ PPFD) also favored *in vitro* root induction. Delayed rooting was observed under high light intensity (30 to 50 $\mu molm^{-2}s^{-1}$ PPFD).

Table 5 Effects of IBA on *in vitro* regeneration of root numbers and length from *in vitro* generated shoots on half strength MS medium

IBA Concentrations (mgl^{-1})	Number of Roots ± SD	Length of Roots (cm) ± SD
0.5	2.2 ± 0.83	1.1 ± 0.70
1.0	6.4 ± 0.14	1.5 ± 0.83
1.5	7.6 ± 0.87	1.7 ± 0.54
2.0	8.6 ± 0.30	2.6 ± 0.00
2.5	11.2 ± 0.21	3.2 ± 0.23
3.0	10.4 ± 0.69	2.8 ± 0.47
4.0	9.8 ± 0.62	2.2 ± 0.63
5.0	7.5 ± 0.73	1.4 ± 0.92

Note: Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with fifteen replicates per treatment

In vitro produced shoots also rooted under *ex vitro* conditions. Shoots of length 3 to 4 cm having 2-3 nodes were harvested and when pulse treated with 200 mgl^{-1} of IBA for 3.0 min, 85% of the shoots rooted after 20-25 days of this treatment. About 1.9 ± 0.66 roots were obtained. IBA is more effective than NAA and NOA in promoting rooting of a wide variety of plants, and it is used commercially for rooting of many plant species worldwide (Phulwaria *et al.*, 2011a).

Hardening in green house

After 25-30 days of acclimatization plantlets measuring 5-6 cm long were successfully transferred from bottles to pots containing sand, garden soil, and organic manure. The cloned plantlets were hardened successfully in the green house and transferred to the pots (Fig. 1D). The hardened plantlets of *O. umbellata* were transferred to the nursery after 45 days of hardening in the green house.

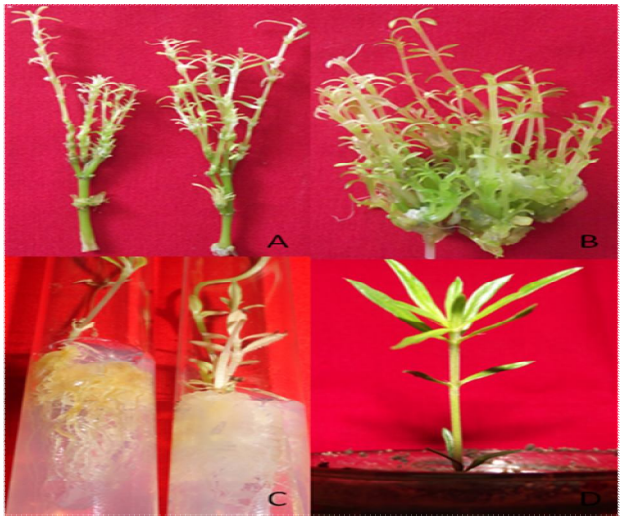

Fig. 1A- Induction of multiple shoots from nodal meristem; **Fig. 1B**- Shoots in multiplication stage in liquid MS medium;**Fig. 1C**- Bunch of roots in IBA through *in vitro* method;**Fig. 1D**- Hardened plant in green house

This is the first report on *in vitro* propagation of *O. umbellata* at mass scale from mature somatic tissues. The cloning process developed and described in this paper offers quick

method of propagation as well as *ex situ* conservation of this medicinal plant.

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