African Journal of Biotechnology Vol. 8 (16), pp. 3777-3781, 18 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Direct *in vitro* regeneration of a medicinal tree *Oroxylum indicum* (L.) Vent. through tissue culture

M. Gokhale and Y. K. Bansal*

Plant Tissue Culture Lab., Department of Biosciences, R. D. University, Jabalpur 482001 (M. P.), India.

Accepted 2 June, 2009

A simple and reliable protocol was developed through apical and axillary bud explants of *Oroxylum indicum* for multiple shoot regeneration. Effect of 2 cytokinins; BAP and KN, was studied. BAP at 4.43 µM proved better than KN with highest frequency of shoot initiation and maximum number of shoots initiated. Axillary bud showed significantly ($p < 0.05$) high shoot multiplication on MS medium with 4.43 µM BAP in subsequent sub culture passages. Elongated shoots were rooted on half strength MS medium with 4.92 µM IBA. Of the various methods used for hardening of *in vitro* regenerated plantlets, maximum plantlets survived after their subsequent transfer through sterilized-distelled-tap water and ultimately into soil:sand mixure.

Key words: Apical bud, axillary bud, 6-benzylaminopurine, *in vitro,* tissue cultue, *Oroxylum indicum* L.

INTRODUCTION

Oroxylum indicum (Linn) Vent. Family Bignoniaceae commonly known as Shivnak, Shyonak, Sonpatha or midnight horror, is a small deciduous, soft wooded tree. Several parts of this tree contain alkaloids and flavonoids (Grampurohit et al., 1994; Chen et al., 2003) of medicinal value used in the cure of several ailments including bronchitis, jaundice, piles, smallpox, leucoderma, cardiac disorder, scabies, enlarged spleen, helminthiasis, gastropathy, hemorrhoids and cholera (Pal and Jain, 1998; Kyo et al., 1998). The plant is used as a constituent of ayurvedic peparation Dashmularisht (Yasodha et al., 2004) and is a traditional herbal medicine in China and Japan (Chiang, 1997).

Owing to the indiscriminate collection, over exploitation and uprooting of whole plants bearing roots, this valuable tree has become vulnerable in Karnataka and Andhra Pradesh and endangered in Kerala, Maharastra, M.P. and Chhatisgarh (Darshan and Ved, 2003; Jayram and Prasad, 2008) and is feared to become endangered soon in other states too. Hence there is an urgent need for its mass multiplication and conservation under *in vitro* culture conditions.

Plant tissue culture offers unconventional techniques for plant improvement. It has become an important tool for conservation and mass propagation of important tree species. Conventionally, *O. indicum* reproduces via viable seeds and roots, but the low percentage of seed viability and destructive collection of roots from trees, limits its natural propagation. Hence alternative methods like *in vitro* techniques could be used to propagate this plant and thereby multiply elite genotypes. *In vitro* regeneration of this tree has been reported (Dalal and Rai, 2004). The present paper describes large-scale propagation of *O. indicum* through apical and axillary buds through tissue culture technology.

MATERIALS AND METHODS

Seeds of *O. indicum* were collected from reserve forest area of Chhindwara (latitude 22.0667) Madhya Pradesh-India. Seeds were germinated on sterile moist cotton *in vitro*. *In vitro* raised seedlings were given a treatment of 1-2 min each of 70% ethyl alcohol and 0.1% mercuric chloride. The explants; apical buds (ApB) (0.5-1 cm) and axillary buds (A x B) (0.7-1 cm) were dissected from the 15-20 days old seedling (8 cm). The explants were dried by placing them on sterile filter paper. Explants were inoculated under aseptic conditions on the sterile culture medium in test tubes on Murashige and Skoog (MS) medium supplemented with 3% sucrose, 0.7% agar and plant growth regulators; benzyl amino purine (BAP, 0.44-22.2

*Corresponding author. E-mail: yogendrakbansal@rediffmail.com. Tel.:0761-2604060 or 09425155199. Fax: 0761-2603752.

Table 1. Effect of different concentrations of cytokinin and explant type on *in vitro* shoot regeneration in *O. indicum* on MS medium (MSM).

Conc. of PGR (µM)	% Shoot regeneration		Shoot number initiated		Shoot length (cm)	
BAP	**Apical bud**	**Axillary bud**	**Apical bud**	**Axillary bud**	**Apical bud**	**Axillary bud**
0.44	33.41 ± 1.86	52.56 ± 1.30	1.0 ± 0.00	1.0 ± 0.00	1.2 ± 0.12	0.97 ± 0.33
2.22	42.92 ± 1.30	57.23 ± 1.60	1.0 ± 0.00	1.0 ± 0.00	1.5 ± 0.72	1.26 ± 0.32
4.43	87.61± 2.44	83.56 ± 1.30	6.83 ± 0.00	7 ± 0.67	2.46 ± 0.75	1.5 ± 0.42
22.2	39.24 ± 2.39	69.90 ± 1.36	3 ± 0.00	10.0 ± 1.0	1.0 ± 0.12	0.6 ± 0.21
44.43	8.66 ± 4.38	41.90 ± 1.91	0.0 ± 0.00	2 ± 0.00	0.51 ± 1.04	0.5±0.00
KN						
0.46	20.0 ± 1.16	50.53 ± 2.87	1.0 ± 0.00	1.0 ± 0.00	1.7 ± 0.06	1 ± 0.29
2.32	12.0 ± 0.87	54.6 ± 1.27	1.0 ± 0.00	1.0 ± 0.00	2.0 ± 0.58	1.56 ± 0.43
4.64	6.77 ± 0.79	43.25 ± 1.91	1.0 ± 0.00	2.0 ± 0.00	2.5 ± 0.58	2.1 ± 0.15
23.2	5.0 ± 1.16	18.91 ± 1.06	3.9 ± 0.67	4.33 ± 0.67	1.8 ± 0.46	2.06 ± 0.35
46.4	2.66 ± 1.01	7.05 ± 1.28	0.0 ± 0.00	1.0 ± 0.00	1.04 ± 0.54	0.5 ± 0.00
F value	1.04		0.22		0.18	
P value	P = < 0.001		P = < 0.001		P = < 0.001	

Values are mean ± SE.
SE = Standard error of the mean of three replicated experiments with 50 explant each.

µM) and Kinetin (KN,0.46-46.4 µM) was tested individually and in combination. The pH of the media was adjusted to 5.7 before adding agar. Medium dispensed in glass test tube (15x125 mm) was autoclaved at a pressure of 15 psi and a temperature of 121°C for 15 min. Before inoculation of autoclaved medium was left at 25°C for 24 h to check if there was no visible microbial contamination.

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25 ± 2°C, relative humidity (RH) of 60-70% and a light intensity of approximate. 1500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 h (light/dark).

The effect of continuous supplementation of PGRs on direct shoot regeneration was observed up to 3 subculture passages each of 20-22 days. Shoot buds from *in vitro* raised shoots for direct multiplication, from primary culture were used as explant for 2 and 3 subcultures. All experiments were completely randomized and repeated at least twice. Each treatment consisted of 50 replicates. The data on various parameters was statistically analyzed for the analysis of variance (ANOVA),'F' test for significance and least significant difference (LSD) was calculated at p = 0.05 ($LSD_{0.05}$). All computations were done by employing computer programme SPSS (Anonymous, 2004).

RESULTS AND DISCUSSION

Shoot initiation

Of the 2 cytokinins used BAP induced significantly higher percentage of shoot initiation and mean number of shoots, whereas higher mean shoot length was obtained in the shoots obtained on media supplemented with KN (Table 1). In the present study, it was found that the axillary bud showed high frequency of shoot initiation and shoot number at moderate concentration of BAP (4.43 µM). The superiorty of BAP over KN has been reported for shoot bud initiation (Satyanarayan et al., 2008). Cytokinins have been known to break dormancy of axillary buds resulting in the formation of microshoots (Devi et al, 1994; Sujatha and Reddy, 1998). In a tree species *Wrightia tomentosa* (Purohit et al., 2004) the percentage of bud breakage was significantly higher on media supplemented with BAP (2.22 µM - 8.86 µM ^{1}) (Figure 1).

Out of the 2 explants cultured on different concentrations of cytokinins axillary bud exhibited the highest frequency of shoot initiation and shoot number whereas apical bud exhibited highest shoot length (SL). In apical bud, shoot length was found to be significantly higher in MS medium with 4.64 µM kinetin. A similar observation was reported in *Anacardium* (Walia et al., 2007). It has been demonstrated that the endogenous auxin synthesized in the developing shoots is essential for the degradation and mobilization of storage cell wall polysaccharides (Santos et al, 2004).

Shoot multiplication

Axillary buds showed the highest number of shoots per responsive explant on MS medium with 22.2 BAP in primary sub culture. However, KN failed to induce multiple shoot formation and produced single shoot. In order to improve the shoot multiplication rate from their apical bud and axillary bud explant, they were cultured on media containing combination of various cytokinins. However, best response in terms of the number of shoots per responsive explant was observed on MS medium with 22.2 µM BAP from axillary bud explant in subsequent subculture passages (Table 2) but the shoots obtained with decreased shoot length. On MS medium containing BAP

Figure 1. (1) Initiation of multiple shoot on MS medium with BAP (4.43 µM). (2-3) Elongation of multiple shoots in passage II. (4) Rooting of *in vitro* raised shoots on MSM±IBA (4.92 µM). (5 - 6) *In vitro* regenerated plantlets in water. (7) *In vitro* regenerated plantlets in soil and sand mixture (1:1).

4.43 µM, longer shoots obtained with low rate of multiplication than on 22.2 µM BAP. Rate of multiplication showed a continuous increase up to third subculture passages significantly with BAP supplementation. In the same tree *in vitro* regeneration was reported on media supplemented with BAP (8.87 µM) from cotyledonary node explant.

Supplementation of BAP alone is reported to be capable for production of multiple shoots efficiently in some other plants (Ananthakrishnan et al., 1999, Ndoye et al., 2003). Concentrations of BAP 4.43 µM evoked shoot proliferation in *Crossandra* (Kathiravan et al., 1995) and citrus (Girija et al., 1999).

Rooting

Although all the 3 auxins (IBA, NAA, IAA), induced roots in *in vitro* raised shoots of *O. indicum*, yet IBA responded best for all parameters of rooting (Table 3). Shoots with highest percentage of root induction, maximum number of elongated roots were developed on MS medium containing IBA (4.92 µM). Long, branched healthy roots were produced on this rooting media. IBA is a potential auxin that induces rooting in *in vitro* regenerated shoots (Iriondo et al., 1995; Rajore and Batra, 2005).

In vitro raised plantlets could not survive if they transferred directly from culture tube to soil:sand mixure. Percentage of survival of acclimatized plants is greatly enhanced after water treatment. Water treatment is a maintanance of *in vitro* raised plantlets in distilled water in conical flasks covered with beaker (first 4 days), then in tap water and finally into soil:sand (1:1) mixure covered with polybags to maintain high humidity. Approximately 83% plantlets survive by this technique. Such plants were transferred to earthen pots after a period of 8 - 10 days, irrigated regularly and than planted in the field.

Hardening and acclimatization

Micropropagated plants often do not survive without acclimatization or they resume growth only a few days after

Table 2. Effects of BAP and subculture passage on *in vitro* shoot multiplication from apical bud and axillary bud of *O.indicum.*

BAP (µM)	Number of shoots per explant (Mean ± SE)			Shoot length (cm) (Mean ± SE)		
	Primary culture	Subculture I	Subculture II	Primary culture	Subculture I	Subculture II
Axillary bud						
0.44	1.0 ± 1.0	4.3 ± 1.18	6.8 ± 0.72	0.9 ± 0.26	0.5 ± 0.0	0.5 ± 0.0
2.22	1.0 ± 1.0	3.2 ± 0.57	7.5 ± 0.53	1.2 ± 0.30	1.0 ± 0.29	0.9 ± 0.25
4.43	7.0 ± 1.0	12.4 ± 1.51	14.6 ± 1.24	1.5 ± 0.40	1.5 ± 0.45	1.5 ± 0.40
22.2	12.0 ± 1.0	20.6 ± 1.56	29.4±1.40	0.5 ± 0.17	0.8 ± 0.25	0.8±0.26
44.43	2.0 ± 1.0	1 ± 0.0	5.4 ± 0.82	0.5±0.00	0.5 ± 0.12	0.5 ± 0.32
P Value = 0.00; F Value = 1.44						
Apical bud						
0.44	1.0 ± 0.0	4.5 ± 0.91	3.4 ± 0.80	1.2±0.44	20 ± 0.32	1.5 ± 0.45
2.22	1.0 ± 0.0	2.1 ± 0.68	4.5 ± 0.90	1.5 ±0.45	1.7 ± 0.38	2.0 ± 0.37
4.43	1.0 ± 0.0	5.4 ± 0.97	6.3 ± 0.55	2.0±0.20	2.0±0.40	3.0 ± 0.49
22.2	1.0 ± 0.0	7.4 ± 0.68	10.7 ± 1.72	1.0±0.26	1.0 ± 0.32	0.8 ± 0.41
44.43	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5±0.0	0.5 ± 0.25	0.5 ± 0.0
P value = 033; F value = 0.48						

Values are mean ± SE.
SE = Standard error of the mean of three replicated experiments with 50 explant each.

Table 3. Effect of auxins (IBA, NAA, IAA) on root induction from *in vitro* raised shoots in *O. indicum.*

IBA (µM)	% of root induction	Mean no. of roots	Mean root length (cm)
0.49	30.24 ± 2.02	3.1± 0.26	1.0 ± 0.25
2.46	57.84 ± 1.09	9.2 ±0.53	2.3 ± 0.25
4.92	80.17 ± 1.13	12.5 ± 1.19	4.0 ±0.42
NAA (µM)			
0.53	39.82 ± 2.0	4.2 ± 0.45	2.5 ± 0.66
2.68	66.34 ± 1.54	7.6 ±1.25	2.8 ± 0.35
5.37	78.45 ± 1.42	7.6 ± 0.63	3.0 ± 0.38
IAA (µM)			
0.57	12.64 ± 1.65	2.1 ± 0.68	0.5 ± 0.10
2.85	19.52 ± 1.95	3.4 ± 0.38	1.2 ±0.21
5.71	16.75 ±1.76	1.0 ± 0.00	1.0 ± 0.25
P value	0.00	0.00	0.014
F value	4.21	2.24	1.07

Values are mean ± SE.
SE = Standard error of the mean of three replicated experiments with 50 explant each.

soil transfer due to sub optimal conditions during the preceding stages of multiplication, rooting and acclimatization (Van Telgen et al., 1992).

The plantlets were covered with polythene bags to maintain high humidity and to prevent excessive loss of water from the leaves cuticle possessing wax (Jagannathan and Marcotrigiano 1986). Interestingly in *O. indicum* a middle stage was required by *in vitro* raised plantlets before transferring them to soil. For this, the plants were initially kept in distilled/tap water in flasks covered with beaker for approx 8 days (4 days in distilled water and 4 days in tap water). The beakers were removed once for 1-2 h duration during each day. Eventually the plantlets were transferred to soil:sand (1:1) cups.

Formation of new roots was initiated in water treatments, which resulted in the rate of survival of *in vitro* raised plantlets to 82%. Cytokinin stimulates stomatal opening, although *in vitro* raised plants showed less frequency of stomata (Table 1). In *O. indicum* stomata are

present only on the abaxial surface of the leaves. Wide stomata in *in vitro* plants of *O. indicum* has been reported (Parveen et al., 2006) but are incapable of controlling water loss.

In conclusion, a simple, efficient and high fidelity protocol for mass propagation of *O. indicum* (L.) Vent. from apical and auxiliary bud explants has been established. Using this protocol it is possible to produce viable, uniform and healthy plants with maximum survival rate.

ACKNOWLEDGEMENT

Gokhale M acknowledges funding received under the scheme "Women Scientist Scholarship Scheme for Societal Programmes (WOS-B), Department of Science and Technology, Government of India" for carrying out this research.

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