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In vitro Propagation of *Ochlandra wightii* (Munro) Fisch.: An Endemic Reed of Southern Western Ghats India

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Abstract: *Ochlandra wightii* (Munro) Fisch., an endemic reed bamboo in the Southern Western Ghats has been multiplied using tissue culture techniques. *In vitro* production of *O. wightii* plantlets has been achieved by using isolated embryos from mature seeds and nodal segments from *in vitro* seedlings. Better germination of isolated embryos (85%) was noticed in BAP (6-benzyl amino purine) (0.5 mg L^{-1}) with 1-2 shoots in 30 days. Half-strength MS medium supplemented with BAP (0.5 mg L^{-1}) and TDZ (Thidiazuron) (0.5 mg L^{-1}) enhanced the rate of shoot multiplication to an average of 9.8 shoots in 60 days. Simultaneous development of roots along with shoots was also noticed. Rhizome induction was achieved from 65% of seedling cultures when treated with KN (Kinetin) (1 mg L^{-1}) and higher concentration of sucrose (4%) in 35 days. Multiple shoots were also induced from nodal explants obtained from *in vitro* seedlings. Average 3.6 shoots were achieved in 40 days when treated with BAP (2 mg L^{-1}) and KN (0.5 mg L^{-1}). Twenty percent of the nodal cultures developed 1-2 roots during shoot development and the rest were rooted in half-strength MS solid medium supplemented with IBA (indole-3-butyric acid) (0.5 mg L^{-1}). Plantlets with healthy roots were hardened and transplanted to the clay pots. They showed 80% survival after 6 months. However, the plantlets with *in vitro* rhizomes showed 100% survival. Development of new culms and rhizomes were noticed after 3 months of greenhouse growth. Around 880 plantlets could be generated in 9 months from 10 embryos isolated from mature seeds using this protocol.

Key words: Bamboo, cytokinin, embryo, *in vitro* rhizome, micropropagation, node, *Ochlandra*, reed, regeneration, tissue culture

INTRODUCTION

Ochlandra wightii (Munro) Fisch. belongs to the family Poaceae is an endemic reed bamboo of the Southern Western Ghats. Its distribution is restricted to the hilly tracts of the extreme Southern regions of Kerala (Kumar, 2002). The genus, *Ochlandra* comprises 11 species, of which ten are endemic to the Western Ghats and one to Sri Lanka (Koshy and Mathew, 2009). The plant consists of rhizomes, culms and culm branches. Rhizomes are continuously grown horizontally and new culms are produced from the lateral buds. Culms are erect and slender up to 5 m in length and 3-4 cm girth. Leaves are oblong-lanceolate, glabrous and the ligule is long and papery. The species is monocarpic which flowers only once in their life span and dies. The flowering cycle of this species is presumed as 15-17 years (Jijeesh and Seethalakshmi, 2011).

Bamboos and reeds have received great attention as a forest produce due to its multifarious uses. The culms of *O. wightii* are widely used in pulp, paper, plyboard and cottage industries. It is also used as a material for fencing, for making flute stake for plantations etc. Leaves are used as fodder and thatching substitutes (Seethalakshmi and Kumar, 1998). The rapidly increasing bamboo-based industries have resulted in severe loss of forest stocks which demands adequate re-plantation of elite bamboo species to strengthen the raw material stock (Mudoi and Borthakur, 2009).

Vegetative propagation in reeds is very difficult due to limited availability of offsets from established culms and continuous harvesting cause damage to the parent culms. Moreover, the offsets are bulky and heavy to dig out and hence it is labour intensive and difficult to transport. Vegetative propagation of reeds by culm cuttings was standardized by Seethalakshmi *et al*. (1990).

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Propagation through seeds is undesirable due to the rapid loss of viability, infection of seeds by pests and diseases during storage and the uncertain flowering cycle. Conventional propagation techniques are insufficient and inefficient for large scale propagation and to satisfy the growing demand of planting materials, *in vitro* propagation is inevitable (Gielis, 1999) . These bottlenecks are also applicable to *O. wightii* for large scale production of planting materials. Numerous reports on tissue culture multiplication of bamboo have been published by several authors (Alexander and Rao, 1968; Huang and Murashige, 1983). However, reports on *in vitro* regeneration of *Ochlandra* spp. are very limited (Philip, 1998). Thus, potential techniques need to be developed to scale up sapling production of this endemic species to meet the requirements of afforestation programmes. The present study elucidated the *in vitro* multiplication of *O. wightii* from embryos isolated from mature seeds.

MATERIALS AND METHODS

Embryo culture: Flowering populations of *O. wightii* were located in Ponmudi, Palode forest range, Kerala. As the species is closely allied to *O. travancorica*, proper taxonomic identification was done and matched with live collections in the bambusetum, TBGRI. Mature seeds of *O. wightii* harvested were washed thoroughly in running tap water and treated with 1% commercial bleach (Ranbaxy, S.A.S. Nagar) and 0.2% labolin (Qualigens, Mumbai) for 30 min. They were rinsed in sterile distilled water and flame sterilized using ethanol. The embryos were carefully isolated by dissecting the seeds and inoculated onto half strength MS (Murashige and Skoog, 1962) solid (0.7% agar) medium supplemented with 2% sucrose and Plant Growth Regulators (PGR) such as BAP and KN at various concentrations and combinations (0.1-1.0 mg L^{-1}). pH of the media was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. All cultures were incubated at 24±2°C and under a photoperiod of 16/8 h provided by cool white fluorescent tubes.

The isolated embryos mostly developed single shoots with one or two leaves and roots in 30 days of incubation. The shoots were decapitated and long roots were trimmed before transferring to liquid basal medium supplemented with TDZ, BAP and KN (Table 1). Data regarding the number of shoots and roots and length of shoots were recorded after 30 days of subculture. The embryo culture is followed by axillary shoot proliferation and multiplication.

***In vitro* nodal culture:** In a separate study, nodal explants of 1.0 - 1.5 cm size isolated from *in vitro* seedlings were inoculated onto half strength MS solid (0.7% agar) medium supplemented with various concentrations and combinations of BAP and KN (Table 2). After 40 days, they were subcultured for rooting.

***In vitro* rhizome production:** Decapitated *in vitro* seedlings were cultured in half-strength MS liquid medium

Table 1: Effect of cytokinins on *in vitro* propagation of *O. wightii* embryos

Treatment*					
BAP	KN	TDZ	No. of shoots**	Shoot length (mm)**	No. of roots**
-	-	-	0.3±0.2^{a}	8.2±1.8^{a}	0.8±0.2^{a}
0.5			5.3±0.4^{i}	25.5±2.1^{def}	1.7±0.3^{abc}
1.0			1.5±0.2^{abc}	26.3±1.8^{ef}	2.7±0.4^{de}
2.0			2.5±0.5^{bcd}	18.0±1.6^{abc}	1.5±0.4^{abc}
	0.5		2.0±0.5^{abc}	24.5±2.2^{cde}	2.3±0.3^{cd}
	1.0		1.8±0.5^{abc}	28.4±3.1^{fgh}	2.8±0.5^{de}
	2.0		1.2±0.2^{ab}	26.2±4.6^{ef}	2.2±0.5^{bcd}
		0.5	3.8±0.5^{efg}	38.8±1.8^{i}	3.3±0.3^{e}
		1.0	2.5±0.2^{bcd}	23.7±2.5^{bcd}	2.2±0.2^{bcd}
0.5	0.5		3.2±0.2^{def}	28.3±1.8^{fgh}	1.3±0.2^{abc}
1.0	0.5		4.0±0.5^{fgh}	36.0±2.0^{hi}	1.0±0.1^{a}
1.0	1.0		4.7±0.6^{ghi}	30.4±1.8^{fgh}	1.3±0.2^{abc}
1.0	2.0		1.0±0.3^{a}	31.0±3.2^{fgh}	1.2±0.3^{ab}
2.0	1.0		2.0±0.5^{abc}	27.8±3.4^{fg}	1.2±0.3^{ab}
2.0	2.0		2.0±0.5^{abc}	27.8±3.4^{fg}	1.2±0.3^{ab}
0.5		0.5	9.8±0.5^{k}	34.4±1.4^{ghi}	1.0±0.3^{a}
0.5		1.0	7.0±0.6^{j}	25.0±1.3^{def}	1.5±0.2^{abc}
1.0		0.5	3.5±0.6^{efg}	18.5±1.7^{abc}	2.0±0.3^{abc}
1.0		1.0	1.0±0.4^{a}	11.0±0.6^{a}	1.5±0.2^{abc}
2.0		0.5	2.8±0.3^{cde}	16.1±1.4^{ab}	2.3±0.2^{cd}
2.0		1.0	1.5±0.2^{abc}	16.6±1.4^{ab}	1.3±0.2^{abc}

*Basal medium: Half-strength MS+2% sucrose and pH 5.7, **Means±SE of 8 replicates after 60 days of culture, Means followed by the same letter do not differ statistically at p = 0.05 according to DMRT

Table 2: Shoot production from nodal explants obtained from *in vitro* seedlings of *O. wightii*

Treatment (mg L^{-1})*		*In vitro* response**	
BAP	KN	Average No. of shoots	Average shoot length (mm)
-	-	0.0	0.0
0.5	-	1.0±0.4^{a}	48.8±0.4^{e}
1.0	-	1.5±0.3^{a}.	34.1±1.3^{c}
2.0	-	1.6±0.4^{a}	28.8±1.3^{b}
-	0.5	1.9±0.4^{ab}	50.0±2.5^{e}
-	1.0	1.0±0.2^{a}	56.8±3.0^{f}
-	2.0	1.6±0.3^{a}	40.3±3.1^{d}
1.0	1.0	2.9±0.4^{cd}	21.4±0.6^{a}
1.0	2.0	3.1±0.2^{cd}	19.3±0.8^{a}
2.0	0.5	3.6±0.4^{d}	28.4±0.8^{b}
2.0	1.0	3.3±0.4^{cd}	21.1±1.4^{a}
2.0	2.0	2.6±0.3^{bc}	20.0±1.8^{a}

*Basal medium: Half-strength MS+2% sucrose and pH 5.7, **Means±SE of 8 replicates after 40 days of incubation, Means followed by the same letter do not differ statistically at p = 0.05 according to DMRT

fortified with different concentrations of sucrose (0, 2%, 3, 4, 5 and 6%) for rhizome production. Effect of cytokinins like BAP and KN on rhizome production was also tested. The rate of *in vitro* rhizome production was recorded after 30 days.

Rooting and field establishment: Bunches of 2-4 shoots isolated from nodal cultures were subjected to *in vitro* rooting in basal MS medium containing IBA (0.5 mg L^{-1}). Well rooted plantlets were washed thoroughly in tap water and treated with 3% commercial fungicide (Indofil M45) before planting in a mixture of river sand and coarse charcoal (3:1). The plantlets were planted (5-10 clumps) in clay pots and kept in semi-shade (50%) and high humid (80-85%) mist house for hardening. After 4-5 week, the plantlets were transplanted to small plastic cups or poly bags and the survival rate was noticed.

Statistical analysis: Each of the growth regulator treatments contains a minimum of 8 replicas. Mean and standard error values were calculated on parameters like shoot number, shoot length and root number. One way analysis of variance was performed ($p<0.05$) and the means were compared using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Embryo culture: Embryos from mature seeds were used to initiate *in vitro* culture. Germination of isolated embryos was noticed after 10-12 days of culture in half strength MS solid medium supplemented with cytokinins (BAP and KN 0.1-1.0 mg L^{-1}). But the rate of germination was varied according to the type and concentration of cytokinins. BAP (0.5 mg L^{-1}) supplemented cultures showed the highest rate of germination (85%). All other treatments showed less response of average 60-80% germination (data not included). Normal seedlings with 1-2 shoots having leaves and roots developed in all the cytokinin treatments. However, medium devoid of growth regulators delayed the germination of isolated embryos to 20 days and showed only 60% response. Low rate of contamination and rapid induction of morphogenic responses have forced to use embryos as the most suitable explants in bamboo tissue culture (Woods *et al*., 1992; Yeh and Chang, 1987; Rout and Das, 1994). Further, multiple shoot induction from mature explants is a difficult task in bamboo tissue culture (Ravikumar *et al*., 1998). However, *in vivo* nodal segments (Ramanayake *et al*., 2006), inflorescence (Lin *et al*., 2005), nodes from *in vitro* raised seedlings (Rout and Das, 1994), shoot apices (Huang *et al*., 1989) and leaf explants (Hassan and Debergh, 1987) have also been successfully tested in different bamboos. Embryos have been utilized for *in vitro* propagation of difficult to propagate woody plants. Isolated embryos without endosperm showed shoot multiplication in *Taxus media* when treated with BAP or 2,4-D (Liao *et al*., 2006). In *Taxus baccata*, BAP and NAA enhanced the rate of production from embryo explant (Abbasin *et al*., 2010).

After 30 days of germination, the seedlings were decapitated and the roots were completely removed and subcultured for further multiplication in 25 mL liquid medium supplemented with cytokinins (Table 1). The suitability of liquid medium for shoot multiplication in *Bambusa tulda* (Saxena, 1990) and *Dendrocalamus strictus* (Nadgir *et al*., 1984) has been reported. Incorporation of agar detrimentally affects the shoot multiplication rate. Saxena (1990) avoided the necessity of a shaker to provide aeration to the shoots of *B. tulda* by reducing the quantity of liquid medium. This method was successfully adopted in the present study in *O. wightii*.

Significant variation in morphogenic response was observed in different cytokinin treatments. The roles of cytokinins on *in vitro* bud break and shoot multiplication is well known. The present study also showed the importance of cytokinin with preferential morphogenic responses. Among the cytokinins tested, KN seems to be least effective whereas TDZ and BAP showed better response. The potential effect of BAP over other cytokinins on shoot multiplication has been reported in several bamboos such as *D. asper* (Arya *et al*., 1999), *Bambusa bambos* (Kapoor and Rao, 2006), *B. vulgaris* (Ramanayake *et al*., 2006) and *Guadua angustifolia* (Jimenez *et al*., 2006). In *O. wightii* combined action of two cytokinins resulted in enhanced shoot multiplication response. Half-strength MS medium supplemented with

BAP (0.5 mg L^{-1}) and TDZ (0.5 mg L^{-1}) recorded the rate of multiplication to a maximum of 9.8 shoots in 60 days after subculture. Further increase in BAP or TDZ concentrations did not show favourable response on shoot production. Synergistic effect of PGRs showed improved morphogenic responses *in vitro*. Thidiazuron, a substituted phenylurea (1-phenyl-3-(1, 2, 3-thidiazo-5-yl)-urea) has been identified for its cytokinin-like activity in various explants cultured *in vitro* (Mok *et al*., 1982). Huetteman and Preece (1993) reported TDZ as the most potential cytokinin-like substance for micropropagating woody plants. Similar to our observation, addition of TDZ along with BAP enhanced the multiplication over 50% in *Amomum hypoleucum* (Bejoy *et al*., 2010). In *Bambusa wamin* (Arshad *et al*., 2005), BAP in combination with KN was found ideal for proliferation of mature nodal explants. Similar synergistic effect of two cytokinins on *in vitro* regeneration has been reported from many species such as *Tamarindus indica* (Farooq and Farooq, 2003), *Eugenia singampattiana* (Pavendan and Rajasekaran, 2011), *Cinnamomum camphora* (Soulange *et al*., 2007), *Boesenbergia pulcherrima,* (Anish *et al*., 2008) whereas, cytokinin and auxin enhanced multiplication was reported from *Taxus baccata* (Abbasin *et al*., 2010) and *Curcuma haritha* (Bejoy *et al*., 2006). The length of shoots was taken as a measure of growth in various cytokinin treatments. In the present study, shoot length was significantly less in presence of BAP and KN whereas TDZ (0.5 mg L^{-1}) showed the highest shoot length (Table 1). Simultaneous development of shoots and roots were also observed in 85-95% of isolated embryos in *O. wightii*. Similar results were reported in other bamboos (Shirgurkar *et al*., 1996; Huetteman and Preece, 1993). However, the rooting response was varied according to the growth regulator treatments (Table 1). Among the different treatments, TDZ supplemented cultures showed better response of 3.3 roots per explant. Murch and Saxena (2001) have reported the enhanced effect of TDZ on rooting in *Pelargonium* x *hortum*.

***In vitro* rhizome production:** Modifications in the *in vitro* culture media can lead to the development of storage and perennating structures like tubers, bulbils, rhizomes, corms etc. *In vitro* rhizomes not only help in early establishment of plants in the field but also culm production (Kapoor and Rao, 2006). The decapitated *in vitro* seedlings of *O. wightii* were cultured onto half-strength MS liquid medium supplemented with various concentrations of sucrose for the induction of *in vitro* rhizomes. Most of the cultures showed 2-4 rhizomes developed from the base of the proliferating shoot which grew horizontally in the medium and later developed into shoots (Fig. 1b). The rate of rhizome production was varied along with the growth regulator and sucrose

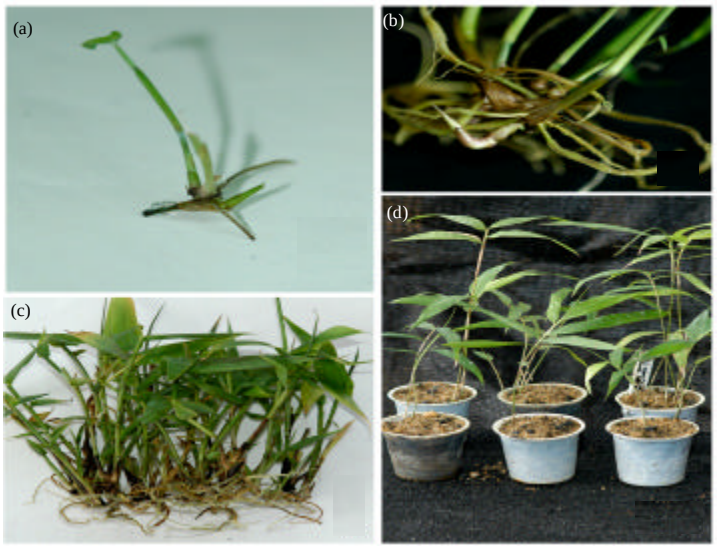

Fig. 1(a-d): *In vitro* propagation and rhizome induction on *Ochlandra wightii*, (a) Multiple shoot induction on ½ MS+BAP (2 mg L^{-1}) and KN (0.5 mg L^{-1}) from node harvested from *in vitro* seedlings, (b) *In vitro* rhizome formation on ½ MS+KIN (1 mg L^{-1})+Sucrose (4%), (c) Deflasked plantlets after 30 days in rooting medium, (d) *Ex vitro* plants of *O. wightii* after hardening

concentration (Fig. 2). This event occurred spontaneously in *D. strictus* during the rooting phase (Shirgurkar *et al*., 1996). In the present study, basal medium without growth regulator and sucrose was found ineffective in rhizome induction. Kinetin proved to be more effective on rhizome development when 3-5% sucrose was used whereas; BAP (1 mg L^{-1}) was less effective and 20 percent of cultures developed rhizomes. Availability of higher levels of sucrose has also influenced rhizome development in *O. wightii*. Four percent sucrose was found ideal for rhizome production in both the cytokinins tested. Half strength MS with KN (1 mg L^{-1}) and 4% sucrose showed the highest rate (65%) of *in vitro* rhizome production in 30 days. Further decrease or increase in sucrose concentration did not show positive response (Fig. 2). *In vitro* rhizome formation has also been reported in *B. bambos* var. *gigantea* with the addition of 5% sucrose along with growth regulators and recorded 100% rhizome formation within four weeks (Kapoor and Rao, 2006). Apart from growth regulators, increased sucrose levels in the medium also have a significant role in the induction of storage organs *in vitro*. Bamboo rhizomes mainly function as storage of starch and fibres (Hsiung *et al*., 1980). Reports revealed that enhanced sucrose concentrations foster the development of storage organs in *Curcuma longa* (Shirgurkar *et al*., 2001), *C. aromatic* (Nayak, 2000), *Bunium persicum* (Grewal, 1996). However, microtubers were achieved on half MS medium supplemented with BAP (2 mg L^{-1}) and NAA (0.1 mg L^{-1}) under normal sucrose (3%) regime (Yamaner and Erday, 2008).

***In vitro* nodal culture:** The use of nodal explants from *in vitro* cultures has certain advantages viz., availability of explants for raising axenic cultures, irrespective of season. Once an aseptic culture system has been developed, it can be utilized for the continuous production of plantlets. In *O. wightii* nodal explants (1.0-1.5 cm) isolated from *in vitro* seedlings were inoculated onto basal medium supplemented with various concentrations and combinations of BAP and KN (Table 2). Initial axillary bud proliferation was observed in 15 days and subsequently more shoots from the base (Fig. 1a). A combination of BAP (2 mg L^{-1}) and KN (0.5 mg L^{-1}) treated cultures showed most number of shoots (3.6) in 40 days. Individual use of the cytokinins showed bud proliferation but rarely multiple shoot development. Though shoot growth was moderate in all the treatments, KN (0.5 mg L^{-1}) exhibited best growth response and obtained 50 mm shoots in 40 days.

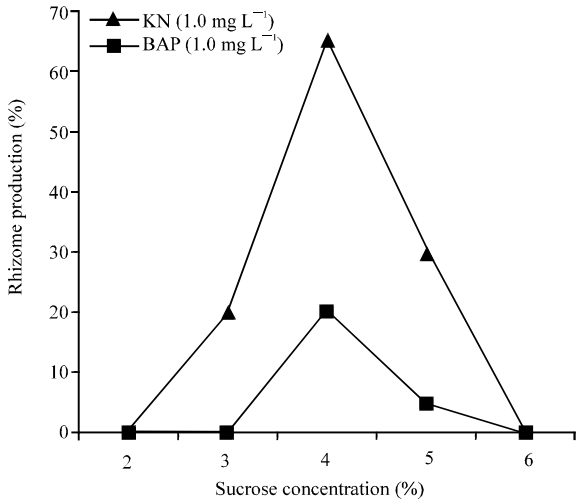

Fig. 2: Effect of cytokinins and sucrose concentration on *in vitro* rhizome production in *O. wightii* seedlings after 30 days of incubation, Basal medium: ½ MS liquid medium

Incidence of callusing or *in vitro* rhizome formation was not observed in any of the treatments. Rout and Das (1994) used *in vitro* nodal explant for callus induction and subsequent somatic embryogenesis in three species of bamboo. Around 880 plantlets could be generated from ten embryos isolated from mature seeds using this protocol (embryo culture followed by axillary bud proliferation and multiplication) in 9 months. In *D. hamiltonii*, *in vitro* shoots raised from adult nodal cultures exhibited profuse multiplication (20 fold) when treated with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Agnihotri and Nandi, 2009).

Rooting and field establishment: Cultures established from embryos showed simultaneous development of roots along with shoots. Similarly, in cultures having *in vitro* rhizomes, roots were developed from rhizome nodes. In these cases a separate rooting phase is not necessary. Similar results were reported in *D. strictus* where *in vitro* shoots rooted in BA containing multiplication medium, in the absence of Auxin (Shirgurkar *et al*., 1996). Twenty percent of the nodal cultures of *O. wightii* developed 1-2 roots during shoot development and the rest were rooted separately. Small clumps of 2-4 shoots obtained from *in vitro* nodal explants were treated in half-strength MS solid medium supplemented with IBA (0.5 mg L^{-1}). Under this treatment 70% cultures developed 2-4 healthy roots in 30 days of incubation. IBA is widely used as rooting hormone in several bamboo species like *B. vulgaris* (Ramanayake *et al*., 2006), *B. wamin* (Arshad *et al*., 2005) and *D. hookeri* (Ramanayake *et al*.,

2008). Agnihotri and Nandi (2009) reported the improved rooting of *D. hamiltonii* microshoots when treated with 20 mg L^{-1} IBA for 10 days followed by hormone-free medium. In yellow bamboo, shoots from adult culms showed only 40% rhizogenic response with 3 mg L^{-1} IBA, while pretreatment with TDZ (0.5 mg L^{-1}) and incubation under continuous light increased the rootability of neo-formed shoots to 100% (Ramanayake *et al*., 2006).

Plantlets with healthy roots were deflasked (Fig. 1c) and treated with a commercial fungicide (3%) for 5 min. before planting in community pots containing river sand and coarse charcoal (3:1). The plantlets were kept in semi-shade (50%) and high humid (80-85%) greenhouse conditions for 3 months for initial establishment. Development of new culms and rhizomes were noticed during hardening. The hardened plantlets were then repotted in plastic cups and recorded 80% survival in 6 months (Fig. 1d). However, *in vitro* seedlings with rhizomes showed 100% survival.

In conclusion, the study clearly demonstrates an efficient *in vitro* protocol for the propagation of *O. wightii* through embryo culture and subsequent axillary shoot proliferation. *In vitro* propagation technique allows the mass production of plantlets within a short period and is less labour intensive. Thus, it offers distinct advantageous over conventional propagation methods to scale up sapling production of this endemic species. The present investigation has the potential of generating many thousands of plants per year to recover cleared natural stands or to start new plantations of *O. wightii*.

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