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Tissue culture and *In-vitro* conservation in *Operculina turpethum* (L.) Silva Manso: A threatened medicinal Plant of South India

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Abstract
An investigation was undertaken on tissue culture and *In-vitro* conservation studies in *Operculina turpethum* (L.) Silva Manso. at the Division of Floriculture and Medicinal Crops, ICAR- Indian Institute of Horticultural Research, Bengaluru during 2020-2021. The experiment was laid out in Completely Randomized Design (CRD) and replicated thrice. Shoot was multiplied using nodal explants cultured on MS medium supplemented with various combinations of growth regulators like BAP, Kinetin and NAA. Among the different combinations, BAP (1 mg/l) + NAA (0.5 mg/l) recorded maximum number of shoots (3.25±0.05) and leaves per explant (4.95±0.04) with minimum days for shoot initiation (9.15±0.06). Whereas, the highest shoot length (4.65±0.05 cm) was noticed on BAP (1 mg/l). The satisfactory results after three months of *In-vitro* conservation were obtained using BAP (1 mg/l) + NAA (0.5 mg/l) in terms of high survival per cent (100), maximum number of shoots (3.30±0.02) and leaves per explant (4.68±0.04). Hence, BAP (1.0 mg/l) + NAA (0.5 mg/l) and BAP (1.0 mg/l) can be considered as the best treatment combination for *In-vitro* shoot multiplication of *O. turpethum*. Further, the protocol can be utilized for effective conservation, utilization and multiplication of this valuable threatened medicinal plant.

Keywords: *Operculina turpethum*, shoot multiplication, nodal segments, *In-vitro* conservation

Introduction
O. turpethum (L.) Silva Manso. is a threatened medicinal plant used in the Indian system of medicine, belonging to the family Convolvulaceae. It is a perennial climber with purplish stem and milky white exudates (Kohli *et al*., 2010) [9]. It is commonly known as “Indian Jalap”, “Trivrit” or “Turpeth” in English, “Nisoth” or “Pitohri” in Hindi, “Bangadaballi” or “Bilitigade” in Kannada, “Tegada” in Telugu, “Caralam” or “Civatai” in Tamil and “Thrikoolpakkonna” in Malayalam (Nafees *et al*., 2020) [13]. The species is distributed across India, Bangladesh, Nepal, China, Srilanka, Australia, Africa, South America, Pakistan, Taiwan, Myanmar, Indonesia, Malaysia, Thailand, Papua New Guinea, Philippines and is naturalized in West Indies (Sharma and Singh, 2012) [24]. In India, it is commonly found in the dry zones of Karnataka and Tamil Nadu (Hoq and Tamanna, 2019) [7]. It is a very potent herb having wide pharmacological actions and therapeutic significance. Roots having α and β turpethin as active principles are used in the treatment of fever, cough, asthma, edema, anaemia, ascites, anorexia, constipation, gout, rheumatism, hepatitis, haemorrhoids, intoxication, ulcers, fistulas abdominal tumors, wounds, worm infestation, scorpion sting and snake bite (Thamizhmozhi and Nagavalli, 2017) [27]. Some of the ayurvedic formulations like Trivrit Arishta, Trivrit Avaleha, Trivritadi Modak, Trivritadi Kalka and Avipattikar Churna contains *O. turpethum* as a vital ingredient (Sharma and Singh, 2012) [24]. But rapid depletion of natural habitat has greatly reduced the distribution of this species and increased the risk of genetic diversity loss (Alam *et al*., 2010) [1]. Apart from this, destructive harvesting of root as a source of raw material for ayurvedic drug preparation and other anthropogenic activities have made the species severely threatened in nature. It is Red-listed (vulnerable) medicinal species by IUCN (International Union for Conservation of Nature). Hence, there is an urgent need for conservation.

Seed propagation suffers from problems like poor seed viability, low percentage of germination, survivability and dormancy (Tiwari *et al*., 2018) [28]. Hence, there is a need to standardize an alternative method to produce true to type plants.

In-vitro propagation is one such tool which can fulfil the conservation as well large-scale multiplication. By exploiting this technique, further they can be subjected to *In-vitro* conservation studies. Therefore, the present study was carried out with an objective to standardize a protocol for *In-vitro* shoot multiplication and conservation of *O. turpethum.*

Material and Methods

Experimental site

The experiment was carried out at ICAR- Indian Institute of Horticultural Research (ICAR-IIHR), Hesaraghatta, Bengaluru. It is located in the eastern dry zone of Karnataka at $13^{0}58^{'}$ North latitude and 78^{0} East longitude. It is situated at an altitude of 890 meters above the mean sea level (MSL). It comes under zone-5 of region-3 among the agro-climatic zones of Karnataka and receives South-West and North-East monsoons.

Plant material and cultural conditions

a) *In-vitro* shoot multiplication

Nodal segments having axillary buds were collected from the mature plants of *O. turpethum* which were grown in the Field Gene Bank of RET (Rare, Endangered and Threatened) medicinal plants, Division of Floriculture and Medicinal crops, ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru. The nodal explants were placed in a beaker covered with muslin cloth. Then they were surface sterilized by washing for 30 minutes under running tap water to remove the adhering dust particles and microbes from the surface. Later, they were treated with a liquid detergent (Tween 20) for another 15 minutes and washed adequately under running tap water to remove the detergent. Thereafter, the explants were dipped in 70 per cent (v/v) ethanol for 1-2 minutes and alcohol was decanted by washing the explants thoroughly with sterile distilled water and surface sterilized with $HgCl_2$ (0.2%) solution for 5-7 minutes under aseptic conditions in a laminar airflow hood. The surface sterilized explants were inoculated on basal MS medium containing different concentrations and combinations of growth regulators. All the cultures were maintained in a Standard Culture Condition (SCC) at a temperature of 26 ± 2^{0}C under incandescent bulb (25W) with a photoperiod of 16-hour white light and 8-hour dark per day. The proliferation of new shoots began within four weeks of inoculation and these nodal explants were subcultured every month. The estimation of growth parameters like days taken for shoot initiation, number of shoots per explant, shoot length and number of leaves per explant were recorded in Standard Culture Conditions (SCC) after eight weeks of inoculation.

b) *In-vitro* conservation

Short term conservation of *In-vitro* raised plants of *O. turpethum* was attempted for three months by providing low temperature (10^{0} C) and low light intensity (2.97 μm^{-2} s^{-1}). In order to accomplish this, equal number of four-week old tissue cultured plants from each treatment (MS medium + hormonal combinations) were taken and kept under low light intensity (2.97 μm^{-2} s^{-1}) in a chamber having ambient temperature maintained at 10 ^{0}C. Equal number of replicates from each treatment were kept under Standard Culture Condition (SCC) to compare it with those kept under Reduced Culture Condition (RCC). Proper subculturing was done before keeping them under SCC and RCC. Observations on growth parameters were recorded at regular intervals.

Statistical analysis

The experiment was laid out in Completely Randomized Design (CRD) with three replications. The data was analyzed statistically by following the procedure outlined by Panse and Sukhatme (1967) [16].

Results and Discussion

a) *In-vitro* shoot multiplication

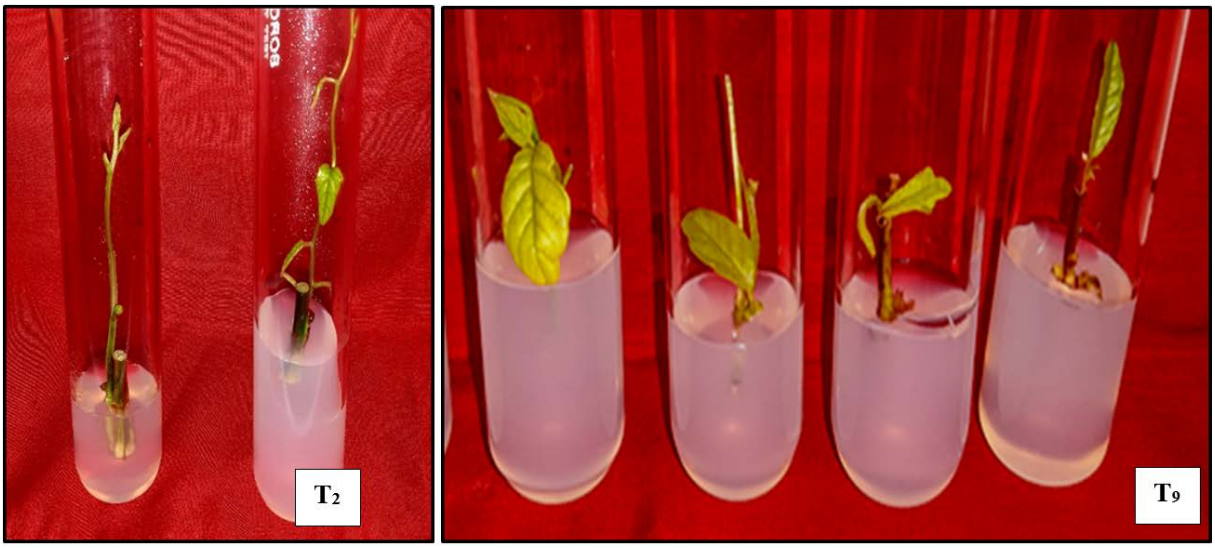
Healthy nodal explants were collected from the Field Gene Bank and cultured on MS medium supplemented with various concentrations and combinations of growth regulators like BAP, KIN and NAA. The details of observations recorded are presented in Table 1.

MS medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l) recorded maximum number of shoots (3.25±0.05) and leaves per explant (4.95±0.04) with minimum (9.15±0.06) days for shoot initiation. Whereas, the highest shoot length (4.65±0.05 cm) was recorded in BAP (1.0 mg/l). MS medium fortified with KIN (1.0 mg/l) reported minimum number of leaves (1.72±0.05) and shoots per explants (1.04±0.04) with decreased shoot length (1.41±0.04 cm). Similar results were obtained by Alam *et al.* (2010) [1], where BAP (1.0 mg/l) alone was responsible for rapid shoot bud proliferation with maximum number of shoots in each bud. BAP is a cytokinin that helps in cell division and organisation of apical meristem. This might be the reason for enhanced cell multiplication in axillary and terminal meristematic zones of nodal explant tissue. The effectiveness of BAP in shoot initiation, shoot formation, release of lateral bud dormancy and to overcome apical dominance has been explained by George *et al*. (1993) [6]. Although the supplementation of MS medium with BAP and KIN can initiate shoots in the culture medium, the enhanced effect on shoot proliferation can be seen only on addition of auxin (NAA). The findings of Bisht *et al*. (2012) [4] showed similar results in *Hedychium coronarium*, when seedlings explants were inoculated on MS medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l). Similarly, maximum shoot proliferation with highest shoot length was obtained through rapid multiplication of *Salacia chinensis* (Majid *et al.*, 2016) [11].

The role of BAP and NAA on multiple shoot induction has been reported in some of the RET medicinal plants like *Chlorophytum borivilianum* (Purohit *et al.*, 1994) [18], *Calophyllum apetalum* (Nair and Seeni, 2003) [15], *Celastrus paniculatus* (Nair and Seeni, 2001; Rao and Purohit, 2006) [14, 21], *Picrorhiza kurrooa* (Chandra *et al.*, 2006) [5], *Saraca asoca (*Subbu *et al*., 2008) [26], *Oroxylum indicum* (Sandesh *et al*., 2018; Tiwari *et al.*, 2007) [22, 29], *Celastrus paniculatus* (Arya *et al*., 2001; Senapathi *et al*., 2013) [2, 23], *Kaempferia galanga* (Kalpana and Anbazhangan, 2009) [8], *Cayratia pedata* var. *glabra* (Sharmila *et al.*, 2020) [25].

Table 1: *In-vitro* shoot multiplication in *Operculina turpethum* (L.) Silva Manso. using different growth regulators

Treatment	Number of days taken for shoot initiation (mean ± SE) *	Shoot length (cm) (mean ± SE) *	Number of shoots per explant (mean ± SE) *	Number of leaves per explant (mean ± SE) *
T_1 - BAP (0.5)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_2 - BAP (1.0)	10.01±0.06	4.65±0.05	3.01±0.01	4.31±0.04
T_3 - KIN (0.5)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_4 - KIN (1.0)	20.33±0.52	1.41±0.04	1.04±0.04	1.72±0.05
T_5 - BAP (0.5) + KIN (0.5)	16.26±0.17	2.78±0.07	1.50±0.06	2.85±0.03
T_6 - BAP (1.0) + KIN (0.5)	13.78±0.08	3.93±0.04	2.35±0.07	2.79±0.05
T_7 - BAP (1.0) + KIN (1.0)	17.05±0.06	3.29±0.04	1.18±0.02	3.47±0.05
T_8 - BAP (0.5) + KIN (1.0)	17.57±0.05	2.43±0.04	2.06±0.06	1.94±0.04
T_9 - BAP (1.0) + NAA (0.5)	9.15±0.06	4.20±0.07	3.25±0.05	4.95±0.04
T_{10} - BAP (0.5) + NAA (1.0)	11.76±0.05	3.99±0.01	2.50±0.03	2.46±0.04
T_{11} - KIN (0.5) + NAA (1.0)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_{12} - KIN (1.0) + NAA (1.0)	11.02±0.12	3.59±0.03	2.92±0.06	3.75±0.06
S.Em±	0.17	0.04	0.04	0.04
CD@1%	0.66	0.16	0.17	0.16



Best treatments in tissue culture of *Operculina turpethum* (L.) Silva Manso. {T2-BAP (1 mg/l), T9: BAP (1 mg/l) + NAA (0.5 mg/l)}

b) *In-vitro* conservation

The *In-vitro* conserved plants at RCC recorded high survival per cent (100) when cultured on MS medium containing BAP (1.0 mg/l), BAP (1.0 mg/l) + KIN (0.5 mg/l), BAP (1.0 mg/l) + NAA (0.5 mg/l) and BAP (0.5 mg/l) + NAA (1.0 mg/l). The maximum number of shoots (3.30±0.02) and leaves per explant (4.68±0.04) were noticed on MS medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l) even after three months of conservation. Similarly, Bhat (2020) [3] and Pooja (2020) [17] carried out an investigation on *In-vitro* conservation of *Alpinia galanga* and *Aristolochia tagala* respectively, and were successful in conserving the *In-vitro* raised plants for a short duration of three months under reduced culture conditions.

The *In-vitro* approach has been successfully utilized for the conservation of other RET medicinal plants like *Tylophora indica* (Rajasekharan *et al.*, 2009) [19], *Nothapodytes nimmoniana* (Rajasekharan *et al.*, 2010) [20], *Decalepis hamiltonii* (Kumar, 2015) [10], *Holostemma ada-kodien* (Tuppad, 2016) [30] and *Salacia chinensis* (Mastiholi *et al.*, 2018) [12] which could be successfully conserved for a period of six months. This depicts that a specific concentration and combination of cytokinin and auxin is most suitable for cell division, resulting in the slow growth of shoots for three months. Hence, the medium containing BAP (1.0 mg/l) + NAA (0.5 mg/l) can be considered as the best, for *In-vitro* conservation of *O. turpethum*.

Comparisons of growth for tissue cultured plantlets kept under Standard Culture Condition (SCC) and Reduced Culture Condition (RCC) after three months.

A comparison study between the tissue culture derived plants kept under SCC and RCC showed that MS medium fortified with BAP (1.0 mg/l) + NAA (0.5 mg/l) reported maximum survival per cent, number of shoots and leaves per explant in both SCC and RCC after three months of *In-vitro* conservation (Table 2). This clearly shows that; this particular combination and concentration of growth regulators can be used for normal growth as well as conservation of tissue cultured plantlets in *O. turpethum*.

In the present study, the *In-vitro* raised plants which were maintained at low temperature (10 ^{0}C) and low light intensity (2.97 $\mu m^{-2}s^{-1}$) showed slow growth when compared with those kept under SCC, after three months of conservation. Reduction in temperature and light intensity is an effective way of conservation with optimal shoot and auxiliary bud formation. These strategies of *In-vitro* conservation help to reduce the growth rate of cultures (Rajasekharan *et al.*, 2009) [19]. Hence, short term conservation can be considered as an useful tool to maintain the *In-vitro* raised plants for a prolonged period of time.

Table 2: Comparisons of growth for tissue cultured plantlets kept under Standard Culture Condition (SCC) and Reduced Culture Condition (RCC) after three months.

Treatment	Survival per cent		Shoot length (cm) (mean ± SE) *		Number of shoots per explant (mean ± SE) *		Number of leaves per explant (mean ± SE) *	
	SCC	RCC	SCC	RCC	SCC	RCC	SCC	RCC
T_2 - BAP (1.0)	80	100	5.01±0.03	4.78±0.06	3.25±0.02	3.13±0.03	4.44±0.05	4.18±0.03
T_4 - KIN (1.0)	55	75	1.70±0.04	1.52±0.05	1.17±0.04	0.99±0.02	1.87±0.07	1.60±0.02
T_5 - BAP (0.5) + KIN (0.5)	65	90	3.19±0.03	2.97±0.03	1.86±0.02	1.63±0.02	3.05±0.07	2.77±0.05
T_6 - BAP (1.0) + KIN (0.5)	80	100	4.32±0.07	4.22±0.09	2.71±0.04	2.50±0.01	2.93±0.05	2.72±0.03
T_7 - BAP (1.0) + KIN (1.0)	70	90	3.71±0.05	3.52±0.06	1.28±0.01	1.17±0.01	3.59±0.05	3.41±0.02
T_8 - BAP (0.5) + KIN (1.0)	60	85	2.72±0.05	2.59±0.04	2.39±0.04	2.16±0.04	2.11±0.08	1.83±0.03
T_9 - BAP (1.0) + NAA (0.5)	85	100	4.67±0.05	4.53±0.04	3.47±0.03	3.30±0.02	5.14±0.07	4.68±0.04
T_{10} - BAP (0.5) + NAA (1.0)	80	100	4.15±0.04	4.00±0.01	2.85±0.05	2.64±0.02	2.57±0.05	2.38±0.03
T_{12} - KIN (1.0) + NAA (1.0)	75	90	4.01±0.02	3.85±0.07	3.15±0.03	2.94±0.03	3.91±0.04	3.71±0.03
S.Em±	2.89	2.14	0.04	0.05	0.03	0.02	0.06	0.03
CD@1%	11.75	8.76	0.18	0.22	0.13	0.10	0.24	0.13

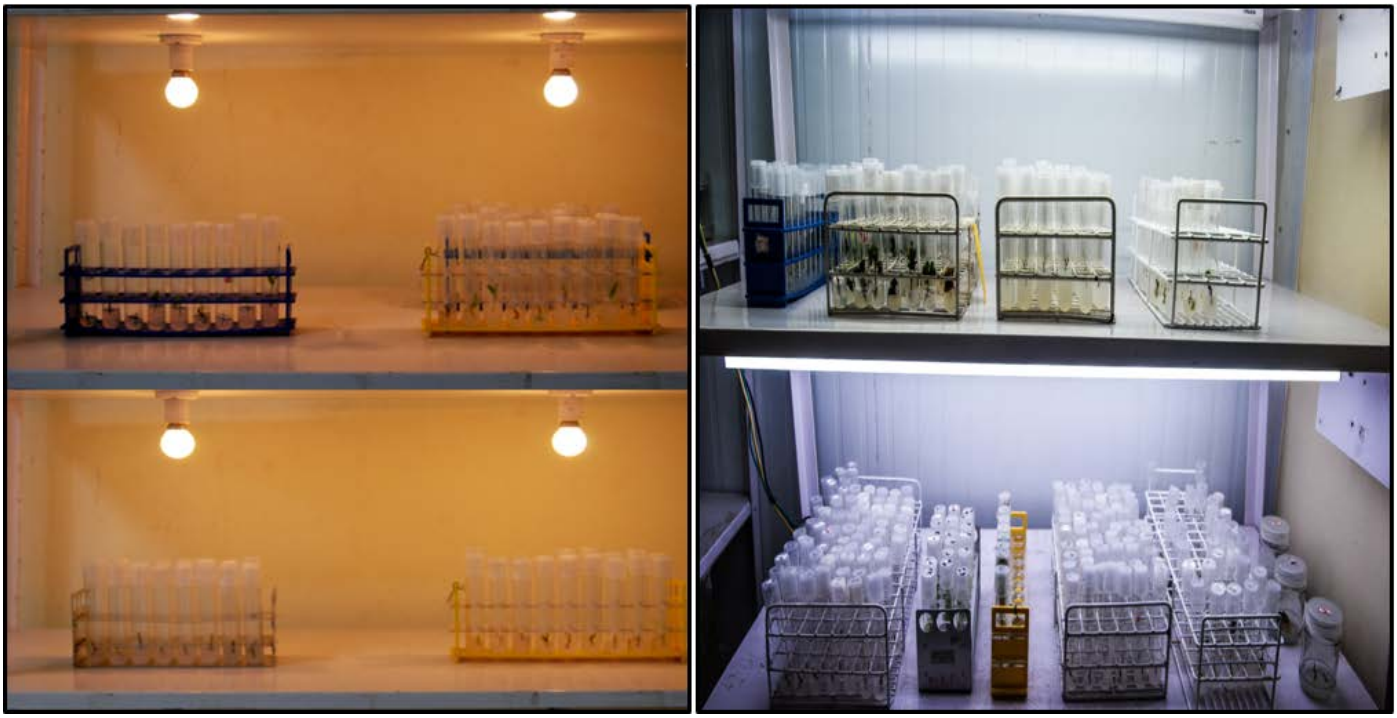


Plate 2: *In-vitro* propagated *Operculina turpethum* (L.) Silva Manso. plantlets kept under RCC and SCC

Conclusion

Multiplication using seed is a problem in this species and there is a need to standardize an alternative method of propagation for the production of true to type plants. *In-vitro* propagation serves as an efficient method that helps to overcome the problem of heterozygosity which in turn contributes to *In-vitro* conservation. In the present investigation, BAP (1.0 mg/l) + NAA (0.5 mg/l) can be considered as an effective treatment combination that helps in large-scale multiplication and conservation of this threatened species.

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