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In vitro propagation through *ex vitro* rooting of a medicinal spice *Piper longum* Linn.

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ABSTRACT

Successful *in vitro* propagation through *ex vitro* rooting mechanism has been achieved in *Piper longum* by nodal shoot segment cultures. Shoot tip explants were less proliferative compared to the nodal meristems. The explants were sterilized using 0.1% $HgCl_2$ and cultured on Murashige and Skoog (MS) medium with various concentrations of growth regulators. MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) was found suitable for bud breaking within four weeks. Kinetin (Kin) was not reported impressive compared to BAP in culture induction response. The shoots were multiplied and elongated on MS medium supplemented with 0.5 mg/L each of BAP and Kin and 0.1 mg/L indole-3 acetic acid (IAA), where the shoots were elongated up to 5.8 cm length. Rooting and acclimatization was achieved by *ex vitro* rooting methods using 300 mg/L indole-3 butyric acid (IBA) for 5 min, and 5.3 roots with 3.2 cm average length were observed. The rooted shoots were transferred to the greenhouse for acclimatization. The hardened plantlets were transferred to earthen pots and maintained in the greenhouse. Normal flowering was observed in micropropagated plants of *P. longum*.

***Keywords*:** *Piper longum*; endangered; MS medium; *Ex vitro* rooting

1. INTRODUCTION

India is the place of origin of many herbal based medicines and these are documented to cure different types of illness (Biswas *et al.,* 2004). *Piper longum* Linn., belongs to the family

Piperaceae, and also reported as an endangered medicinal plant (Nair, 2000; Chaudhuri, 2007), native of Indo-Malaya region and found throughout the hotter parts of India from central to the north-eastern Himalayas. It is commonly known as Pippali and long pepper (Shastry, 2001).

It is scandent perennial aromatic shrub with jointed branches. The leaves are cordate in shape and the flowers grow on solitary spikes. The male and female spikes are produced on different plants. The fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes (Williamsons, 2002). The roots are thick and branched. Medicinally the roots and fruits are potentially important and called *pippali-moolam* (Ashalatha and Rekha, 2015). It is a prominent drug of Indian systems of traditional medicines (Anonymous, 2004). It grows wild in the tropical rain forests of India, Nepal, Indonesia, Malaysia, Sri lanka, Rhio, Timor and the Philippines. In India, it is seen in Assam, West Bengal, Uttar Pradesh, Madhya Pradesh, Maharashtra, Kerala, Karnataka and Tamil Nadu (Viswanathan, 1995).

The major active constituents are piperine, piperlongumine, and methyl-3,4,5-trimethoxycinnamate (Chatterjee and Dutta, 1963). Volatile oil, starch, protein and alkaloids, saponins, carbohydrates, and amygdalin are also present (Dasgupta and Dutta, 1980). The fruits contain piperonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, refractomide A, pregumidiene, brachystamide, brachystamide-A, brachystine, pipercide, piperderidine, longamide and tetrahydropiperine, tetrahydropiperlongumine, dehydropipernonaline piperidine etc. (Madhusudan and Vandana, 2001) The roots are known to possess trimethoxy cinnamoyl-piperidine and piperlongumine, sesamin, pulvuatilol and fargesin (Lee *et al.*, 2001; Khushbu *et al.*, 2011).

In Ayurveda, this plant is used in 30 different types of medicinal formulations (Joshi *et al.*, 2013). The roots are pungent and having heating, stomachic, laxative, anthelmintic, hepato-protective, abortificient, haematinic, diuretic, digestive and carminative properties. It improves the appetite and useful in bronchitis, abdominal pain, spleen diseases and tumors (Arya, 1995). It also cures inflammation of the liver, pains in the joint, lumbago, snakebite, scorpion-sting and night-blindness (Heera *et al.*, 2014).

The plant possess anti-inflammatory (Sharma and Singh, 1980), cough suppressor, antibacterial, antifungal (Bhargav and Chauhan, 1968), insecticidal, antiamoebic (Sawangjaroen *et al.*, 2004), antiasthmatic (Kulshresta *et al.*, 1971), antimalarial, CNS stimulant, antitubercular, anti-helminthic, antidiabetic, antispasmodic, antigiardial, immunostimulatory (Devan *et al.*, 2007), hepatoprotective (Shah *et al.*, 1998), analeptic, antinarcotic, antiulcerogenic (Agarwal *et al.*, 1994), antioxidant (Natarajan *et al.*, 2006), analgesic (Vedhanayaki *et al.*, 2003), anti-cancer (Pradee and Kuttan, 2002; Selvendiran and Sakthisekaran, 2004) and antidepressant (Seon *et al.*, 2005) activity.

Micropropagation of medicinal plants in fact support the *in situ* conservation activities, facilitates population enhancement of species where natural propagation is hindered due to destructive harvesting and reproductive barriers (Krishnan *et al.*, 2011). It has been reported that the industrial demand of *P. longum* is gradually increasing, which resulted in depletion of this valuable germplasm from its natural habitat (Heera *et al.*, 2014). It is important to develop a micropropagation protocol of *P. longum*, because Pippali is possessing high demand in Indian market, due to its medicinal properties. It is one of the high trade sourced medicinal plant in India and the annual consumption of *P. longum* (fruits and roots) has crossed 1737 Metric Ton. But the maximum amount of the consumptions are fulfilled through

import only. In the year 2004-2005; about 9,067,191 Kg Pippali was imported to India (NMPB-FRLHT, 2008).

Ex vitro rooting is more efficient way of rooting of *in vitro* propagated shoots as it simplifies tissue culture protocols and provides opportunity to improve the micropropagation efficiency from both economic and biological point of view. *Ex vitro* rooted plantlets are easy to acclimatize than *in vitro* rooted plantlets and avoid possible root damage when transplanting (Ranaweeraa *et al.*, 2013). *Ex vitro* rooting implies better hardening and improved survival rate than in those developed from *in vitro* rooting method (Patel *et al.*, 2014a). This rooting process in micropropagation technique could overcome the problems during acclimatization prior to transplanting in the field conditions (Yan *et al*., 2010), and rooting and acclimatization could be achieved simultaneously (Baskaran and Van Staden, 2013).

During last five years, *ex vitro* rooting was successfully achieved in many medicinal plants like *Lawsonia inermis* (Ram and Shekhawat, 2011), *Maytenas emarginata* (Meena and Shekhawat, 2012), *Arnebia hispidissima* (Phulwaria and Shekhawat, 2013), *Neoregelia concentrica* (Martins *et al.*, 2013), *Caralluma edulis*, (Patel *et al.*, 2014a), *Passiflora foetida* and *Artemisia absinthium* (Shekhawat *et al.*, 2015c, d), *Cadaba fruticosa* (Lodha *et al.*, 2015) and *Jatropa curcas* (Rathore *et al.*, 2015).

Several attempts have been made on *in vitro* regeneration of *P. longum*. Heera *et al*. (2014) studied the pharmacognostical and phytochemical evaluation of spikes of *in vitro* and wild plants *P. longum*. Soniya and Das (2002) reported micropropagation of *P. longum* through shoot tip explants. The present study reports the *in vitro* response of different explants of *P. longum*, their reproducibility and reliable techniques for shoot multiplication, *ex vitro* rooting within short period of time.

2. MATERIAL AND METHODS

2. 1. Collection of explants and surface sterilization

The plants of *Piper longum* were procured from the coastal region of Pondicherry, India during January 2014 to December 2014 and transplanted in the Nursery. Shoot tips and nodal segments with axillary buds obtained from the nursery grown plants were used as explants for the establishment of the cultures. The explants were rinsed thoroughly under running tap water, pre-soaked in liquid detergent (Tween® 20, Sigma Aldrich, India) for 15 min and treated with 0.1% (w/v) Bavistin (a systemic fungicide; BASF India Ltd., India) for 5 min. Surface sterilization was rendered with 0.1% (w/v) $HgCl_2$ (disinfectant, Hi-Media, India) for 5-6 min under laminar air flow cabinet (Technico Pvt. Ltd. Chennai, India) to maintain aseptic conditions. These were finally rinsed with sterile distilled water for 8-10 times.

2. 2. Culture medium and *in vitro* environment

MS basal medium (Murashige and Skoog, 1962) was used for shoot regeneration and proliferation. The medium was supplemented with 30 g/L sucrose, 8.0 g/L agar, additives (50 mg/L ascorbic acid, 25 mg/L each of adenine sulphate, citric acid and arginine; Himedia, India) and dispensed into culture vessels. The pH of the medium was adjusted to 5.8 ±0.02 using 0.1 N NaOH or HCl before sterilizing in autoclave at 121^{o}C for 15 min. Cultures were maintained in a growth room at 25 ±2 °C with a 12/12 h light/dark photoperiod at a

photosynthetic photon flux density (PPFD) of 50–55 µmol m^{-2} s^{-1} from cool white fluorescent tubes (Philips India Ltd.).

2. 3. Culture induction and multiplication

For the establishment of cultures, selected explants were cultured on solidified MS medium. The medium was augmented with BAP and Kin (1.0-5.0 mg/L) alone or in combination (0.1-2.0 mg/L) with IAA (0.5 mg/L) for shoot proliferation experiments. To examine the significance of types of explants for the establishment of cultures, proliferated shoots with mother explants were transferred to fresh medium with same concentration of growth hormones for 2-4 passages.

2. 4. Induction of *ex vitro* roots and acclimatization of plantlets

Experiments were conducted to attain *ex vitro* rooting from the dissected ends of *in vitro* raised shoots to achieve rooting and acclimatization simultaneously. Healthy and sturdy microshoots were separated single from 4 weeks-old cultures of multiplication stage and thoroughly washed with distilled water to remove any adhering agar. The microshoots were pulse treated by dipping the cut ends of the shoots in various concentrations of (100-500 mg/L) auxins (IAA, IBA, Naphthalene acetic acid and Naphthoxy acetic acid) solutions for 5 min. Different concentrations of auxins solution were prepared by dissolving the auxin powders in 5% (v/v) NaOH and made up to desirable quantity with the help of sterile distilled water. The pulse treated microshoots were placed 1.5-2.0 cm deep in ecofriendly horticultural plastic cups/pots (size 150 ml; Vandana Paper Products, Chennai, India) and culture bottles containing 55gm autoclaved soilrite® (a mixture of perlite, Irish Peat moss and exfoliated vermiculite; KelPerlite, Bangalore, India), moistened with 10 ml aqueous $1/4^{th}$ MS salts solution by the interval of one week and maintained in the greenhouse for five weeks. All the plantlets were covered with polyethylene bags with minute puncture and water was sprayed continuously to avoid wilting.

The set up was maintained in th egreenhouse at a day temperature conditions (25 to 28 °C) and relative humidity (80-85%). Control experiments were conducted at the same time by immersion in distilled water. The *ex vitro* rooted plantlets were acclimatized in paper cups which were covered with transparent polythene cups to provide enough space for gas exchange. The *in vitro* rooted plantlets were acclimatized by gradual loosening and then completely removing the transparent caps of the bottles. These plantlets were subsequently transferred to polybags/earthen pots containing soilrite®, garden soil and organic manure (1:1:1) in the greenhouse for further acclimatization process. The plantlets were transferred to the field after 5 week.

2. 5. Data collection and statistical analysis

All the experiments in the present investigation were conducted with a minimum of 10 replicates per treatment and repeated thrice. Observations were recorded after the time period of 4 weeks of interval. The results were expressed as mean ± SD of triplicates. The data were subjected to analysis of variance and the significance of differences among mean values was carried out using Duncan's Multiple Range Test (DMRT) at $P < 0.05$ using SPSS software, version 16.0 (SPSS Inc., Chicago, USA).

3. RESULTS

3. 1. Establishment of cultures

Surface sterilization of explants with 0.1% $HgCl_2$ for 4 and ½ min was most effective in regeneration of contamination free cultures. Shoot tips and nodal segments of *P. longum* were cultured on MS medium supplemented with cytokinins (BAP and Kin). The basal end of the explants was found swollen and produced microshoots from the nodes within two weeks of inoculation on BAP 1.0 mg/L (Figure 1A, 1B and Table 1).

The young nodal segments were responded better for bud breaking as compared to mature and stout explants, therefore the mature shoots as explants avoided for the further experiments. The percentage response, number of shoots and average shoot length were controlled by the type and concentration of the growth regulator employed. However, there was no response observed in hormone free (control) MS medium from any types of explants. Soniya and Das (2002) regenerated shoots using shoot tips. But in present study nodal shoot segments performed better than shoot tips in multiple shoot induction. Sharon and Maurya (2008) regenerated shoots using lateral buds as explants.

Table 1. Effect of different concentration of cytokinins (BAP and Kin) on responses of bud breaking from nodal explants of *P. longum*.

BAP (mg/L)	Kin (mg/L)	Response (%)	Number of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
0.00	0.00	0	0.0 ± 0.00^{a}	0.00 ± 0.00^{a}
0.50	-	79	2.0 ± 0.29^{d}	1.32 ± 0.41^{b}
1.00	-	90	4.2 ± 0.73^{f}	3.01 ± 0.26^{e}
1.50	-	87	3.4 ± 0.19^{e}	2.19 ± 0.14^{d}
2.00	-	74	2.9 ± 0.22^{d}	1.26 ± 0.15^{a}
2.50	-	66	2.1 ± 0.29^{d}	1.14 ± 0.19^{a}
3.00	-	60	1.0 ± 0.47^{b}	1.30 ± 0.10^{b}
-	0.50	59	1.0 ± 0.28^{b}	1.50 ± 0.22^{c}
-	1.00	73	1.2 ± 0.30^{c}	1.93 ± 0.27^{c}
-	1.50	67	1.0 ± 0.16^{a}	1.60 ± 0.19^{c}
-	2.00	59	1.0 ± 0.25^{b}	1.48 ± 0.15^{b}

-	2.50	44	1.0 ± 0.39^{b}	1.39 ± 0.30^{b}
-	3.00	40	1.1 ± 0.33^{c}	1.20 ± 0.24^{a}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 2. Induction of shoots from apical shoot tips and nodal explants

Apical shoot tips showed elongation up to 3.2 cm on MS medium supplemented with 1.0 mg/L BAP within 4 weeks, but multiple shoots were not observed (Figure 1D). There was no elongation with higher concentrations of BAP, and the shoot elongation was not considerable in lower concentrations of BAP and all the concentrations of Kin tested (Figure 1C). The regeneration capacity of nodal shoot explants than shoot tip explants were reported in *Capsicum chinense* (Kehie *et al.,* 2012), *Withania somnifera* (Fatima and Anis, 2012) and *Salvadora oleoides* (Phulwaria *et al.,* 2014).

Multiple shoot regeneration from nodal explants was observed on MS medium supplemented with BAP at different concentrations. Kinetin at the range 0.5-3.0 mg/L induced maximum one shoot from the nodal meristem explants within four weeks. The increase in concentration of BAP decreased shoot induction from the nodal meristems of the explant. Full strength MS medium supplemented with 1.0 mg/L BAP was observed suitable for bud breaking from nodal shoot segments. Maximum 90% explants were responded with 4.2 ±0.73 shoots from each nodal explant in this medium. Kinetin was reported comparatively less effective than BAP for shoot induction from nodal explants. BAP has gained importance as a potential plant growth regulator for inducing bud break. The positive efficacy of BAP over Kin in bud breaking response and culture induction was reported in *Rubia cordifolia* (Ghatge *et al.,* 2011), *Marsilea quadrifolia* (Shekhawat, 2015), *Turnera ulmifolia* (Shekhawat *et al.,* 2014) and *Cadaba fruticosa* (Lodha *et al.,* 2015). The explants harvested during June-November were found most suitable for the initiation of cultures of *P. longum*.

3. 3. Multiplication and elongation of microshoots

Newly regenerated shoots were separated and subcultured continuously on fresh MS medium fortified with cytokinins and auxin. The shoots were elongated up to 5.8±0.20 cm when the explants were incubated for 4 weeks on MS medium fortified with 0.5 mg/L each of BAP and Kin + 0.1 mg/L IAA (Figure 1E). In this experiment, nodal shoot segments with axillary buds performed better and responded well than shoot tips as explants, former shown highest number of micro-shoots induced from the axillary buds (Table 2). Cent percentage cultures showed multiple shoots on this concentration. Similar response was observed with same concentration of BAP, Kn and NAA with fragile shoots. Healthy and sturdy shoots were obtained in the medium combination with IAA. Cultures established through apical shoot tips were hindered the multiple shoot regeneration at multiplication stage also. Combined effect of auxins and cytokinins in shoot multiplication was reported in *Dioscorea rotundata* (Ezeibekwe *et al.,* 2009), *Vitex negundo* (Rathore and Shekhawat, 2011), *Cardiospermum halicacabum* (Shekhawat *et al.,* 2012), *Terminalia bellirica* (Phulwaria *et al.,* 2012), *Celastrus paniculatus* (Phulwaria *et al.,* 2013) and *Leptadenia reticulata* (Patel *et al.,* 2014b).

Figure 1. A and B. Induction of multiple shoots from the nodal explants on MS medium + BAP, C. Multiple shoots on MS medium + Kin, D. Elongated single shoot when apical tips was used as explants, E. Multiplication of shoots *in vitro*, F. *Ex vitro* rooting experiment in the greenhouse, G. Hardened plant in earthen pot, and H. Normal flowering in the tissue culture raised plant.

Table 2. Combined effect of cytokinins and auxin on multiplication of shoots of *P. longum*.

BAP (mg/L)	Kin (mg/L)	IAA (mg/L)	Shoot numbers (Mean ± SD)	Shoot length (cm) Mean ± SD
0.1	0.1	0.1	3.0 ± 1.00^{b}	3.9 ± 0.74^{b}
0.5	0.5	0.1	5.1 ± 1.49^{c}	5.8 ± 0.20^{d}
1.0	0.5	0.1	3.5 ± 0.29^{b}	4.3 ± 0.39^{c}
1.5	0.5	0.1	2.9 ± 0.40^{a}	3.7 ± 0.26^{b}
2.0	0.5	0.1	2.3 ± 0.33^{a}	3.0 ± 0.10^{a}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 4. *Ex vitro* rooting

The basal end of *in vitro* produced micro-shoots were given the treatment (5 mins) of auxins and transferred to the greenhouse environment. Plants rooted under *ex vitro* environment are better suited/adapted to the natural climate and easy to harden. These have more vigor to tolerate stresses experienced during hardening stage. The cut ends (5 mm) of the *in vitro* regenerated shoots evaluated with 300 mg/L of IBA exhibited about 96% of rooting. A maximum of 5.3 ±0.29 roots per shoot with 3.2 ±0.39 cm root length was produced within 4 weeks (Figure 1F and Table 3).

Table 3. Effect of auxins *ex vitro* root induction in *Piper longum.*

IAA (mg/L)	IBA (mg/L)	NAA (mg/L)	NOA (mg/L)	Response (%)	Number of Roots (Mean ± SD)	Length of Roots (cm) (Mean ± SD)
100	-	-	-	43	1.2 ± 0.23^{a}	1.8 ± 0.15^{b}
200	-	-	-	47	2.5 ± 0.31^{c}	2.0 ± 0.30^{b}
300	-	-	-	52	3.0 ± 0.29^{d}	2.3 ± 0.28^{b}
400	-	-	-	50	2.6 ± 0.20^{c}	1.5 ± 0.44^{b}
-	100	-	-	66	2.0 ± 0.19^{b}	2.0 ± 0.23^{b}

-	200	-	-	74	3.9 ± 0.22^{e}	2.7 ± 0.10^{c}
-	300	-	-	96	5.3 ± 0.29^{f}	3.2 ± 0.32^{c}
-	400	-	-	31	3.0 ± 0.17^{d}	2.0 ± 0.20^{b}
-	-	100	-	40	1.5 ± 0.52^{a}	1.4 ± 0.14^{a}
-	-	200	-	43	2.6 ± 0.76^{c}	2.0 ± 0.16^{b}
-	-	300	-	49	3.0 ± 0.39^{d}	2.2 ± 0.11^{b}
-	-	400	-	46	2.4 ± 0.12^{b}	1.0 ± 0.40^{a}
-	-	-	100	33	1.7 ± 0.36^{a}	1.7 ± 0.37^{b}
-	-	-	200	38	2.4 ± 0.20^{b}	2.0 ± 0.19^{b}
-	-	-	300	45	3.5 ± 0.27^{d}	2.0 ± 0.30^{b}
-	-	-	400	41	2.0 ± 0.15^{b}	1.8 ± 0.22^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

Poor rooting performance was recorded on all concentrations of other root inducing hormones (IAA, NAA and NOA) as compared to IBA. The efficacy of IBA on *ex vitro* rooting over other auxins were reported in number of species such as *Morinda coreia*, *M. citrifolia* (Shekhawat *et al.*, 2015a, b) and *Pyrus elaegrifolia* (Aygun and Dumanoglu, 2015).

There were no roots observed on auxin untreated shoots (control) on soilrite®. All the auxins at the concentration of 300 mg/L resulted in maximum number of root induction under *ex vitro* condition. Only 50% of shoots responded when the shoots were pulsed with other auxins. Increased concentration of auxins hindered the response as well as number of roots under *ex vitro* environment.

3. 5. Acclimatization and field transfer

After 4 weeks the *ex vitro* rooted plants were acclimatized efficiently in the greenhouse. The plantlets were sprayed with $1/4^{th}$ strength aqueous MS macro salts at the interval of 2 days for proper initial growth. After 15 days, the transparent polythene cups were removed for 5-10 minutes and plantlets were monitored closely. Wilting was observed on prolonged exposure initially. In this way, plantlets were exposed for few hours every day by removing the cover of transparent cups. This period of low humidity exposure was gradually increased to harden plantlets so that they can withstand to normal environmental conditions. Acclimatization was carried out in mist chamber and maintained the humidity between 80 ±

10 % (30 sec misting at 15 min interval). The hardened plantlets were then transferred to polythene bags containing soilrite®, garden soil and organic manure (1:1:1) and maintained in the greenhouse. These plantlets were irrigated with ordinary water. After a month, these were transferred to earthen pots containing organic manure, garden soil and red soil (1:1:1) and maintained in shade house (Figure 1G). Such plantlets have been transferred to the field after proper hardening in shade house (1 month).

Profuse branching of root system was observed in the *ex vitro* rooted plantlets while transferring to the field. It resembled the root system of mother plants in nature. Flowering was normal in hardened plants (Figure 1H) as in the wild. The hardened plantlets were successfully transferred to the field with 96% survival rate.

4. CONCLUSIONS

An effective protocol for the micropropagation of *P. longum* was established using nodal shoot meristems in this study. Nodal shoot segments were capable of producing multiple shoots *in vitro*. The i*n vitro* multiplied shoots were successfully rooted using *ex vitro* methods under the greenhouse conditions using exogenous auxin pulsing which saved cost, energy and time for micropropagation of plants. This can be used as an effective conservation strategy for the conservation of valuable medicinal spice *P. longum*. This is the first report on *ex vitro* rooting in *P. longum*.

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