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Regeneration of plantlets of *Piper longum* L. through *in vitro* culture from nodal segments

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ABSTRACT

Plantlet regeneration in *Piper longum* L. has been achieved from nodal segments excised from *in vivo* grown plantlets cultured on MS medium supplemented with growth regulators. The present investigation was carried out to regenerate plantlet of *Piper longum* L through in vitro culture. Nodal segments from one year old plants of field grown *Piper longum* were used as explants for initial culture. The nodal explants were cultured on MS medium supplemented with different concentration and combination of cytokinines and auxines for primary shoot proliferation. The best shoot proliferation was observed in MS medium containing 1.0 mg/l Kinetin and 1.5 mg/l BAP where 98 % of explants showed proliferation with highest rate of shoot multiplication (5-6 shoots per explant). Callus induction occurred in (1 mg/l) BAP and (0.5 mg/l) Kinetin and 10-15 days of callus subculture initiation of greenish white shoot buds was observed. For rooting, the *in vitro* micro shoot were inoculated to MS basal media supplemented with 0.5 mg/l IAA and rooting was more profuse. The regenerated plantlets were successfully established in soil with survival rate 90%. The protocol described is simple, rapid efficient for in vitro propagation of *P. longum* (L.) from nodal explants and soil establishment of plantlets.

1. INTRODUCTION

Piper longum L., a perennial, dioecious plant species, well known for the medicinal value of almost all its parts especially roots, stems and fruits which are used in the treatment of diseases of respiratory tract like bronchitis and asthma. *Piper longum* L. is a member of family Piperaceae, commonly known as Pippali. Male and female plants differ in the morphology of their spikes. The principal pharmacological constituents are piperine and piplartine [11]. As the plants are excessively extracted from its natural resource, the species has now become very rare in the forests. *Piper longum* is also comes under this category. Traditional or normal methods of propagation of plants through cuttings have some problems like poor seed viability, low percentage of germination and scanty or delayed rooting. Therefore, to mitigate this problem tissue culture technique could be applied. Comparing with traditional methods of producing medicinal plant, in vitro micropropagation have many advantages such as the independent of seasonal variation, mass production, identification and production of clones with desired characteristics, conservation of threatened plant species, production of new and improved genetically engineered plant, preservation of genetic material through cryopreservation and production of secondary metabolites. Not much tissue culture work has been done in this plant except a few reports on regeneration [1, 8, 10-13].

In this present paper, *in-vitro* clonal propagation of field grown plants by nodal explant is reported. *In vitro* method of vegetative multiplication of *Piper longum* would have considerable benefits for the medicinal trade and germplasm conservation. The application of plant tissue culture offers valuable ways to overcome all the problems that's found in natural propagation. The present study describes a suitable method for in vitro plantlet regeneration of *Piper longum* through nodal vine culture. The methodology definitely helpful for obtaining large scale diseases free seedlings and ensure adequate supply of quality plantlets of *Piper longum* to meet different purposes and conservation and domestication of the wild species.

2. MATERIALS AND METHODS

2.1 Preparation of explants

Healthy nodal segments with active buds were collected from one year old plants of *Piper longum* maintained in the medicinal garden of M. S. Swaminathan Research Foundation, Jeypore, Koraput. The nodal segments were cut in to 2 to 3 cm length with single bud intact.

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These nodal vine cuttings were washed with 5% (v/v) tween-20 solution (Himedia, India) for 15 minute and rinsed several times with running tap water. Then the source explant were surface sterilized with 20% commercial sodium hypo chloride for 10 to 15 min with repeated washes with sterile water (three washes, 5 min each).

These nodal cuttings were surface sterilized with copper oxy chloride 0.3 % (w/v) for 10 minutes each and then washed with sterile distilled water and transferred to laminar air flow cabinet. In the laminar chamber the nodal segments were again treated with 70% ethyl alcohol for 10 second followed by repeated washings in double distilled water then another treatment in 0.1% (w/v) Mercuric chloride ($HgCl_2$) for another 5 minutes. Finally, the nodal segments were washed thoroughly 3 to 4 times with sterile distilled water and soaked with sterile blotting paper and used as explants for in vitro cultures.

2.2 Culture medium and conditions

The sterilized blotted explants were inoculated in Murashige and Skoog's, 1962 [5] agar-gelled medium fortified with various concentrations/combinations of growth hormones. For shoot induction, the medium was supplemented with Kinetin and BAP. For root induction in vitro raised shoots measuring about 3–4 cm length grown in multiplication medium were excised and cultured on MS basal medium supplemented with either IAA (Indole 3- acetic acid) or IBA (Indole3-butyricacid) in various concentrations. The pH of the medium was adjusted to 5.8 prior to gelling it with 0.8% agar (Plant tissue culture grade, Hi-media, India) and autoclaved at 15 psi pressure and 121°C temperature for 15 - 20 minute. All the culture vessels containing 20 ml media was autoclaved. All cultures were incubated in 16 h light/8 h dark photoperiod (cool, white fluorescent light). The cultures were incubated at 25 ± 2°C in diffused light under 60 - 70% relative humidity in the culture room. The cultures were maintained by regular subcultures at 15 days intervals on fresh medium with the same compositions.

2.3 Shoot organogenesis and elongation

To facilitate multiple shoot growth induction, the explants were transferred to shoot proliferation medium [MS medium containing B5 vitamins supplemented with BAP (1.5 – 2.25mg/l), kinetin (1.0 -1.75mg/l) in combination]. After 4 to 5 weeks, percentage of explants produced multiple shoots and numbers of shoots per explants were recorded. After 4 to 6 wk of induction culture, the explants with regenerated shoots (around 1-2 cm) were transferred onto the shoot elongation medium containing half strength MS salts, B5 vitamins, L-glutamine (400 mg/l) supplemented with BAP at different concentrations (1.5 – 2.25mg/l). The cultures were maintained 2 to 3 weeks in above medium without subculturing.

2.4 Acclimatization

Rooted plantlets were removed from the culture tube and the roots were washed under running tape water to remove the agar. Then the plantlets were transferred to small plastic cups containing compost, sand, soil mixture (1:1:1) and applied MS media liquid thrice in a week and maintained inside poly house. After three weeks they were transplanted to earthen pots containing mixture of soil, sand and manure in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization soil for a period of three weeks for acclimatization.

2.5 Statistical analysis

Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed by using standard ANOVA procedures and differences between the means were compared using the Fisher's least significant different test (LSD). For this SPSS V 16.0 software was used to analyze the significance difference among the treatments.

3. RESULTS

3.1 Shoot proliferation and multiplication

Surface sterilized nodal explants were cultured on MS media supplemented with various concentration and combinations of cytokinins and auxins. The response of nodal explants cultured on different shoot proliferation media over a period of six weeks is presented in Table 1.

MS medium Supplemented with growth regulator supplements produced better results in terms of percentage explants response, shoots /explant, average shoot length and average number of nodes produced per shoot. In such media combinations bud break was noticed within 10 days of culture (Figure 1, Table 1). Of the combination tested MS medium supplemented with Kinetin (1.0 mg/l) +BAP (1.5 mg/l) elicited optimal response in which an average of 8.5 ± 0.7 shoot lets (Table 1) with a mean shoot length of 5.1 ± 0.24 cm per explant was recorded. In this combination, the shooting response was observed as 98%.

The second best shoot multiplication i.e. 6.4 ± 0.1 was obtained in the medium MS medium supplemented with Kinetin (1.75mg/l) and BAP (2.25 mg/l) with a mean shoot length of 4.8 ± 0.21 cm. Higher concentration of Kinetin (2.5 mg/l) and BAP (3.0 mg/l) showed fewer number of shoots and the shoots were stunted with a mean shoot length of 3±0.21cm (Table 1).

Multiple shoots started arising from nodes after 18-22 day of inoculation. In the present study remarkable callus induction rate was observed when nodal explants were used and the appearance of *Piper longum* L. callus was globular and light green in color. The explants were enlarged within 12-14 days of inoculation; however callus formation was started after 20-25 days. Rapid callus growth (90%) response was observed in the MS medium supplemented with 1.5 mg/l BAP + (1.0 mg/l) Kinetin and (1.5 mg/l) BAP + (0.5 mg/l) IBA.

Table 1: Effect of various concentration of BAP, Kinetin and IBA on shoot formation(20 culture tube/treatment, data scored after 6 weeks).

Sl.no.	Hormonal Compositions (mg/l)			% of response	Days of bud break	Mean no. of shoot/explants (M±SE)	Mean shoot length in cm ± SE	Remarks
	BAP	Kinetin	IBA					
1	0	0	0	90	12-15	1.1±0.3	3.1±0.2	-
2	1	0.5	-	75	10	3.45±0.16	3.5±0.23	-
3	1.25	0.75	-	85	10	2.45±0.73	3.5±0.31	-
4	1.5	1	-	98	10	8.5±0.7	5.1±0.24	Callus
5	1.75	1.25	-	55	10	5.75±0.8	3.9±0.31	-
6	2	1.5	-	70	10	2.3±0.84	4±0.31	-
7	2.25	1.75	-	80	10	6.46±0.1	4.8±0.21	-
8	2.5	2	-	90	10	2.9±0.83	4.5±0.2	-
9	2.75	2.25	-	85	10	4.35±0.85	4.4±0.51	-
10	3	2.5	-	95	10	2.23±0.74	3±0.21	-
11	0.25	-	0.25	55	12	1±0	1.9±0.6	-
12	0.5	-	0.25	75	10	1±0	2.3±0.47	-
13	1	-	0.25	75	10	1±0	3.5±0.43	-
14	1.5	-	0.25	85	10	1.5±0.31	2.8±0.37	-
15	2	-	0.25	90	10	2.0±0.38	3.6±0.2	-
16	2.5	-	0.25	95	10	1.9±0.3	3.1±0.21	-
17	3	-	0.25	70	10	1.1±0.49	3±0.41	-
18	0.25	-	0.5	80	12	1.3±0.41	3.5±0.37	-
19	0.5	-	0.5	75	10	1±0	1.8±0.5	-
20	1	-	0.5	85	10	2.1±0.35	2±0.35	-
21	1.5	-	0.5	80	10	3.3±0.3	3.8±0.15	Callus
22	2	-	0.5	90	10	3.2±0.48	3.2±0.23	-
23	2.5	-	0.5	95	10	3.0±0.48	3.4±1.23	-
24	3	-	0.5	65	10	2.1±0.51	3.3±1.0	-

(20 replicate per treatment; repeated three times. Means are calculated at P<0.05 level of significance , S.E. Standard error of mean).

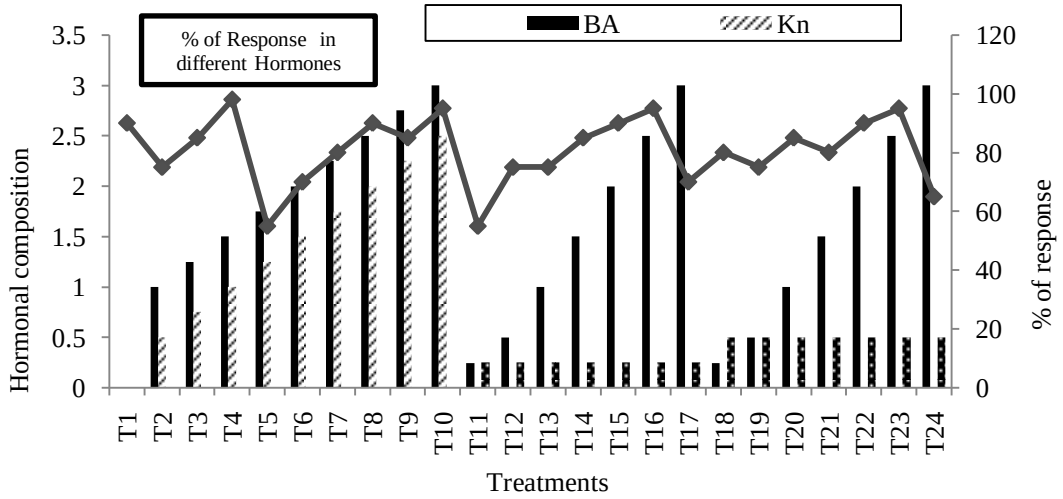

Fig. 1: Shooting medium: % of response in different treatments of BA, Kinetin and IBA.

3.2 Induction of rooting

For root induction, healthy individual shoots (>3 cms long) were separated and transferred to half strength MS medium containing IAA or IBA. The rooting responses of shoots on different media, which included mean number of roots/shoot and mean root growth over a period of four weeks were different (Table 2). Rooting was also observed in case of shoot planted on auxin free basal medium (control). Similarly, at lower level of IAA treatments there was hardly any rooting in the cultured shoots during the four weeks of observation period. However in 2.5 mg/l concentration of IAA and in higher concentration of IBA tested responded well. Rooting was better in the culture in the combination of MS with 2.5 mg/l of IAA where about 90% cultures responded with an average number of 8.8 ± 0.65 roots per plantlet and an average root length 4.8 ± 0.31 cm was recorded (Table 2) .The second highest response (80%) was recorded at (3.0 mg/l) of IBA. It was observed that root primordial emerged from the shoot base starting from day 6 to 8 days after shoot inoculation and soon after that the root growth was rapid. IAA has more effective then IBA in induction of rooting as days required to rooting was only 6 to 8 days in case of IAA and10 to15 days required for similar response in case of IBA.

Table 2: Effect of different concentrations of Auxin on adventitious root formation. (20 culture tube/treatments, data scored after 4 weeks).

Growth regulators	Concentration (mg/l)	Average no. of Roots M±SD	Root length (cm) M±SD
Without hormones	0	3.60±0.96	1.01±0.96
IAA	1	1.18±0.26	2.43±1.63
	1.5	2.36±0.26	3.23±0.82
	2	4.49±1.63	3.40±0.41
	2.5	8.8±0.65	4.8±0.32
	3	4.4±0.82	4.0±0.21
IBA	1	4.37±0.82	2.11±0.58
	1.5	4.43±0.82	3.15±0.77
	2	3.20±0.26	4.21±0.32
	2.5	5.41±0.26	4.43±0.21
	3	6.81±0.26	5.31±0.89

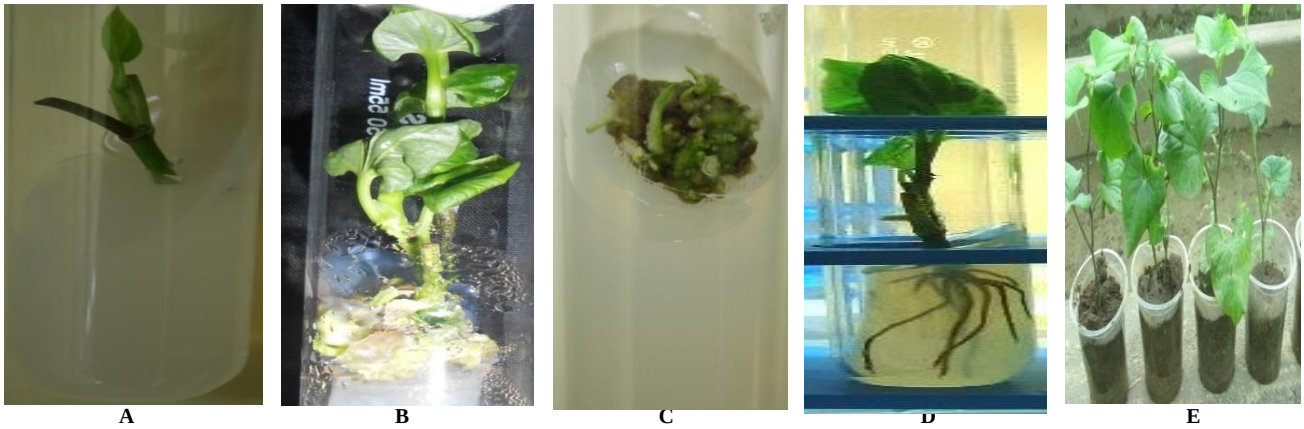

Fig. 2 (a-e): a- Nodal explant of piper longum after 10days of bud opening, b- Shoot induction after 4 week of culture, c- Shoot induction in callus, d- Root induction in invitro sub cultured plant of *Piper longum* and e- Hardening of rooted plant lets in plastic pots).

3.3 Acclimatization and field establishment

In vitro rooted plantlets were washed thoroughly to remove the adhering gel, transplanted to sterile poly pots (small plastic cups) containing autoclaved compost, sand, soil mixture (1:1:1) and applied MS liquid media thrice in a week and maintained inside poly house. After three weeks they were transplanted to earthen pots containing mixture of soil, sand and manure in (1:1:1) ratio and kept under shade house for a period of three weeks for acclimatization. Survival rate of the plantlets were recorded after four weeks. About 90% of the rooted plantlets established in the greenhouse within 2 to 3 weeks of transfer. The plants grew well and attained a 10 to 15 cm height within 4 weeks.

4. DISCUSSION

As the tissue culture protocol for mass proliferation and conservation offers a viable alternative. Many regeneration protocols for *Piper longum* have been reported earlier from shoot tips, root, leaf, node, internode, and petiole [9]. *In vitro* shoot regeneration from callus cultures through organogenesis [2] or direct shoot regeneration from leaf bases [12] or through axillary bud break [7] have been reported in *Piper longum*. This has also been recently reported in the case of micro propagation of other piper species like direct somatic embryogenesis from in vitro cultured leaf segments was also reported in *Piper colubrinum* [13], morphogenetic potential of root, leaf, node and internode explants *P. longum*, *P. betle* and *P. nigrum* [1]. In the present study, nodal vine explants of *Piper longum* showed significantly higher response in the medium with the combination of Kinetin (1.0 mg/l) +BAP (1.5 mg/l) +IBA (0.0mg/l). The quality of shoots and the overall growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recorded when BAP and IBA was added in different combinations in the medium. Review of literature indicates that the addition of either IAA in the culture medium improved the response in a number of species in terms of shoot growth. Dominguez et al. 2006 [4] reported that proliferating callus and shoot cultures derived from leaf tissue explants of *Piper auritum* in MS medium supplemented with 2.0 mg/l (2,4-D)+ 1.5mg/l kinetin and elongated shoots were successfully rooted (100%) on half-strength MS medium supplemented with 2.0 mg/l IAA. In our study two cytokinins were taken for higher shoot multiplication. These results are in agreement with previous findings where levels of cytokinins promoted shoot bud multiplication but prevented shoot elongation [3]. Production of plantlets with profuse rooting in *in vitro* is important for successful establishment of regenerated plants in soil [6]. The auxins IAA and IBA were used singly to induce rooting from *in vitro* raised shoot lets. A range of concentration was tested (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) for rooting. In the present study 1/2 strength MS basal medium and the two different auxins (IAA and IBA) were tried, the maximum results on rooting were obtained on half strength MS with IAA (2.5 mg/l) then IBA (3.0 mg/l). The auxins, IAA were used singly by Dominguez et al. 2006 [4] to induce

rooting from *in vitro* raised shootlets of *Piper auritum*. In their study half strength MS basal medium with IAA (2.0 mg/l) has profuse rooting response. The well rooted plants were transferred to plastic cups containing vermiculite for hardening and kept under controlled condition (Fig.2.d). When *in vitro* plant lets transferred to the vermiculite medium, plants started producing fresh shoots and roots after two weeks of transplanting. Later they were transferred to the field and the survival rate was 90%. The efficient micro propagation technique described in this study may be highly useful for mass multiplication of quality planting material of *Piper longum* for commercial and off season cultivation which is not only help the ex-situ conservation but also helpful in the restoration of germplasm of the species.

5. CONCLUSION

Micropropagation can be achieved in a short time and space and it help in mass multiplication of plants starting from a single individual protoplast to different plant parts as an explant. Micropropagation has wide commercial applications which help in conservation of germplasm of threatened species, secondary metabolite production in important plant species and production of disease free quality planting material for commercial cultivation. The reproducible, quick and large-scale micropropagation protocol reported here could be used for the conservation of this valuable medicinal herb. Axillary shoot proliferation from node was dependent on the interaction between plant growth regulator concentrations in the medium. A rapid multiplication rate could be obtained by a reduced cytokinin ratio. This protocol has great potential for improvement of this crop by biotechnological approaches such as genetic transformation and production of secondary metabolites. The establishment of protocols for in vitro propagation of *P. longum* through shoot multiplication and direct regeneration offers a potential system for improvement, conserving and mass propagation of this important medicinal plant.

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7. REFERENCES

1. Bhat SR, Chandel KPS, Malik SK, Plant regeneration from various explants of cultivated Piper species. Plant Cell Reports. 1995; 14:398-402.
2. Bhat SR, Kackar A, Chandel KPS, Plant regeneration from callus cultures of *Piper longum* L. by organogenesis. Plant Cell Reports. 1992; 11:525-528.
3. Bhatt I, Dhar U, Factors controlling micropropagation of Myrica esculenta buch.-Ham. Ex D. Don: A high value wild edible of Kumaun Himalaya. African Journal of Biotechnology. 2004; 3:534–540.
4. Domínguez F, Lozoya X, Simon J, Tissue Culture Regeneration of a Medicinal Plant from Mexico: *Piper auritum* Kunth. Hortscience. 2006; 41(1):207–209.
5. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology of Plant. 1962; 15: 473–497
6. Ohyama K, Tissue culture in mulberry tree. Japan Agricultural Research Quarterly. 1970; 5:30-34.
7. Parida R, Dhal Y, A study on the micro-propagation and antioxidant activity of *Piper longum* (An important medicinal plant). Journal of Medicinal Plants Research. 2011; 5:6991-6994.
8. Philip S, Banerjee NS, Das MR, Genetic variation and micropropagation in three varieties of *Piper longum* L. Current sciences. 2004; 78:169–173.
9. Rani D, Dantu PK, Direct shoot regeneration from nodal, internodal and petiolar segments of Piper longum L. and in vitro conservation of indexed plantlets. Plant Cell Tissue and Organ Culture. 2012; 109:9–17.
10. Sarasan V, Nair GM, Tissue culture of medicinal plants: morphogenesis, direct regeneration and somatic embryogenesis, in Prakash, J. and Pierik, R.L.M. (eds.) Horticultur- New Technologies & Applications , 1991; PP 237–240. Dordrecht, The Netherlands: Kluwer Academic Publishers.
11. Sawangjaroen N, Sawangjaroen K, Poonpanang P, Effects of *Piper longum* fruit, *Piper sarmentosum* root and *Quercus infectoria* nut gall on Caecal amoebiasis in mice. Journal of Ethnopharmacology. 2004; 91:357-360.
12. Soniya EV, Das MR, In vitro micropropagation of *Piper longum*– an important medicinal plant. Plant Cell Tissue and Organ Culture. 2002; 70:325–327.
13. Yusuf A, Tyagi RK, Malik SK, Somatic embryogenesis and plantlet regeneration from leaf segments of *Piper colubrinum*. Plant Cell Tissue and Organ Culture. 2001; 65:255–258.

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