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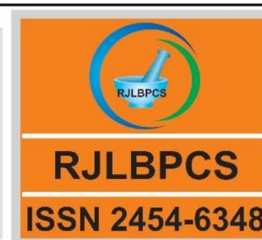

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EFFICIENT METHOD OF REGENERATION FROM NODAL EXPLANTS OF *PIPER LONGUM* L. (PIPERACEAE)

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ABSTRACT: *Piper longum* L. is an important medicinal plants belongs to the family Piperaceae. Present study is aimed towards the regeneration of shoot lets through *in vitro* culture of nodal explants. Murashige and Skoog (MS) basal medium supplemented with different concentrations and combinations of 6-benzyl amino purine (BAP), Kinetin (Kn) and α- naphalene acetic acid (NAA) were employed in order to induce effective multiplication of shoots. The maximum percentage of shoot induction was achieved on the medium fortified with 2.0 mg/L of BAP. The initiated shoots were sub cultured on fresh medium with the same composition and also with the addition of 0.2 mg/L of NAA for the further multiplication of shoots. Among the various combinations used, the medium enriched with 1.5 mg/L of BAP and 0.2 mg/L of NAA was found to be the most suitable one towards the maximum percentage of shoot multiplication with the highest number of shoots (8.00±0.57) and mean shoot length of 6.46±0.03 cm. The multiple shoots produced roots in the multiplication stage itself; therefore no separate media was employed for rooting stage. The developed protocol is rapid and efficient one for the regeneration of shoots and roots simultaneously. Hence this could be useful for the large scale production of multiple shoots of this important medicinal plant.

KEYWORDS: *Piper longum* L., nodal explants, regeneration, medicinal plant.

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1.INTRODUCTION

Piper longum. L is an important medicinal plant, commonly known as long pepper belonging to the family Piperaceae and also reported as an endangered medicinal plant [1-3]. The male and female spikes are produced on different plants. The fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes. The roots are thick and branched and medicinally the roots and fruits are potentially important and called *pippali-moolam* [4]. It is a prominent drug of Indian systems of traditional medicines [5]. *P. longum* grows wild in the tropical rain forests of India, Indonesia, Nepal, Malaysia, Rhio, Sri lanka, Timor and the Philippines. In India, it is seen in Tamil Nadu, Assam, Kerala, West Bengal, Uttar Pradesh, Karnataka, Madhya Pradesh and Maharashtra [6]. The major active compounds are piperlongumine, piperine and methyl-3,4,5-trimethoxycinnamate [7]. Volatile oil, protein, starch and alkaloids, saponins and amygdalin are also present [8]. The roots are known to possess trimethoxy cinnamoyl-piperidine and piperlongumine, pulvuatilol, sesamin and fargesin [9,10]. Almost all parts of the plants including the stems, roots and fruits are medicinally used in the treatment of diseases of respiratory tract like asthma and bronchitis [11]. *P. longum* is used in thirty different types of medicinal formulations in Ayurveda medicine system [12]. The bio active properties of roots are pungent and having heating, laxative, stomachic, anthelmintic, abortificient, hepato-protective, haematinic, digestive, diuretic and carminative properties. It induce the appetite and useful in bronchitis, abdominal pain, tumors and spleen diseases [13]. The plant possess bio active potential such as anti-inflammatory [14]; antibacterial, antifungal [15]; insecticidal and antiamoebic [16]; antiasthmatic [17]; antimalarial, CNS stimulant, antitubercular, anti-helminthic, antidiabetic and immunostimulatory [18]; antioxidant [19] and antidepressant [20]; analgesic [21] activity. As the plants are excessively exploited from its natural resource, the species has now become very rare in some forests of Kerala [1]. India is not self – sufficient in its requirement and in order to meet its local requirements, huge quantities of the produce is being imported from different countries like Indonesia, Sri Lanka and Malaysia. Hence, there is an immense need for the commercial cultivation of this plant with view to reduce the import. The requirement of such a huge volume of plants with quality cannot be easily achieved by conventional propagation method (vine cuttings). Conventional propagation method always have some problems like, poor seed viability, low percentage of germination and scanty, delayed rooting of vegetative cuttings etc., Nowadays tissue culture micropropagation is widely applied to produce the economically important crops in a rapid manner with a larger volume. Hence this technique can also be used as a tool to increase the availability of planting material of *Piper* longum. There are very few reports on micro-propagation of *P. longum* by Bandana [22]; Sarasan *et al.,*[23]; Parida and Dhal, [24]; Bhat *et al.,*[25]; Rani and Dantu [26] and Ravindran et al., [27]. The present study was aimed towards the regeneration of quality shoot lets from the nodal explants.

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2. MATERIALS AND METHODS

2.1. Source of mother plant and preparation of explants

The healthy mother plants of *P. longum* for the present study were collected form herbal nursery of Government Siddha Hospital, Tamil Nadu, India and maintained in the garden of Pachaiyappa's College, Chennai, Tamil Nadu. The mother plants were identified and authenticated by Taxonomist in Department of Botany, Pachaiyappa's College, Chennai. The nodal segments were excised and used as explants for the study. The collected nodal segments were washed with running tap water to remove the dust particle on the surface of the plant material and add few drops of soap solution (Teepol) and wash thoroughly with tap water. The washed explants were transferred in to Laminar Air Flow chamber (LAF) where the surface sterilization was carried out with the help of disinfectant mercuric chloride ($HgCl_2$). The nodal explants were subjected to surface sterilization with 0.1% of (w/v) of $HgCl_2$ for 2-3 minutes. Under the aseptic condition the nodal explants were resized about 0.5-1.0 cm long for inoculation.

2.2. Nutrient Medium

In the present study, Murashige and Skoog medium were used as basal medium for induction and multiplication of shoots. The MS basal medium fortified with different concentrations of 6-benzyl amino purine (BAP) (0.5 - 2.0 mg/L) and Kinetin (Kn) (0.5 - 2.0 mg/L) was used individually for shoot induction .In addition to this combination, 0.2 mg/L of α- naphalene acetic acid (NAA) is also added with the other combinations to achieve successful multiplication of shoots. The pH of the media was adjusted to 5.8 and all the media used were autoclaved for 20 minutes at 121°C and 15 psi of pressure. In this study no separate medium with PGRs used for rooting.

2.3. Inoculation of explants and Culture conditions

The nodal explants were excised and resized was about 0.5-1.0 cm length and inoculated on MS basal medium fortified with different concentrations and combinations of PGRs. All the cultures were incubated under the temperature of 25 ± 2ºC and the light intensity of 2000 - 4000 Lux. The photoperiod regime for cultures was 16 hr light and 8 hr dark and the relative humidity was maintained between 50 - 60%.

2.4. Initiation of shoots and Subculture

Nodal explants were inoculated in MS basal medium fortified with different concentrations of BAP (0.5 - 2.0 mg/L) and Kn (0.5 - 2.0 mg/L) towards the initiation of shoots. The young initiated multiple shoots were removed and then sub cultured on a fresh medium with same composition and also with 0.2 mg/L of NAA for further multiplication of shoots. The cultures were incubated and observed regularly and the results were recorded. The percentage of induction and multiplication of shoots, number and the length of shoots were recorded in 4 weeks old culture to assess the best treatment for regeneration of shoot lets from nodal explants.

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2.5. Statistical Analysis

All the tests were carried out in triplicates and the data were analyzed statistically using the SPSS 16.0 software (SPSS Inc., Chicago, USA) and the mean values are expressed as Mean ± SE. The significance of differences among means was carried out at $P < 0.05$ probability level using Duncan's Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

3.1. Initiation of shoots

The sterilized nodal explants were inoculated on MS basal medium supplemented with various concentrations of BAP (0.5 - 2.0 mg/L) and Kn (0.5 - 2.0 mg/L) for the initiation of shoots from the nodal explants. The medium supplemented with 2.0 mg/L of BAP was showed the maximum percentage (83.33±0.88)of initiation and produced 4.33±0.88 shoots with mean length of 4.66±0.16cm (Fig 1a & 1b; Table 1).

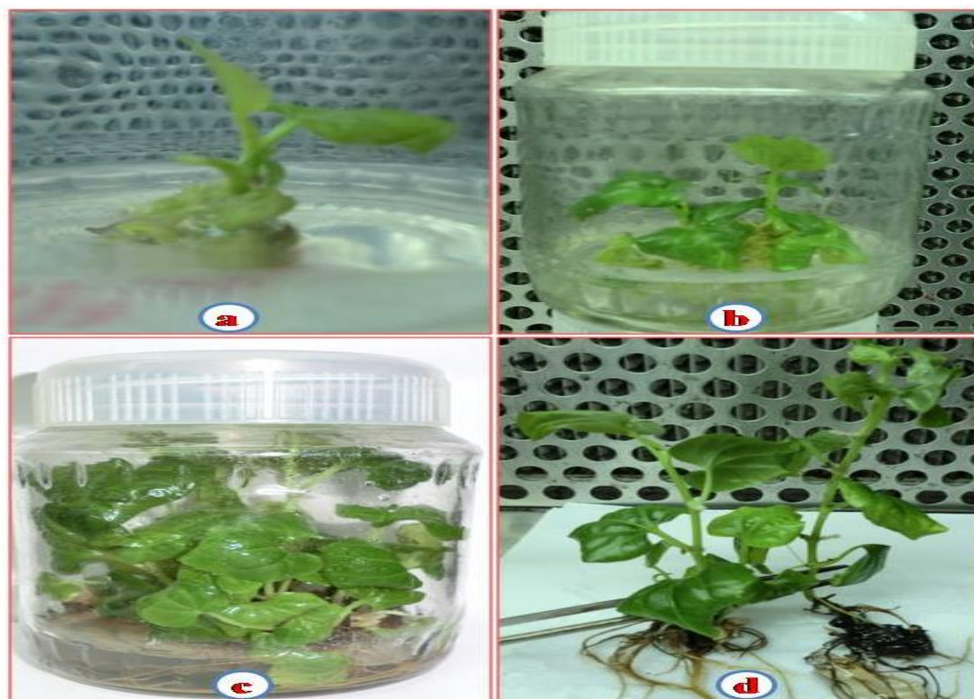

Figure 1: Regeneration of *Piper longum* L.

a- Shoot induction, **b-** Multiple shoots, **c-** Multiple shoots with roots**, d-** Well rooted plantlets.

In the present study, among the different concentrations of PGRs, the medium supplemented with BAP was comparatively more efficient than that of Kn in terms of maximum percentage of shoot induction with highest number of shoots per explant. Hence, the medium enriched with 2.0 mg/L of BAP was identified as a suitable one for the successful initiation of shoots. Ravindran *et al.,*[27] reported that the 1.0 mg/L of BAP was found suitable for bud breaking which is coincide with the current work but few reports on *P. longum* were contrary with reference to the shoot induction. The effect of BAP over Kn in bud breaking response and shoot induction was reported in various species like *Rubia cordifolia* [28] and*Marsilekhea quadrifolia* [29].

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Table 1: Effect of plant growth regulators on shoot initiation

Medium	Plant Growth Regulators		Percentage of Shoot induction (%) (Mean±SE)	Number of Shoots per node (Mean±SE)	Length of Shoots (cm) (Mean±SE)
	BAP (mg/L)	Kn (mg/L)			
MS-1	0.5	-	35.00±1.15[a]	1.33±0.33[ab]	3.10±0.20[ab]
MS-2	1.0	-	45.33±0.33[c]	3.33±0.88[cd]	3.40±0.57[ab]
MS-3	1.5	-	74.66±0.88[e]	3.00±0.00[bcd]	4.23±0.14[c]
MS-4	2.0	-	83.33±0.88[f]	4.33±0.88[d]	4.66±0.16[c]
MS-5	-	0.5	32.33±1.45[a]	1.00±0.00[a]	3.00±0.28[a]
MS-6	-	1.0	40.33±1.45[b]	1.33±0.33[ab]	3.10±0.20[ab]
MS-7	-	1.5	52.66±1.45[d]	2.00±0.00[abc]	3.66±0.16[b]
MS-8	-	2.0	56.66±0.88[d]	3.00±0.57[bcd]	3.50±0.00[ab]
F-Value			267.246	5.263	11.205
P-Value			0.000	0.003	0.000

The values represent the Mean ± SE of ten replicates and all experiments were repeated thrice. Means with different letter within column are significantly different from each other at $P \leq 0.05$.

3.2. Multiplication of shoots

The initiated multiple shoots were sub cultured on fresh medium with same composition used for the initiation. In addition to these concentrations 0.2 mg/L of NAA was also used to identify the effective combination for the further multiplication of shoots. Even though the multiplication of shoots was observed in all the media combinations, the morphogenetic responses in terms of number of shoots per mother culture and length of shoots varied with different formulations (Table2). The maximum percentage (87.66±0.33) of shoot multiplication was noticed on the medium enriched with 1.5 mg/L of BAP and 0.5 mg/L of NAA. This medium produced highest number of shoots (8.00±0.57) with the mean length of 6.46±0.03cm (Table2; Fig1c). The medium fortified with BAP and NAA was more efficient than the Kn and NAA combination in terms of shoot multiplication. This result is not in tandem with the available previous report of Ravindran *et al.,*[27] in *P.longum*. They reported a different combinationviz., BAP (0.5 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L) as a suitable one for the maximum multiplication of shoots. Parida and dhal, [24] reported that 7.5 shoots were produced on MS medium fortified with 1.0 mg/L of BA and 1.0 mg/L of IAA in *P. longum*. Bandana [22] reported that the efficient shoot proliferation achieved in *P.longum* by nodal segment culture on MS medium supplemented with 1.0 mg/L of Kn and 1.5 mg/L of BAP which is coincide with the present report. Plant regeneration in *P.longum* was achieved by various workers through direct and indirect adventitious shoot production [23]. Regeneration was also achieved through callus culture of *P. longum* by Bhat *et al.,*[25].

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Table 2: Effect of plant growth regulators on multiplication of shoots

Medium	Plant Growth Regulators			Percentage of Shoot Multiplication (%)	Number of Shoots (M±SE)	Length of Shoots (cm) (M±SE)
	BAP (mg/L)	Kn (mg/L)	NAA (mg/L)			
MS-1	0.5	-	-	27.66 ± 1.45^{b}	1.33 ± 0.33^{ab}	3.10 ± 0.20^{a}
MS-2	1.0	-	-	31.66 ± 0.88^{cd}	1066 ± 0.33^{ab}	3.43 ± 0.23^{ab}
MS-3	1.5	-	-	37.66 ± 1.45^{e}	4.33 ± 0.33^{cdef}	5.00 ± 0.28^{ef}
MS-4	2.0	-	-	34.66 ± 0.88^{d}	3.00 ± 0.00^{abc}	5.26 ± 1.45^{f}
MS-5	-	0.5	-	22.00 ± 1.15^{a}	1.00 ± 0.00^{a}	4.10 ± 0.20^{bc}
MS-6	-	1.0	-	27.66 ± 0.88^{b}	1.33 ± 0.33^{ab}	4.83 ± 0.16^{cdef}
MS-7	-	1.5	-	31.66 ± 0.88^{cd}	4.33 ± 1.85^{cdef}	4.50 ± 0.28^{cde}
MS-8	-	2.0	-	29.00 ± 1.15^{bc}	3.00 ± 0.00^{abc}	4.16 ± 0.44^{cd}
MS-9	1.0	-	0.2	75.00 ± 1.15^{h}	4.00 ± 0.57^{cde}	4.66 ± 0.33^{cdef}
MS-10	1.5	-	0.2	87.66 ± 0.33^{j}	8.00 ± 0.57^{g}	6.46 ± 0.03^{g}
MS-11	2.0	-	0.2	83.00 ± 1.15^{i}	6.33 ± 0.33^{fg}	5.43 ± 0.23^{f}
MS-12	-	1.0	0.2	61.66 ± 0.88^{f}	3.33 ± 0.33^{bcd}	5.33 ± 0.16^{f}
MS-13	-	1.5	0.2	68.33 ± 0.33^{g}	6.00 ± 0.57^{ef}	4.93 ± 0.06^{ef}
MS-14	-	2.0	0.2	66.00 ± 1.15^{g}	5.33 ± 0.88^{def}	4.86 ± 0.06^{def}
F-Value				511.463	10.591	13.439
P-Value				0.000	0.000	0.000

The values represent the Mean ± SE of ten replicates and all experiments were repeated thrice. Means with different letter within column are significantly different from each other at $P \leq 0.05$.

In the present study, roots are developed simultaneously in the multiplication stage itself and hence no separate medium was employed towards the root induction (Fig1d). This facilitates the reduction in the cost of production in addition to the reduced time and it will make value addition to this protocol when it is considered for the large scale production. The simultaneous root induction may be due to the presence of auxin (NAA) in the multiplication medium. Soniya and Das [30] used separate MS medium supplemented with 2.46 µM indole butyric acid (IBA) for rooting. This result is in contrary with Bandana, [22] who reported that the root induction was achieved in *P. longum* on the medium fortified with 0.5 mg/L of IAA. Among the various media combinations used, BAP with NAA was found be the most favorable one when compare to Kn + NAA for the successful multiplication of shoots. This combination BAP and NAA combination was markedly enhance the percentage of multiplication of shoots, number of shoots besides the appreciable length.

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4. CONCLUSION

The regeneration of shoots of *P. longum* was carried out using nodal segments as explants source in this study. The nodal segments were capable of producing multiple shoots *in vitro*. This can be used as an effective conservation strategy for the conservation of valuable medicinal spice *P.longum*. The regeneration of shoots and roots was achieved in the same multiplication medium itself is an added advantage of this protocol. Hence this protocol can be applied to develop the large scale production of disease free quality planting material of *P. longum* and other medicinal plants as well.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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