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Original Article

Micropropogation and *in vitro* conservation of two medicinally important species of long pepper (*Piper longum L. and P. Chaba* Hunt)

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ABSTRACT

Two medicinally important species of long pepper, *Piper longum* and *P. chaba* were successfully micropropagated on McCown's Woody Plant Medium (WPM) supplemented with BAP and kinetin. WPM with 3 mgl^{-1} BAP and 1 mgl^{-1} kinetin was found to be ideal for shoot regeneration and their subsequent growth from both leaf and stem explants either with or without intervening callus phase in both the species. These shoots developed good root system when growth regulators were removed from the culture medium. When rooted plantlets of *P. longum* were grown in culture medium with 3 mgl^{-1} BAP and 1 mgl^{-1} kinetin there was conversion of root meristem to shoot meristem which subsequently developed into shoots and then plantlets. Over 90% of the regenerated plantlets could be easily established in soil. Shoot tips could be conserved under minimal growth conditions with yearly subculture in WPM without any growth regulators. The plantlets could be multiplied normally after one year of storage and the rooted plantlets were successfully planted out.

Keywords: conservation, *in vitro* propagation, long pepper, micropropagation, *Piper chaba*, *P. longum*

INTRODUCTION

Many species of *Piper* are economically important and are used in indigenous medicine. Three species of *Piper*, *viz.*, *P. longum* L., *P. peepuloides* Roxb. and *P. chaba* Hunt (Syn. *P. retrofractum* Vahl) contribute to the long pepper 'pipalmul' of commerce in India. Of these *P. longum* is native to India and *P. chaba* is native to Java and hence, are known as Indian and Java long peppers, respectively. Both these species are related to *P. nigrum*, the black pepper of commerce, which is the most important spice of the world (Purseglove *et al.* 1981). Fruits of these species are used as spices and also in pickles and preserves. They have stimulant and carminative properties and are used in traditional medicine. The roots and thickened parts of the stem are cut, dried and used as the important drug 'pipalmul' in Ayurvedic and Unani systems of medicine.

Developing micropropagation protocols in these two predominantly vegetatively propagated species will help in production of large scale disease free planting material and also in crop improvement programmes. India is the centre of diversity for many spices. However, disturbances in their natural habitats have resulted in loss of these valuable materials. Conservation of the germplasm in *in vitro* gene bank is a viable method to augment the conventional conservation strategies like evolution gardens or field gene banks. There were a few attempts earlier to micropropagate *Piper* species reporting difficulties in regenerating plantlets from callus (Broome and Zimmerman, 1978; Chua, 1981, Mathews and Rao, 1984; Fitchet, 1988a; 1988b). To our knowledge there is only one earlier report on successful plant regeneration from shoot tip derived callus of *P. longum* (Bhat *et al.* 1992). There are no earlier reports of micropropagation of *P. chaba*. This paper reports micropropagation of *P. longum* and *P. chaba* through plant regeneration from leaf, stem and root tissue either directly or involving intermediary callus phase and conservation of these medicinally important species, *in vitro*.

MATERIALS AND METHODS

Source of explants

Tender leaf and shoot explants were taken from orthotropic shoots of field grown plants of *P. longum* (Fig. 1) and *P. chaba* (Fig. 2) and were washed in running water for 20 minutes. They were surface sterilized with 1% sodium hypochlorite for 5 to 10 minutes and then rinsed in three

changes of sterile water. One more cycle of surface sterilization was done with 0.1% mercuric chloride ($HgCl_2$) solution for 5 minutes, and the explants were rinsed again in 3 changes of sterile water. Surface sterilized shoot tips (1-2 cm long) and tender whole leaves with a portion of the petiole or small sections (1 cm^2) of leaf lamina were inoculated into the culture medium under aseptic conditions.

Culture initiation and multiplication

The basal nutrient medium used was that of Woody Plant Medium, WPM (Mc Cown and Amos, 1979). This was supplemented with 3% sucrose, N^6Benzyl aminopurine (BAP) at 0.5, 1.0, 2.0 and 3.0 mgl^{-1} and kinetin (kin) at 0.5, 1.0, 2.0 and 3.0 mgl^{-1} concentrations singly and in various combinations. The pH of the medium was adjusted to 5.8 before autoclaving at 1.0 kg,cm^2 pressure at 121°C for 20 minutes. The medium was solidified with 0.7% 'Qualigens' bacteriological grade agar. All the cultures were incubated at 25±2°C with 14h photoperiod provided by cool white fluorescent tubes giving light intensity of 2500-3000 lux. Each treatment was replicated in 20 tubes.

In vitro rooting, hardening and planting out

Rooted plantlets of both *P.longum* and *P.chaba* were transferred to soil (garden soil, sand and perlite mixture in equal proportions) in thermocol cups or polypropylene bags and kept in humid chamber for 20-30 days for hardening. Hardened plants were transferred to nursery and after required growth, they were transferred to field.

In vitro conservation

Shoot tips of about 2 cm size with 1 or 2 opened leaves were excised from *in vitro* grown cultures of both *P.longum* and *P.chaba*. Studies on different concentrations of basal medium, carbon source, osmoticum and vessel closure types were made. In each treatment 10 cultures were replicated. The cultures were incubated at 22±2°C with 12h photoperiod of 2500 lux. Observations were made on growth rate, vigour of the cultures, symptoms of drying, vitrification, exhaustion of media, optimum period of storage, viability and survival after storage and soil establishment.

RESULTS AND DISCUSSION

Establishment of cultures

Various explants *viz.*, shoot tips, whole leaves with portion of leaf lamina etc. were cultured on WPM. Contamination mainly due to endogenous bacteria, was the major problem. Similar observations were reported earlier in tissue cultures of *Piper* species (Chua 1981; Fitchet 1988a; 1988b; Bhat *et al*, 1992). When the uppermost segments of tender actively growing orthotropic shoots were used as explants, about 35% of the cultures were clean though in some of them the bacteria appeared much later. The contamination was less when leaves from these topmost segments were used as explants. Contamination was more prevalent in *P.longum* (40%) cultures than in *P.chaba* cultures (30%). Browning of tissues due to phenolic exudates occurred and was detrimental. Frequent transfers to fresh medium overcame this during initial stages and subsequently such transfers were made every 20-30 days.

In vitro propagation

All explants responded readily to WPM supplemented with BAP (0.5, 1.0, 2.0 and 3.0 mgl^{-1}). Callus production and shoot regeneration was noticed to certain extent in all the combinations of BAP and kinetin. However, WPM with 3 mgl^{-1} BAP and 1 mgl^{-1} kinetin yielded the optimum result and hence was used for further studies.

Shoot explants

When shoot tip explants of *P.longum* were cultured on WPM with 3 mgl^{-1} and 1 mgl^{-1} kinetin, there was initial growth of compact and globular callus in 70% of the cultures. Within another 20-30 days, organogenesis in the form of numerous (10-100) shoot primordial could be obtained from the callus. Over 40% of these primordial showed good elongation and continued normal development. Some of them developed roots from the aerial nodes. In 30% of the cultures on WPM supplemented with 3 mg l^{-1} BAP and 1 mgl^{-1} kinetin, direct organogenesis in the form of shoot primordial without intervening callus phase was noticed from the explants (Fig. 3). These primordial also developed into normal shoots in the medium of same composition. Shoot elongation was much higher when growth regulators were completely removed from the medium after organogenesis. Precocious branching and production of multiple shoots (upto 7) were observed in 30% of the cultures. Rooting of the shoots was also excellent in growth regulator free medium and all the shoots inoculated developed good root system. Even the shoot tip explants from field grown plants developed excellent root system in this medium.

In *P.chaba*, shoot cultures responded similarly to the cuture mentioned above. Multiple shoots could be regenerated both directly from the explants (Fig. 4) and from the callus (Fig. 5). However, the response was slower and shoot regeneration efficiency was lower (5-15 plantlets in *P.chaba* compared to 10-100 in *P.longum* (Table 1) but there was no problem of shoot elongation in *P.chaba*.

Leaf explants

Leaf explants are most responsive than shoots for organogenesis and plant regeneration in both *P.longum* and *P.chaba*. Leaf explants with a portion of the petiole were highly morphogenic. In both species, leaf explants showed callus formation at the region of contact with the culture medium especially from the petiolar regions, on WPM with 3 mgl^{-1} BAPand 1 mgl^{-1} kinetin. After 30-45 days of culture on the same medium numerous shoot primordial developed from the callus of both species. In *P.longum*, direct development of shoot primordial from the leaf margins without any callus formation was also observed. These primordia eventually developed into shoots, which could be rooted easily in growth regulator free medium.

Table 1: *In vitro* responses of *P.longum* and *P.chaba* to WPM

Piper spp.	Culture Medium			
	WPM +BAP 3mgl^{-1}+kin 1mgl^{-1}		WPM	
	Shoot	Leaf	Root	Shoot
P.longum	Callus induction, development of multiple shoots and regeneration of plantlets	Production of callus, regeneration of plantlets either directly from leaf tissue or from callus	Production of plantlets from swollen roots	3-5 roots per shoot
P.chaba	Production of hard callus, multiple shoots and regeneration of plantlets.	Production of callus and regeneration of plantlets directly and via callus	-	2-4 roots per shoot

Conversion of root meristems into shoots

When shoot explants of *P. longum* with 2-3 roots were transferred to WPM with 3 mgl^{-1} BAP and 1 mgl^{-1} kinetin, roots showed swelling at the tips and developed many small shoot primordial after about 40 days of culture. Another 30 days of culture in the same medium resulted in development of shoots from these primordial. In many orchids, entire portion of the root also showed swelling which later developed into numerous shoots (Steward and Button 1978; Kerbauy 1984a; 1984b) tomato (Norton and Boll 1954) etc. Conversion of root tips into shoots without the intervention of callus phase was reported in *Vanilla planifolia* (Nirmal Babu *et al.*, 1997; Philip and Nainar 1988), *V.aphylla* (authors' laboratory), *Anthurium longifolium* (Patterson 1975; Champagnat 1971). Philip and Nainar suggested that in *Vanilla*, under certain circumstances, such as lower auxin levels in and around the quiescent center, a fundamental switch could occur in the organizational activity of the meristem, which may result in the development of shoot meristem instead of root meristem. This may be the reason for the development of shoots from the roots in *P.longum* also.

In vitro rooting

The excised shoots could be rooted in WPM basal medium devoid of any growth regulators in both *P. longum* (Fig. 6) and *P. chaba*. Addition of charcoal to the medium enhanced root induction.

In vitro conservation

Protocols for *in vitro* conservation by slow growth of black pepper and its related species *viz.*, *P. barberi*, *P. colubrinum*, *P. betle* and *P. longum* were standardised (Geetha *et al.*, 1995) by maintaining cultures at reduced temperatures, in the presence of osmotic inhibitors, at reduced nutrient levels, or by minimising evaporation loss by using closed containers. It was observed that use of sealed culture tubes (screw cap, polypropylene cap and aluminium foil) minimized moisture loss and thereby reduced the rate of media exhaustion to a greater extent. Even after 360 days the retention of medium was high resulting in green and healthy cultures due to the availability of nutrients. In sealed culture tubes, only 25% of the medium was lost after 360 days whereas in the tubes closed with cotton plugs 75% of the medium evaporated within 120-180 days. Though cotton plugs allowed better gaseous exchange, it was thus not suitable for long term storage of *in vitro* cultures.

Shoot tips of *P.longum* could be stored upto one year in full strength WPM basal medium supplemented with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol with 70–80% survival (Fig. 7). In *P. chaba* shoot tips could be stored for one year in full strength WPM basal medium supplemented with 20 gl^{-1} sucrose with a rate of 60 – 70% survival. In *P. longum* further increase in mannitol resulted in yellowing of cultures, shoot tip necrosis and death of cultures after six months.

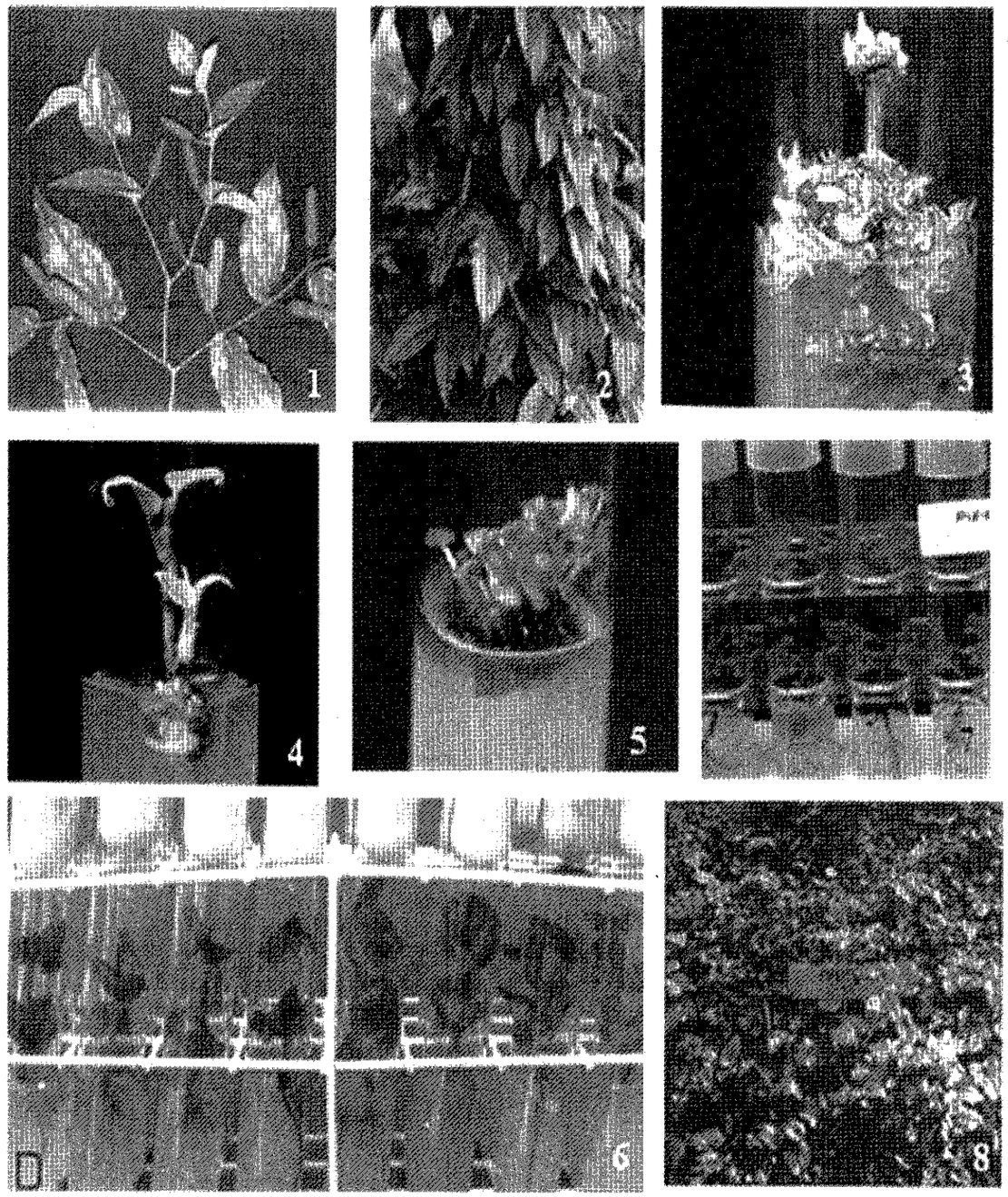

Fig. 1-8: *In vitro* propagation and conservation of *Piper longum and P.chaba*
1: *Piper longum* – habit; 2: *P.chaba*- habit; 3: *P.longum* – direct regeneration from nodal explants; 4: *P.chaba* - Shoot regeneration; 5: *P.chaba* -Callus regeneration; 6: *In vitro* rooting in *P.longum;* 7*: In vitro* conservation of *P.longum;* 8: Field evaluation of tissue cultured *P.longum*

Table 2: *In vitro* conservation of *P. longum* and *P. chaba*

Species	Basal Medium	Carbon source (gm/l)	Growth	Survival period (months)	Survival (%)	Establishment in nursery (%)
P.longum	WPM	20S+10M	Slow	15	70	80
P.chaba	WPM	20S	Slow	12	60	50

The cultures could be directly transferred to soil with about 70 – 80% survival and when transferred back to multiplication medium, more than 90% of the cultures multiplied normally within 60 – 90 days. The normal sized plantlets when transferred to soil established with over 80% success (Table 2). They developed into normal plants without any deformities and were morphologically similar to mother plants.

Hardening and planting out

The well rooted plantlets of both *P.longum* (Fig. 8) and *P.chaba* were transferred to soil (garden soil, sand and perlite mixture in equal proportions). The establishment in soil is over 95%, when the transferred plantlets were kept in humid chamber for 20-30 days for hardening.

Thus, WPM was found to be an excellent basal medium for *in vitro* culture of *Piper longum* and *P.chaba.* Such results were also noticed in other *Piper* species like *P.nigrum, P.colubrinum* and *P.betle,* by the authors (Nirmal Babu *et al.* 1993; 1997). Micropropagation of long pepper was also reported earlier (Bhat *et al.* 1992, Sarasan *et al.* 1993). The difficulties explained by the earlier workers in regeneration of plantlets in this group of crops may be due to improper choice of the basal medium coupled with high rate of contaminated cultured. Other workers (Bandana, 2015) have used either MS (Murashige and Skoog 1962) or SH (Schenk and Hildebrandt 1972) media, whereas WPM was used as basal medium in this study.

CONCLUSION

The results reveal that *P.longum* and *P.chaba* can be micrpropagated from leaf and stem tissues and in addition *P.longum* could be propagated from root tissues also. WPM is highly suitable as a basal medium. No growth regulartors are required for *in vitro* rooting of shoot, and cytokinins (BAP and Kin) are sufficient to bring about organogenesis and plantlet regeneration from shoot and leaf explant of *P.longum* and *P.chaba* and root tissues of *P.longum*). Conversions of root meristem into shoot meristem and its subsequent development to plantlets were reported in *P. longum.* Micropropagated plantlets of these species are being evaluated for their field performance at Indian Institute of Spices Research (IISR), Calicut and preliminary observations indicated that they are on par with clonally propagated plants.

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