Plant regeneration in *Piper longum* L. (Piperaceae) through direct and indirect adventitious shoot development

V SARASAN, ELIZABETH THOMAS, BEENA LAWRENCE & G M NAIR[1]
Plant Tissue Culture Laboratory, Department of Botany
University of Kerala, Kariavattom Campus
Trivandrum - 695 581, India.

ABSTRACT

Methods for large scale multiplication of *Piper longum* are reported. Shoot tips cultured on Murashige & Skoog's medium supplemented with 0.5 mgl^{-1} benzyl adenine or kinetin in combination with 0.5 mgl^{-1} 1-naphthalene acetic acid developed multiple shoots at very low frequency. Multiple shoots originated in large numbers,only from nodes of vertically planted explants, while shoots developed *de novo* from all over the surface, when planted horizontally in the same medium containing 2-5 mgl^{-1} benzyl adenine. Leaf segments also developed adventitious buds in large numbers in Murashige & Skoog's medium containing 3 mgl^{-1} benzyl adenine, 1mgl^{-1} kinetin and 10% (v/v) coconut water. Callus induced from leaf and stem explants in Murashige & Skoog's medium with 1-2 mgl^{-1} 2, 4- dichlorophenoxy acetic acid in combination with 1mgl^{-1} benzyl adenine developed shoot buds when transferred to medium containing 3 mgl^{-1} benzyl adenine. All the shoots were rooted in Murashige & Skoog's basal medium. The isolated plantlets, hardened in vermiculite were transferred to pots containing garden soil and sand.

Key words : direct organogenesis, long pepper,multiple shoots, *Piper longum,* regeneration.

Abbreviations

BA : benzyl adenine
CW : coconut water (liquid endosperm)
2, 4-D : 2,4-dichlorophenoxy acetic acid
2-ip : 2-isopentenyl adenine
Kin : kinetin
MS : Murashige & Skoog (1962)
NAA : 1-naphthalene acetic acid

[1]Correspondent author

Introduction

Tissue culture techniques in recent years have made easy propagation of many economically and medicinally important plants. Such techniques are also successfully used for experimenting and standardizing methodologies for improvement of crops. Many of the tropical spices earning a large revenue have not received adequate attention for improvement of their yield and performance utilizing tissue culture techniques, except for a few main spices (Hosoki & Sagawa 1977; Kuruvinashetti, Haridasan & Iyer 1982; Rao *et al.* 1982; Nadgauda, Mascarenhas & Madhusoodanan 1983; Mathews & Rao 1984; Kumar *et al.* 1985; Babu, Samsudeen & Ravindran 1992; Sarasan 1992).

Piper longum L. (Long pepper, Piperaceae), an unisexual perennial climber commonly found in Asian countries, is a valuable source of active substances of medicinal value besides being used as a spice (Anonymous 1969). The fruits and roots contain major alkaloids such as piperine and piplartine in addition to sterols and glycosides (Anonymous 1969). The conventional method of propagation of this plant is through layering of mature branches which is time consuming. In the present paper we describe rapid multiplication of this species through direct and indirect organogenesis.

Materials and methods

Shoot tips, nodal, internodal and leaf segments from greenhouse grown mature-phase *P. longum* plants were used as explants. The explants were washed throughly in running water for 2 h and soaked in Teepol (a commercial neutral detergent) solution before surface sterilization with 0.1% (w/v) mercuric chloride solution. Shoot tips and young leaves were sterilized for 7 min and stem segments for 10 min. The explants, after sterilization were washed several times in sterile water. The culture medium consisted of MS nutrients (Murashige & Skoog 1962) containing 2% sucrose and soldified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min at 15 lbs per sq inch pressure. The cultures were incubated at 25±2°C under 16 h photoperiod at light intensity of 3000 lux from cool white fluorescent tubes.

Shoot tips (0.5-1.0 cm) were inoculated on MS basal medium supplemented with 0.1-1 mgl^{-1} NAA and 0.1-1 mgl^{-1} Kin or BA. Nodal segments and internodes of 1-1.5 cm were cultured on MS medium supplemented with 2-5 mgl^{-1} BA alone. The nodal segments were planted either vertically or horizontally in the medium to find out differences in response, if any. One cm long stem segments and 0.5 cm^2 leaf discs were cultured on MS medium supplemented with 1-4 mgl^{-1} 2, 4-D and 1 mgl^{-1} BA, 2-3 mgl^{-1} BA+1 mgl^{-1} Kin/2-ip with 10% CW for callus regeneration and adventitious bud induction. After 30 days of primary culture, the explant/callus was subcultured in medium containing 0-2 mgl^{-1} 2, 4-D and1-2 mgl^{-1} BA. Morphogenic callus, selected from these cultures, was subcultured in a medium containing 2-3 mgl^{-1} BA for shoot induction while leaf discs with developed adventitious buds were transferred to MS medium containing 3mgl^{-1} BA, 1mgl^{-1} Kin and 10% CW. The regenerated shoots from different experiments were subcultured in MS basal medium (full and half strength)

for rooting. The plantlets rooted and hardened for 15 days each in basal liquid medium and vermiculite were subsequently transplanted to garden soil and sand mixture (1:3). The plants were initially covered with plastic bags (minimum 5 days) to retain humidity and nourished with 1/10 strength modified Hoagland's solution (Epstein 1972).

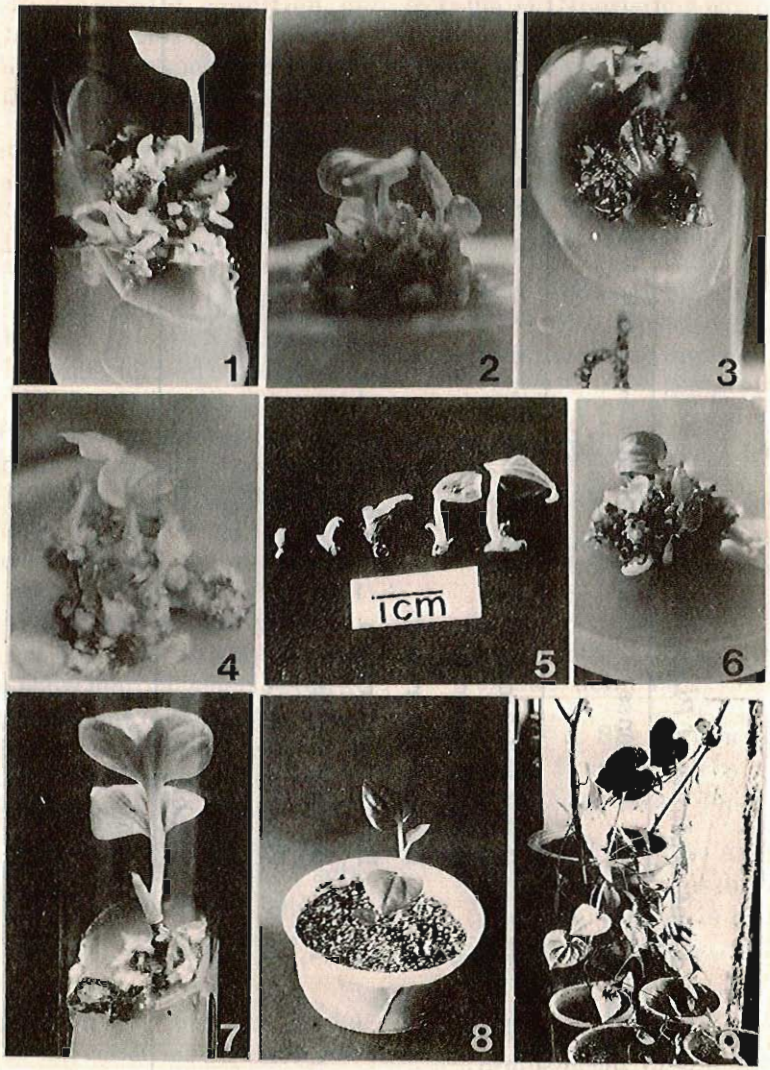
Results and discussion

Shoot tips cultured on MS medium supplemented with 0.4-1 mgl^{-1} NAA in combination with 0.1-0.4 mgl^{-1} BA or Kin developed multiple shoots after 20 days. Further proliferation of shoots and development of roots occurred simultaneously upon transferring to MS containing 0.5 mgl^{-1} BA or Kin in combination with 0.5 mgl^{-1} NAA. The number of multiple shoots developed was less (2 or 3) in each subculture. This may be because of the strong apical dominance present in this plant due to high levels of endogenous auxins. Apical dominance as reasons for reduction in shoot producing cultures is reported in few systems (Newbury 1986; Selva *et al.* 1989).

Multiple shoots developed within 25 days from nodal segments explanted on MS medium fortified with 2-5 mgl^{-1} BA and elongated further in about 40-50 days(Fig. 1). Shoots originated from the nodal region only when the explant was oriented vertically in the medium. However, buds originated adventitiously from all over the surface of the explant when planted horizontally in the medium after an initial phase of slight callussing (Fig. 2). The initiation of buds from the horizontally placed nodal segment occurred after 45 days and there was a quicker establishment into shoots after this. Among the concentrations tried, 3 mgl^{-1} BA was the most effective in producing maximum shoots (Table 1). The isolated shoots readily developed roots when tranferred to MS basal medium. The multiple shoots also developed roots when left in the same medium for a longer period (45-90 days after shoot initiation). The number of regenerated plantlets was maximum in horizontally planted explants.

Generally, multiple shoots are established only around the nodal portion. However, *P.longum* appears to have a natural tendency of developing buds *de novo* from the explant irrespective of a node, if placed horizontally in the medium. This may be due to a reversal of polarity because of altered position and also to a certain extent triggered by phytohormones in the medium.

Leaf discs cultured in MS medium supplemented with 3 mgl^{-1} BA, 1mgl^{-1} Kin and 10% CW developed small buds on the petiolar region initially and subsequently along the main vein within 40 days (Fig. 3). These cultures when divided and subcultured into the same medium developed a large number of shoots (Figs. 4 & 5). Direct organogenesis is reported from explants of juvenile phase (Wong & Loh 1987; Nair *et al.* 1984) and from mature phase in a few plants (Choo 1988; Mark & Lineberger 1988). Multiple shoots were also induced directly from leaf segments in *P. longum* using a combination of BA and CW in MS medium (Sarasan & Nair 1991). The regeneration of plantlets from mature phase explants is reliable only when it is required to select plants of superior quality for regeneration (Rao & Lee 1986). Such explants can give rise to regenerants of the same phenotype if the parent plant was not subjected to



Figs. 1-9. *In vitro* response of various explants of *Piper longum*

1. Nodal segments planted vertically in MS medium containing 3 mgl^{-1} BA showing multiple shoots developed from the node. 2. Stem segments planted horizontally on MS medium containing 3mgl^{-1} BA showing profuse shoot development from the entire surface of the explant. 3. Leaf segment cultured in MS medium with 3 mgl^{-1} BA, 1mgl^{-1} Kin and 10% CW showing initial development of adventitious buds from the petiolar and midrib regions. 4. Later stages of the same showing large number of developed shoots. 5. Isolated *de novo* developed shoots at various stages of development. 6. Callus developed in 2mgl^{-1} BA and 1 mgl^{-1} 2, 4-D cultured in 3mgl^{-1} BA containing medium showing development of shoots. 7. Isolated shoot rooted in MS basal medium. 8. Plantlets in vermiculite. 9. Plantlets in pots after 3 months.

Table 1. ***In vitro*** **response of stem and leaf explants of** ***Piper longum***

Medium	Explant	Hormone (mgl^{-1})		Response	No. of plantlets regenerated* Mean ± SD	Rate of survival (%)**
		2, 4-D	BA			
MS	S	0	2	Direct regeneration	32.9 ± 3.8	85.6
	S	0	3	Direct regeneration	47.3 ± 5.5	88.1
	S	0	4	Direct regeneration	37.6 ± 4.7	98.9
	S	0	5	Direct regeneration	27.6 ± 3.7	99.1
	S and L	1	1	Nodular callus	20.7 ± 3.7	74.2
	S and L	2	1	Profuse nodular callus	22.9 ± 3.6	73.9
	S and L	3	1	Friable callus	9.4 ± 2.9	69.0
	S and L	4	1	Friable callus	9.4± 2.9	69.0

* From single cultured explant
** After 6 months in the field
S - stem; L - leaf

altered environmental condition or stress. Organogenesis from mature phase explants in this species is important because of its dioecious nature.

Stem segments and leaf discs produced white callus from the cut ends after 20 days in MS medium containing 1-2 mgl^{-1} 2, 4-D and $1mgl^{-1}$ BA. Callus induced from stem segments and leaf discs in MS basal medium containing 3-4 mgl^{-1} 2, 4-D with 1 mgl^{-1} BA was friable. Nodular callus subcultured in 2 mgl^{-1} BA with 0-1 mgl^{-1} 2, 4-D turned green, and subsequently produced shoot buds when transferred to MS medium containing 3-4 mg l^{-1} BA (Fig. 6). It is interesting to note that 2, 4-D at lower concentrations induced compact nodular callus which on subculturing produced shoot buds, while callus derived at higher concentrations of 2, 4-D was always friable and did not show morphogenesis even at altered combinations of hormones. This confirms the earlier observations that 2, 4-D at higher concentration blocks morphogenesis (Halperin & Jenson 1967; Negrutiu 1978). Shoot buds produced from the callus when isolated and subcultured in MS basal medium (both full and half strength) rooted within 7 days (Fig. 7).

Transplantation of *in vitro* grown plantlets to field conditions is an important step in any tissue culture work. There are several techniques for aclimatization of *in vitro* grown plantlets from the tube to the pots and subsequently to the field (George & Sherrington 1984; Selvapandiyan *et al.* 1988). Plantlets of *P. longum* transplanted directly to the soil became stunted, probably due to lack of sufficient root development. However, the percentage of survival was much higher when transplanted initially to vermiculite and subsequently to soil (Figs. 8, 9 & Table 1). Nourishing the plants with 1/10 strength Hoagland's modified solution while in vermiculite enhanced their initial establishment.

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