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Original Research Article

Meristem culture for rapid regeneration in Black pepper (*Piper nigrum* Linn.)

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Abstract: The shoot apical meristem for production of plantlets from black pepper variety Sreekara was assessed. Meristem extension was achieved in liquid medium containing Murashige and Skoog with 0.1 mgl^{-1} kinetin and 0.5 mgl^{-1} GA_3, subsequent direct shoot induction in ½ MS with 3 mgl^{-1} BA and 1 mgl^{-1} IAA followed by shoot growth and development in ½ MS +0.5 mgl^{-1}Indole butyric acid. The meristems of 2mm resulted in shoot induction and proliferation via direct organogenesis. Successful rooting of meristem derived shoots was achieved in half strength WPM with 3 mgl^{-1} BA and 1 mgl^{-1} Kinetin. *In vitro* multiplication from the meristem derived plants shows the possibility for mass multiplication. The developed protocol was effective with 40% regeneration frequency of meristems, simple and rapid protocol for regeneration with 66-70days and subsequent micro propagation for the production of plant stock. This is the very first report on meristem culture in blackpepper. The protocol will be of immense importance in rapid mass multiplication of elite germplasm, as well as for conservation of this important species.

Key words: Blackpepper; Meristem culture; Direct organogenesis.

Introduction

Black pepper (*Piper nigrum* L.) originated from Western Ghats of India (Ravindran 2000) belonging to *Piperaceae*. The family Piperaceae is unique for its unique genome to produce large kind of secondary metabolites with the major production of piperin and other essential oils. Among biotic and abiotic stresses responsible for its lower productivity, non availability of healthy planting material is the major factor (Sharma & Kalloo 2004). The disease caused by Piper yellow mottle virus belonging to badna virus (PYMoV) (Bhat *et al.,* 2005; Hareesh & Bhat 2008) makes it important due to their wide spread and yield loss. Upon PYMoV infection, the leaves become leathery, mottled, distorted with reduced size with the shortening of internodes. Diseased vine produce short spikes with poor filling leading to yield loss (de Silva *et al.,* 2002; Bhat *et al.,* 2005). The primary spread of this virus is through vegetative, by stem cuttings from infected vines for fresh planting (de Silva *et al.,* 2002) which demands the need for the production of virus free mother plant for vegetative propagation. Meristem culture in vitro is proved to be most efficient method for obtaining virus free material from wide range of horticultural crops in case the virus is not in the integrated form in the genome(Helliot *et al.,* 2002) , as the meristematic dome with ½ leaf primordial are usually free of Virus (Kane 2005). Among many varieties, black pepper variety sreekara is most susceptible to this virus. Almost all the plants in this variety harbor the virus. Hence, the objective of this study was to develop a new meristem culture protocol for the rapid production of plantlets and also to check for the presence of virus. The main attention was devoted to standardizing the conditions for growth medium for plantlet production and further mass multiplication *in vitro* as in vitro culturing of black pepper is a challenging due to high amount of phenolic production and vitrification. Since the explant was from virus infected plants the examination of virus free nature of the derived plants were also aimed.

Materials and Methods

Blackpepper variety Sreekara showing viral symptom were collected and maintained in greenhouse in polybags. The shoot tips (0.5cm-0.75cm) from these plants were harvested for meristem excision. The shoot tips about 30-35 numbers were transferred to a conical flask, 2-3 drops of teepol and rinsed with distilled water for 15 min, then treated with 100 ml 0.2% copper oxychloride for 15minutes, washed twice with distilled water. The explants were transferred into sterile conical flask and added with 100 ml 0.1% Bavisitin and treated for 15 min under the laminar flow, rinsed twice with sterile distilled

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water. The explants were surface sterilized with 100 ml 0.1% mercuric chloride for 5 min, rinsed twice with sterile distilled water.

Meristem tips of 2mm size were dissected from the surface sterilized shoot tips and placed in liquid media. The floating medium for meristem extension was prepared with MS (Murashige & Skoog 1962) salts plus GA3 (0.1, 0.5) mgl^{-1}, Kinetin (0.1, 0.5 mgl^{-1}) in combinations + 0.2% sucrose. The petridishes having explants floating in the liquid medium were kept under 25+_1ºC with 16 h photoperiod. The petridishes were given with gentle shaking once in a day manually so as to avoid the accumulation of phenol around the meristems. The extended meristems were transferred to shoot induction media. The media was prepared with half strength basal salts and vitamins of MS with 3 mgl^{-1} BA and 1 mgl^{-1} IAA alone and in combination plus sucrose 30g/l, agar 8g/l. Induced shoots were transferred to shoot development medium containing half strength basal salts and vitamins of MS with 0.5 mgl^{-1} IBA plus sucrose 30g/l, agar 8g/l. Two leaved shoots were transferred ½ strength basal salts and vitamins of WPM (Lloyd & Mccown)+ 3mgl^{-1} BA + 1mgl^{-1} kinetin + Sucrose 30g/l, Charcoal 2g/l, agar 8g/l for rooting and subsequent growth of plants (media standardized in our lab for rooting of blackpepper). Nodes from the meristem plants were excised and transferred to ½ WPM+3 mgl^{-1} BA+1 mgl^{-1} Kinetin for the in vitro multiplication and development of full plant. Statistical design adopted was CRD for selection of liquid media composition.

Results and Discussion

The explants below 2mm resulted in blackening. Among the media assessed for the meristem extension MS with 0.5mg^{-1} kinetin +0.1 mgl^{-1} GA_3, MS with 0.1 mgl^{-1} kinetin +0.5 mgl^{-1} GA_3 supported the meristem extension, while the meristems in other two medium were found to be drying (Table 1). Within the media which supported the extension MS+0.1 mgl^{-1} Kin+0.5 mgl^{-1} GA_3 proved significantly superior to other media (Fig.1). Among the media tested, shoot induction was achieved in medium having ½ MS+3 mgl^{-1}BA +1 mgl^{-1}IAA; the other media did not give induction. The induction was observed as pale basal bulging of the meristem and increase in size. The induced shoots were then transferred to MS+ 0.5 mgl^{-1}IBA for shoot development (Fig. 2, 3). The developed shoot was one per induced shoot, no multiple shoots were formed. The developed shoots when transferred to ½ WPM +3 mgl^{-1} BA+1 mgl^{-1} Kin resulted in direct roots without any callusing phase within a week (Fig. 4). For any micropropagation protocol, successful rooting of micro shoots is a pre-requisite to facilitate their multiplication in mass amount. All the shoots were rooted at 6-7 days all around the base of the shoot (Fig. 5). The further growth of plants as shoot elongation and root development was achieved in the same medium with sub culturing at 10 days interval resulted in 100% plantlet regeneration (Fig 6). In *Piper nigrum* stem portion of shoot tip (2mm) was induced into direct shoots in liquid MS with 1.5 mgl^{-1}BA+ 3uM adenine sulfate, subsequent proliferation in MS+ 1.5 mgl^{-1}BA+ 3mgl^{-1} IBA and rooting in ½ MS+ 1.0 mgl^{-1} NAA (Philip *et al.,* 1992), nodal ring was used to induce direct shoot with B5+ 10uM BA, proliferation in B5+ 0.5uM BA and rooting in B5+ 1.0uM IAA (Bhat *et al.,* 1995). From nodal ring, direct shoot induction (Nazeem *et al.,* 2004) was done by in ½ MS+ 1.0 mgl^{-1} BA, subsequent proliferation in ½ MS+ 1 mgl^{-1} BA+1 mgl^{-1} IAA and rooting in ½ MS +5 mgl^{-1} IBA (Babu *et al.,* 1996). In *Piper colubrinum* that plantlets can be regenerated from stem, leaf and root tissues by direct organogenesis as well as through callus phase on WPM + 3 mgl^{-1} BA + 1 mgl^{-1} Kinetin. From our study 40% regeneration capacity was observed from meristems of 2mm length. After the plants attained 3 leaf stages with 3 internodes, *in vitro* multiplication was done to produce the plants in multiple numbers. The shoots with one inter node were excised and inoculated in the ½ WPM+3 mgl^{-1} BA+1 mgl^{-1} IAA. Within 6 days root initiation started and root development was achieved in all the cultures (Fig. 7, 8, 9). Plantlets with fully expanded leave and well developed roots were successfully established in sterile soil for one month (Fig. 10, 11). The ex-vivo survival rate of the plants after transfer to sand: soil (1:3) in greenhouse was observed to be 100% (Fig. 12) with 4-5 internodes within 60days.

Table 1: Effect of media on meristem extension in blackpepper (*Piper nigrum* L.)

Treatments	Media	Transformed mean
T1	MS + Kin 0.5 mgl^{-1} + GA_3 0.1 mgl^{-1} + 0.2% Sucrose	36.33(40)
T2	MS + Kin 0.1 mgl^{-1} + GA_3 0.5 mgl^{-1} + 0.2% Sucrose	81.62(93.33)
T3	MS + Kin 0.5 mgl^{-1} + GA_3 0.5 mgl^{-1} + 0.2% Sucrose	1.65(0)
T4	MS + Kin 0.1 mgl^{-1} + GA_3 0.1 mgl^{-1} + 0.2% Sucrose	1.65(0)

CD (5%) = 26.71. Original value is described in the paranthesis

Figure 1-3:

Figure 4-6:

Figure 7-9:

Figure 10-12:

From Left to right:

1st row: 1. Fully extended meristem in floating media: 2-Direct shoot induction: 3-Shoot development

2ndrow: 4. Root initiation: 5. Root development: 6-Full grown plants with 2/3 nodes

3rd row: 7-Full plant before *invitro* layering: 8-Invitro layered shoot with developed roots: 9-Invitro layered well grown plants.

4th row: 10. Hardening: 11.Hardened plant: 12. Plants with 4-5 internodes in green house.

Conclusion

Meristem culture from 2mm size from black pepper variety Sreekara achieved through direct organogenesis seems to be very promising and rapid technique with only 66-70 days for *in vitro* micro propagation of black pepper though the plants showed the presence of virus by PCR with virus specific primers. The plants will be screened for absences of virus periodically to test any escape in due course of its growth. The use of floating medium paved the way for reducing phenolic exudation and the after the meristem extension, fungus contamination was found to be nill till the plantlet development, thereby yielding remarkable regeneration of plantlets. The quick induction of roots from the *in vitro* stem cuttings with one node shows the possibility of mass multiplication of plants from meristem derived plants *in vitro* in a rapid manner. Along with this generated protocol application of cryotherapy / chemothrepy would yield virus less plants if the virus is not an integrated virus. Apart from this the developed protocol will be of great importance in rapid mass multiplication of elite germplasm, as well as for conservation of this export oriented medicinally important spice crop.

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