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*Full Length Research Paper*

# Feasible plant regeneration in black pepper from petiole explants

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**A feasible plant regeneration protocol was established for economically important plant, black pepper. Callogenesis was induced from petiole explants of potted plants incubated on murashige and skoog (MS) - medium supplemented with different concentrations of several phytohormones (PGRs). The best callus induction (85%) was observed for MS-medium supplemented with 0.5 mg $l^{-1}$ 6 - benzyladenine (BA) after 04 weeks of culture. Subsequent transfer of callus to MS medium containing similar PGRs induced shoot regeneration. Highest shoot regeneration (92%) was recorded for 0.5 mg $l^{-1}$ BA after 5 weeks of transfer. Furthermore, 8.1 shoots/explant were recorded for 0.5 mg $l^{-1}$ BA. Addition of 2.0 mg $l^{-1}$ of indole-3-butyric acid (IBA) produced 5.1 cm long shoots with 85% shoot organogenesis. Shoots produced healthy plantlets when transferred to MS medium containing several concentrations of indole butyric acid IBA. Regenerated plantlets were transferred to pots for acclimatization.**

**Key words:** *Piper nigrum,* callus, organogenesis, petiole, 6-benzyladenine.

## INTRODUCTION

King of spices, Black pepper (*Piper* (*P.*) *nigrum* L.) is one of the potent members of family Piperaceae (Abbasi et al., 2010), is native to India and mostly cultivated in tropical and sub-tropical regions (Ahmad et al., 2010). *P. nigrum* is widely used in cooking and processing of food and perfumery. The quality of peppercorn can be judged from its pungency contributed by active component, piperine (Philip et al., 1992; Bhat et al., 1995). Piperine is free from microbial contamination and biodeterioration, and preferred in processing of food items (Srinivasan, 2007). The secondary metabolites that exhibit medicinal qualities also contribute in antioxidative defense system of plants. Two important and novel compounds of *P. nigrum* viz., β-caryophyllene and nerolidol are having anaesthetic activity and flavouring, respectively (Santra et al., 2005).

Pipine is another unique volatile compound and an ideal odorant detected in field grown plants of Black pepper (Jayalekshmy et al., 2003). *P. nigrum* has pronounced antimicrobial, antimutagenic and antioxidant effects (Gulcin, 2005; Saxena et al., 2007; Ravindran, 2000). An interesting study has shown effects of piperine on mood and cognitive disorder (Wattanathorn et al., 2008; Bhardwaj et al., 2007).

Medicinal plants are harvested from the wild, which causes habitat destruction leading to a depletion of the irreplaceable genetic diversity. Systematic cultivation of

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medicinal plants has been proposed and adopted quite often as a substitute to those collected from the wild and has several advantages (Arora et al., 2010; Nair and Gupta, 2006). To evade these production issues, advanced methods of gene transfer and protoplast technology may be helpful (Guo et al., 2007; Liu et al., 2006; Sarma and Kalloo, 2004; Kelkar and Krishnamurthy, 1998). In order to obtain elite germplasm with enhanced qualities conventional plant breeding methods have been extensively employed to improve agronomical as well as medicinal traits and molecular marker assisted breeding has been used with substantial returns on investment. The success of these advanced techniques mainly depends on the performance of the *in vitro* propagation systems. Mass multiplication and germplasm conservation has been reported for several elite medicinal plant species including *Piper* (Dominguez et al., 2006; Kelker and Krishnamurthy, 1998; Kelker et al., 1996; Bhat et al., 1995; Ahmad, 2010; Hussain et al., 2011).

Few reports are available for *P. nigrum* on *in vitro* regeneration from somatic embryos and different explants (Ahmad et al., 2010; Nair and Gupta, 2006; Nair and Gupta, 2003; Joseph et al., 1996; Philip et al., 1992). However, no reliable regeneration protocol from pot-grown petiole explants is reported for this elite plant species.

Therefore, the overall objective of the current research was to develop an efficient and reproducible protocol for *in vitro* multiplication of *P. nigrum* through petiole explants.

## MATERIALS AND METHODS

### Explant preparation, media and culture conditions for regeneration

Petioles were collected from 2 months old plantlets of *P. nigrum* maintained inside the greenhouse. Before use, the explants were given a quick rinse in 70% ethyl alcohol for ~ 45 s, then immersion in 0.10% mercuric hypochloride in water for 1 min, followed by several rinses with sterile-distilled water. The aseptic explants were cut and cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with/without BA (0.5, 1.0, 1.5, 2.0 mg $l^{-1}$), 2, 4-D (0.5, 1.0, 1.5, 2.0 mg $l^{-1}$) or IBA (0.5, 1.0, 1.5, 2.0 mg $l^{-1}$). The phases of shoot induction shoot elongation and rooting were sub-cultured/carried out in similar medium. Data on response of explants, number of shoots per explants were collected after day - 15 and 40, respectively, and regenerated shoots were excised and sub-cultured on similar medium after day - 40. Data regarding root organogenesis was recorded after 20 days of sub-culture. The rooted plantlets were transferred to potting soil mixture and maintained under controlled conditions.

All cultures were maintained in controlled environmental conditions with a 16/8 photoperiod under cool fluorescent light (~ 50 µmol $m^{-2}$ $s^{-1}$). The design of all experiments was a complete randomized block, and each experiment consisted of 6 explants per culture flask and 05 replicate culture flasks per treatment.

For statistical analyses, all the experiments were repeated once, and ANOVA and DMRT were used for data analyses.

## RESULTS AND DISCUSSION

The overall objective of the current study was to develop a feasible and reliable regeneration protocol for black pepper. Traditional approaches to plant production have been applied in some species of *Piper* (Nair and Gupta, 2006; Philip et al., 1992; Bhat et al., 1995; Madhusudhanan and Rahiman, 2000), but endogenous microbial contamination causing severe impede to establishment of *in vitro* aseptic cultures (Bhat et al., 1995). To overcome such issues, the explants were successfully decontaminated by treating with ethyl alcohol and 0.1% mercuric chloride and no contamination was observed subsequently (Kelkar et al., 1996). Similar protocol for decontamination of explants was successfully used for other plants by Makunga et al. (2003).

This regeneration protocol is efficient and feasible for continuous production of black pepper*;* containing biologically active compounds at a comparable levels to green house grown plants (Ahmad et al., 2010).

### Callus induction

Several PGRs were applied to evaluate their efficiency on induction of organogenesis (Figures 1 to 4). Callus induction was observed for all of PGRs tested (Figure 1). Highest explants response was observed for murashige and skoog (MS) medium containing 0.5 mg $l^{-1}$ BA (85%). Callus induction recorded for benzyladenine (BA) at lower concentrations was significantly higher than other PGRs, and no callus was observed on MS0, however application of indole butyric acid (IBA) and 2, 4-D induced callus but at lower levels (Figure 1). In previous reports Sukhumpinij et al. (2010) also observed the effect of BA on callogenesis in leaf explants of *Piper rapaceum* L.

Findings of Ahmad et al. (2010) are in agreement with our data. BA has been shown optimum response in callus induction in many elite medicinal plant species (Abbasi et al., 2010). However, average callus induction in petiole explants was considerably higher in all concentrations of PGRs exploited than leaf and shoot explants. Conversely, Kelker and Krishnamurthy (1998) observed concentration dependent effects of BA on different petiole explants of *Piper colubrinum*.

### Shoot organogenesis

Percentage frequency of shoot formation was recorded after day - 40 of culture. Nil shooting response was observed at MS0, 2 and 4-D. However, approximately 92% shooting response was recorded for 0.5 mg $l^{-1}$ of BA (Figure 3). Similar concentration of BA was also reported for many plant species (Nayak et al., 2009; Tiwari and Tuli, 2009; Rangsaytorn, 2009). Shooting response was inversely related to different concentrations of BA and

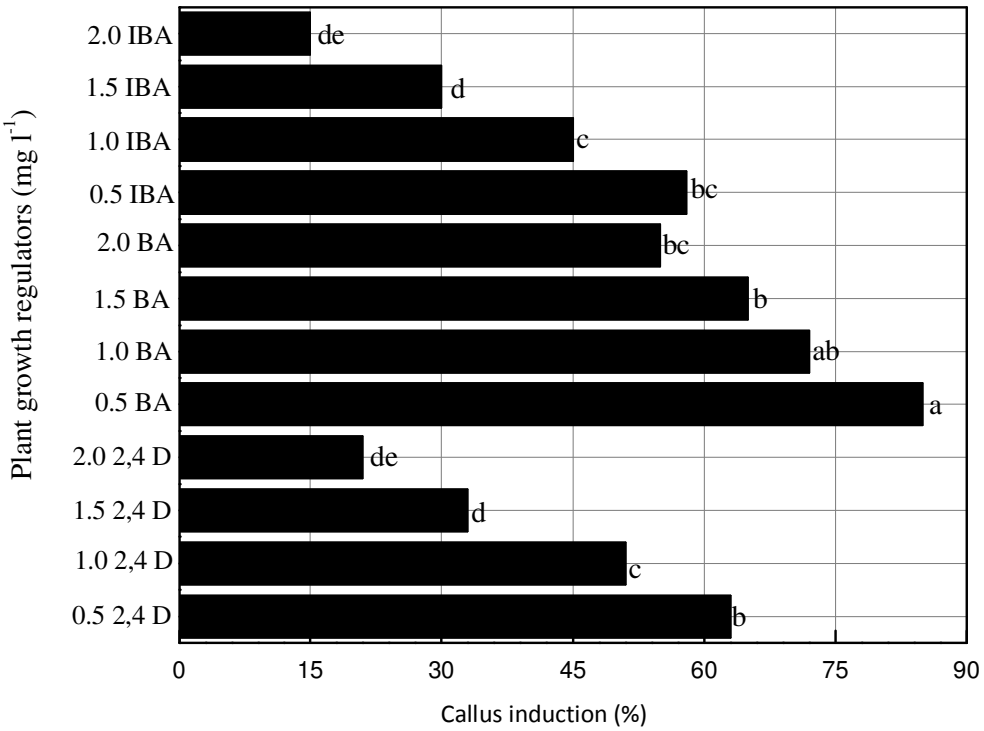


**Figure 1.** Effects of various concentrations of 2, 4 - D, BA and IBA on percent callus induction in *P. nigru.* Data were collected after 4 weeks of culture. Values are means of 5 replicates columns with common letters are not significantly different at P<0.05.

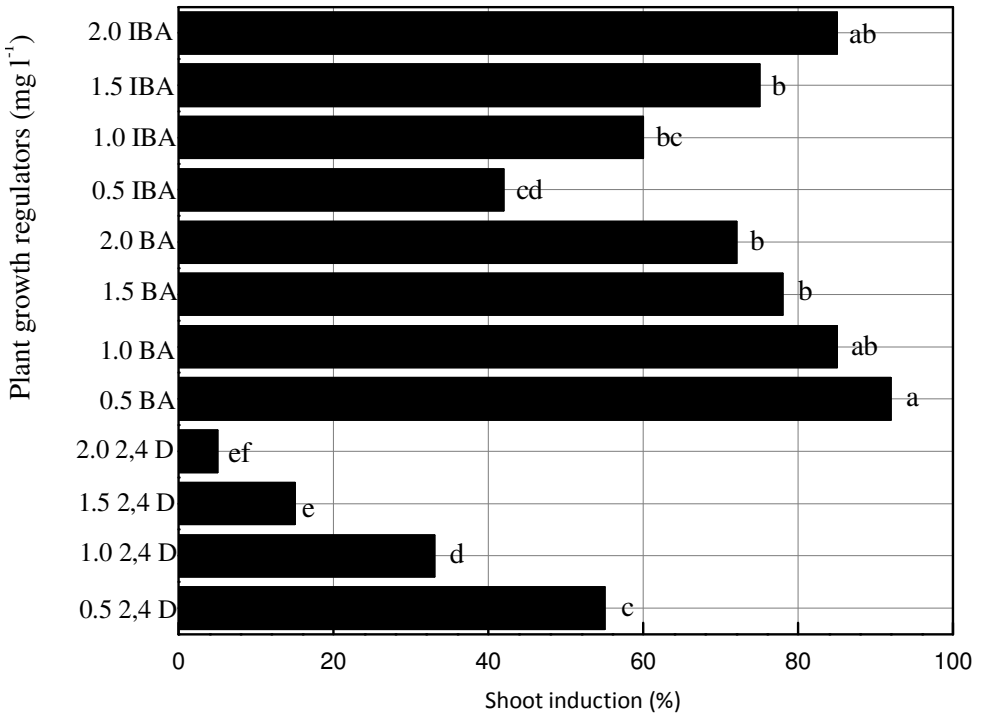


**Figure 2.** Effects of various concentrations of 2, 4-D, BA and IBA on percent shooting in *P. nigrum*. Data were collected after 5 weeks of sub-culture, values are means of 5 replicates, columns with common letters are not significantly different at P<0.05.

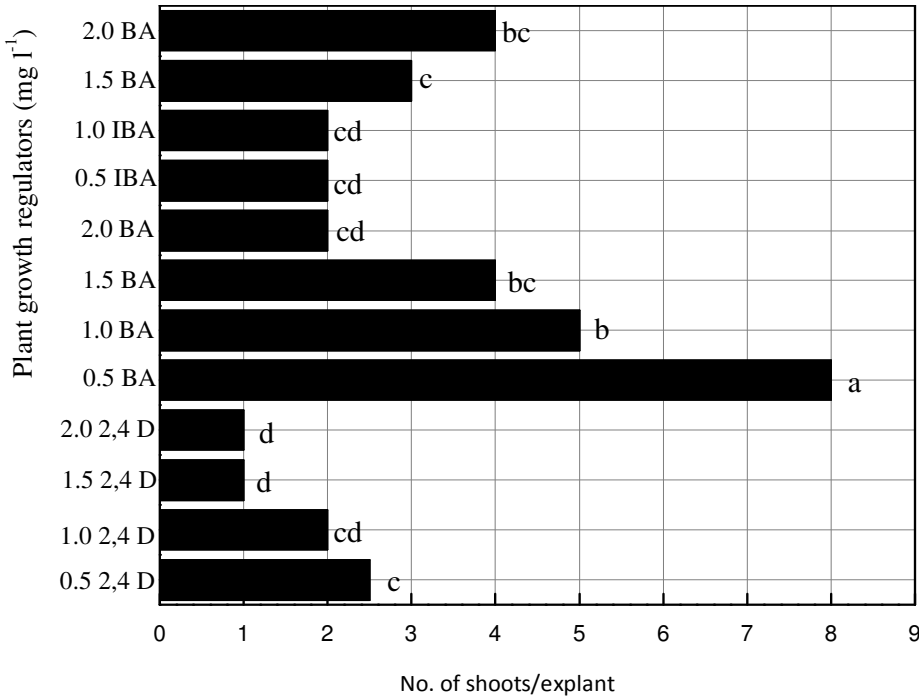


**Figure 3.** Effects of various concentrations of 2, 4 - D, BA and IBA on number of shoots per explant in *P. nigrum*. Data were collected after 5 weeks of sub-culture to MS media with similar composition of plant growth regulators. Mean values in each column with common letters are not significantly different at *P<0.05*.

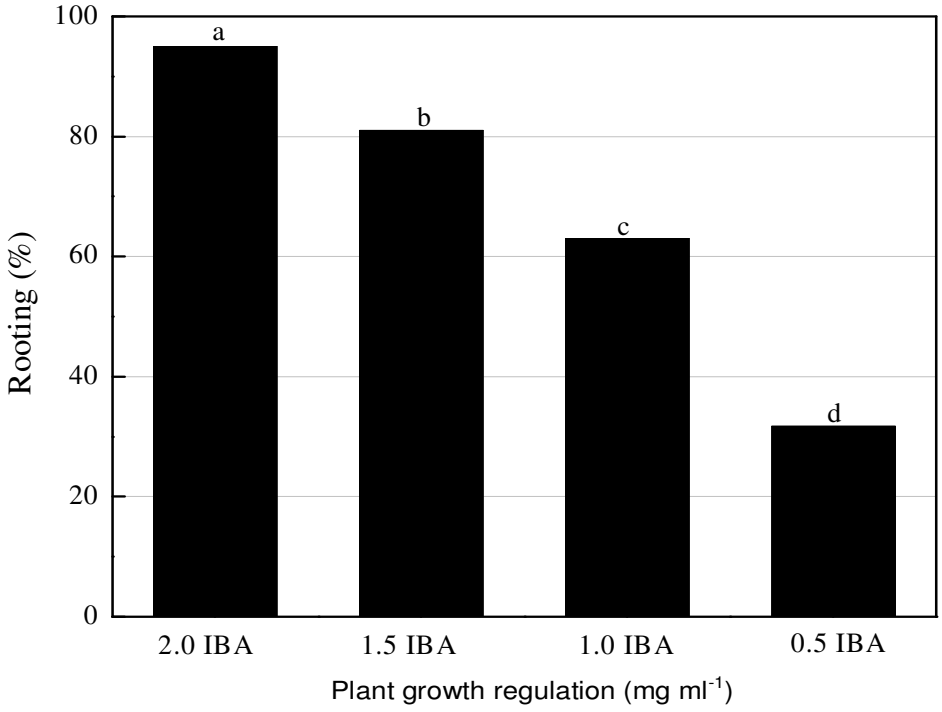


**Figure 4.** Effects of various concentrations of 2, 4 - D, BA and IBA on percent rooting in *P. nigrum*. Data were collected after 5 weeks of sub-culture. Mean values in each column with common letters are not significantly different at *P<0.05*.

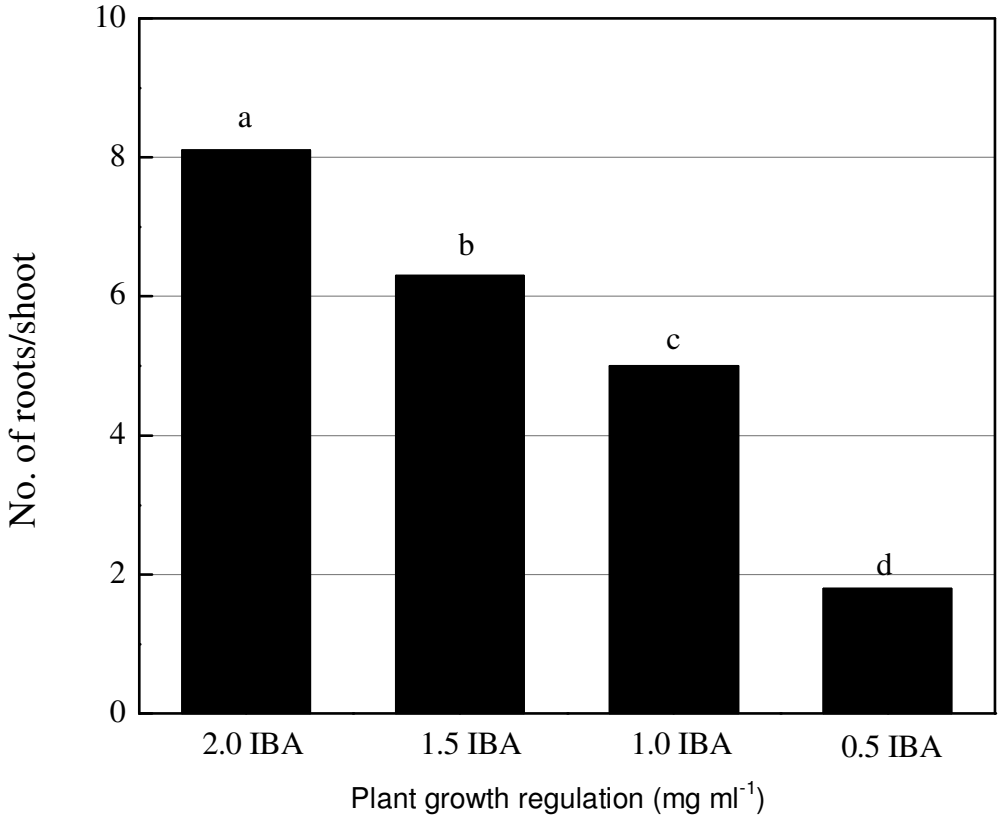


**Figure 5.** Effects of various concentrations of IBA on nunmber of roots/shoot in *Piper nigrum*. Data were collected after 5 weeks of sub-culture. Mean values in each column with common letters are not significantly different at *P<0.05*.

IBA. This data showed the frequency of shooting on different media with or without PGRs, but irrespective of the explant types. This data was similar to the findings of Kelkar and Krishnamurthy (1998), who concluded from their work on *P. colubrinum* that BA markedly affected the efficiency of regeneration. However, Bhat et al. (1995) also concluded that BA was more effective than kinetin for different *Piper* spp. Data for number of shoots per explant showed that up to 8.1 shoots per petiole explants was recorded at 0.5 mg $l^{-1}$ of BA (Figure 4). Tilkat et al. (2009) and Zheng et al. (2009) also reported that BA considerably increase shoot per explant in other plant species. Current results are in agreement with Philip et al. (1992), who found higher concentration of BA to be optimum for shoot regeneration in *P. nigrum*. However, number of shoots per explant remains lower at all concentrations of IBA than BA. Overall, the response of petiole explant was better than leaf and shoot on different concentrations of BA and IBA than combination of BA, 2 and 4-D, however lower concentrations of BA and higher concentration of IBA induced more shoots in all explant types and vice versa (Figure 4). Similar concentration dependent effect of BA was also reported for other species of *Piper* (Bhat et al., 1995).

## Root organogenesis and acclimatization

Healthy shoots were transferred to MS medium containing several concentrations of IBA for rooting (Figure 4). Highest (95%) rooting, maximum number (8.1) of roots/shoot was recorded for 2.0 mg $l^{-1}$ IBA (Figure 4 to 5). Healthy rooted plantlets were transferred to potted soil for acclimatization. Direct relationship between rooting and auxin concentration has been previously reported for other plant species (Tilkat et al., 2009; Ahmad et al., 2010).

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