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IN VITRO REGENERATION OF *PIPER NIGRUM* L.

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Abstract

Direct plant regeneration protocol was developed for an important spice plant *Piper nigrum* L. Shoot regeneration was induced from nodal explants on MS medium supplemented with different plant growth regulators. The best response towards multiple shoot formation was obtained on MS with 1.0 mg/l of BAP and 1.0 mg/l IAA (60%) where mean number of shoot per explant was 4. Healthy roots developed from the base of the 80% shoots cultured on half strength of MS medium containing 1.5 mg/l IBA. The plantlets were successfully transplanted and acclimatized in soil. Ninety per cent transplanted plantlets survived after transplantation in soil.

Piper nigrum L. commonly known as black pepper cultivated for its fruit, which is usually dried and used as a spice and seasoning (Joseph *et al.*1996), considered as the 'King of Spices' due to its trade in the international market (Srinivasan 2007, Mathew *et al*. 2001). In Bangladesh, it is mostly cultivated in Moulvibazar, Sylhet and used as a spice as well as to produce herbal medicine (Yusuf *et al.* 2009).

Black pepper is, conventionally propagated through cuttings with 2 - 6 nodes for nursery and field plantations. Among the major weakness responsible for low productivity of black pepper, non-availability of healthy planting materials and crop losses due to biotic and abiotic stresses are of foremost importance (Sharma and Kalloo 2004). In this context, plant tissue culture is the most efficient and reliable method for rapid and mass scale production of disease free, genetically stable and identical progeny of black pepper throughout the year (Hu and Wang 1983). Considering these facts, the objective of the present study was aimed at developing a protocol for *in vitro* propagation of *Piper nigrum* L. for mass scale exploitation in Bangladesh.

Black pepper plants were collected from Bangladesh Forestry Department, Moulvibazar, Sylhet and then planted in BCSIR experimental field. The explants such as nodes and shoot tips were collected from healthy plants. Explants were sterilized following the protocol described by Khan *et al.* (2016) and cultured on MS (Murashig and Skoog 1962) solidified agar medium supplemented with various concentrations of plant growth regulators. All media contained 3% sucrose and with 9.5 g/l agar with pH 5.8 adjusted before autoclaving. All *in vitro* grown cultures were maintained under illumination on a 16 hrs photoperiod at 25 ± 2^{o}C. Regenerated shoots (3.5 - 4.5 cm long) were excised and transferred to half strength of MS medium supplemented with different concentrations of IBA for induction of roots. Rooted plantlets were successfully transferred to soil.

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A series of experiments were conducted to induce shoot regeneration (Table 1). Several workers have reported multiple shoot induction with only cytokinins in the growth medium (Hiregoudar *et al*. 2003, Hossain *et al.* 1994, Shailendra *et al.* 2005 MS with BAP and IAA showed best multiple shoot regeneration from nodal explants (Table 1).

Table 1. Effect of different growth regulator compositions with MS medium for *in vitro* shoot regeneration of *Piper nigrum*.

Growth regulators supplemented medium (mg/l)			% of responsive explants	Days required for shoot regeneration	Mean number of shoots per explants
BAP	Kn	IAA			
0.5	-	-	33.3	23 - 32	1.0
1.0	-	-	40	22 - 28	1.6
1.5	-	-	60	18 - 24	2.25
2.0	-	-	46.6	20 - 30	2.00
2.5	-	-	40	25 - 35	1.0
1.0	0.5	-	40.00	23 - 32	2.0
1.0	1.0	-	33.33	25 - 32	2.2
2.0	0.5	-	60.00	18 - 30	2.8
2.0	1.0	-	73.33	15 - 25	4.0
1.0	-	0.5	60.0	15 - 26	2.9
1.0	**-**	**1.0**	**80.00**	**10 - 20**	**4.0**
2.0	-	0.5	66.6	15 - 22	2.8
2.0	-	1.0	60	16 - 24	3.3

Approximately 80% shooting response were recorded in MS + 1.0 mg/l BAP + 1.0 mg/l IAA (Table 1). In BAP and IAA supplemented medium 10 - 20 days were required for shoot initiation (Fig. a) and in same medium combination multiple shoot formation and elongation occurred (Fig. b&c). When explants were cultured on BAP and Kn supplemented medium 15 - 25 days were required for shoot initiation (Fig. d) and multiple shoot formed within two to three subculture (Fig. e, f). In this combination mean number of shoots/explants were 4.0 after 60 days of culture (Table 1). While traditional method gave only 64.24% of success after 90 days of cutting (Bhuyan *et al*. 2015). Mathews and Rao (1984) found best result from shoot tip culture of *P. nigrum* on same concentration of BAP and IAA with MS medium which is in conformity with the present investigation. Though the results contradict to Rubluo and Barroso (1992) who reported BA and IAA considerably ineffective in shoot formation.

In the present investigation 2.0 mg/l BAP and 1.0 mg/l Kn showed good regeneration response. Anand and Rao (2000) observed up to 88% response for 4.43 µM BAP and 2.32 µM Kn. However, Bhat *et al.* (1995) also concluded BA was more effective than kinetin for different *Piper* spp. which is contradictory to the present study.

Shoots were transferred to half strength of MS medium supplemented with different concentrations of IBA for root induction (Table 2). The excellent rooting (80%) was obtained when shoots cultured on half strength of MS medium with 1.5 mg/l IBA. Healthy roots were obtained in this medium after 14 - 25 days of culture (Fig. g). In contrast, Philip *et al.* (1992) obtained rooted plantlets through shoot-tip cultures of *P. nigrum* on medium containing 1 mg/l NAA. In a study on another *Piper* species, rooting was achieved on medium containing IAA (Bhat *et al*. 1995). Ahmad *et al.* (2010) found 89% rooting response on 2 mg/l IBA. Chandrasekara *et al.*

2011 reported highest number of roots on 1.0 mg/l IBA in MS medium. Rooted plantlets were successfully transferred to pots (Fig. h) and survival rate was found about 95%. Anand and Rao (2000) reported the use of growth chamber under high humidity conditions for acclimatization of black pepper plants.

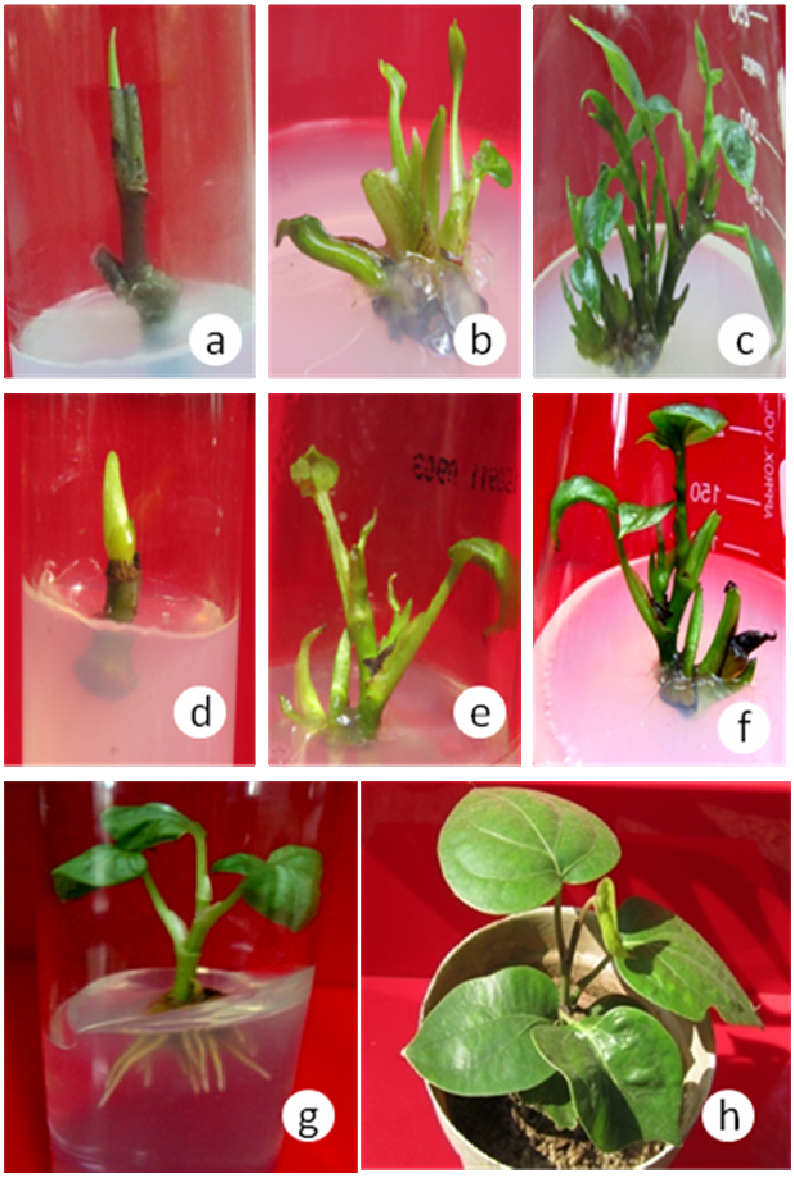

Fig. 1(a - h). Different steps of *in vitro* regeneration of *Piper nigrum:* (a) Shoot formation on MS medium with 1.0 mg/l BAP and 1 mg/l IAA. (b) Multiple shoot formation on same medium mentioned in Fig. a. (c) Multiple shoot elongation on the same medium as in Fig. a, (d, e, f) shoot initiation and multiple shoot formation on MS medium with 2.0 mg/l BAP +1.0 mg/l Kn. (g) *In vitro* root formation on half strength MS medium with 1.5 mg/l IBA. (h) Acclimatization of *in vitro* rooted plants into plastic pot.

Table 2. Effect of different concentrations of IBA on half strength of MS medium for initiation of roots in *Piper nigrum*.

Concentration of IBA	No. of explants inoculated	% of shoots forming roots	Days to initiate roots	Days required to get well developed roots
0.5	15	-	-	-
1.0	15	40	20-25	27-30
1.5	**15**	**80**	**14-25**	**26-30**
2.0	15	75	18-25	27-30
2.5	15	65	20-28	26-32

The *in vitro* regeneration protocol described here is easily reproducible. Moreover, the regeneration of plantlets was achieved without the intervention of callus and this clearly indicated the possibility of obtaining true to type plantlets. The technique described here appears to be readily adaptable for mass propagation of *Piper nigrum*.

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