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In vitro plant regeneration from cotyledon explants of black pepper (*Piper nigrum* L.)

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Abstract

A regeneration method of black pepper (*Piper nigrum* L.) has been developed using cotyledon explants. MS basal medium supplemented with 1.5 mg/l IAA + 0.5 mg/l BA induced the explants to produce green and compact nodular callus. When these calli were transferred to MS medium containing a higher level of BA (1.5 - 3.0 mg/l) with a lower level of IAA or NAA (0.5 mg/l) produced adventitious shoots. Maximum response was achieved on MS medium containing 2.5 mg/l BA and 0.5 mg/l IAA. Regenerated shoots were excised and the best root formation (90%) were achieved in medium with 1.5 mg/l IBA. Rooted plantlets were successfully transferred into the soil.

Keywords: Callus induction, adventitious root, black pepper, *Piper nigrum*, Plant regeneration

Introduction

Black pepper (*Piper nigrum* L.) belongs to the family of Piperaceae and is one of the most important spice crops and has unique position in spice trade. Black pepper is much valued as dried spice and for pepper oil distilled from the fruits for use in perfumery. It is still considered as the king of spices in the world market. The spice is produced from the fruits of this vine. It is a native of the Malabar Coast of south-western India. It has been widely introduced throughout the tropics and the major producers are India, Malaysia, Indonesia and Srilanka (Purseglove *et al.* 1981). It is also produced by the Philippines, Vietnam and Thailand. In recent years pepper production in Brazil has increased considerably (Kumar *et al.* 1986). Black pepper is grown successfully in Bangladesh (Haque and Hossain, 1985) and it is produced in very small quantity which is not recorded in the official statistics (Ahmad, 1985). As the spice is still in great demand in developed countries, it has great potentialities of earning valuable foreign exchange through export.

Sixty-four cultivars have been identified in India. Bangladesh Agricultural Research Institute has released only one variety of black pepper named "Jaintia golmarich" in 1987 in Bangladesh. Improvement of this spice in necessary in order to make it quite good with respect to size and pungencies through plant biotechnological method. The introduction of new characters into black pepper by means of genetic manipulation is of great potential value, especially of the traits that would confer resistance to disease and pests. The usefulness of cell and tissue culture techniques often depends upon the development of an efficient *in vitro* plant regeneration system. However, very little information is available on *in vitro* culture of black pepper (Philip *et al.*, 1992; Rahman *et al.* 2000). This paper report the results of establishment of an efficient protocol for *in vitro* plant regeneration from cotyledon explants of *Piper nigrum* L.

Materials and Methods

Seeds of *Piper nigrum* var "Jaintia golmarich" were collected from Citrus Research Station, Jaintiapur, Sylhet. The seeds were surface sterilized with 5% commercial sodium hypochlorite solution for 15 min. and were thoroughly washed with sterilized distilled water for 4 - 5 times. The seeds were placed in sterile petridishes lined with water soaked filter paper and incubated at 28^{0}C for germination. The cotyledons were excised from 3-day-old germinating seeds and cut transversely into two pieces and were cultured on MS (Murashige and Skoog,1962) medium supplemented with various concentrations and combinations of benzyladenine (BA), α-napthalene acetic acid (NAA) and indole-3-acetic acid (IAA) for callus induction. After two weeks, the calli were sub-cultured on media containing auxin and cytokinin combination for shoot induction. Elongated shoots (>2 cm long) were rooted in MS medium supplemented with IBA and IAA in different concentrations. The pH of the medium was adjusted to 5.7 ± 0.1 before addition of agar (BDH) at 0.8% concentration. After melting, the media were dispensed into the culture tubes and autoclaved for 20 min. The cultures were maintained at 26 ± 1^{0}C with a 16h photoperiod.

Results and Discussion

Preliminary experiments revealed that growth regulators were essential for the induction of callus from cotyledon explants and no callus was induced by basal medium alone. Differential morphogenic responses of cotyledon explants in MS medium supplemented with different concentrations and combinations of cytokinin and auxin were observed and the results are presented in Table 1.

When cotyledon explants were cultured on MS medium supplemented with BA singly or in combination with NAA or IAA, the explants swelled within one week and callus formation started from cut ends within two weeks of incubation. In media containing only BA produced less amount of callus, which was light green in colour. On the other hand, media containing BA in combination with NAA or IAA yielded moderate and abundant amount of callus, respectively, which was green in colour. Maximum callus was obtained in media containing 0.5 mg/l BA + 1.5 mg/l IAA. The callus appeared as hard, compact regions which produced nodular structure within 3 - 4 weeks of culture.

These nodular structure developed into shoot bud when calli were sub-cultured in auxin and cytokinin containing media. But calli cultured in the basal medium without any growth regulator failed to develop any shoot. When calli were sub-cultured to medium with 1.5 - 3.0 mg/l BA, the nodular structures developed into shoot buds at a low frequency (9-12%, data not shown in Table). To increase the frequency of differentiation, various concentration of BA + NAA or BA + IAA were examined (Table 2). Among the combinations used, BA + IAA was more effective for shoot proliferation, with a higher concentration of BA and lower concentration of IAA (Fig.A). The highest percentage of (90%) calli formed shoots in media with 2.5 mg/l BA and 0.5 mg/l IAA, and maximum number of shoots per explants was 18.8, whereas 84.18% calli formed shoots in media with 2.5 mg/l BA and 0.5 mg/l NAA.

Table 1. Effect of growth regulators on callus induction from cotyledon explants of *Piper nigrum*

Growth regulators (mg/l)			Nature and degree of callusing
BA	NAA	IAA	
0	0	0	-S
0.1	0	0	+LG
0.5	0	0	+LG
1.0	0	0	+LG
0.1	1.0	0	+ +GC
0.5	1.0	0	+ +GC
1.0	1.0	0	+ +GC
0.1	1.5	0	+ +GC
0.5	1.5	0	+ +GC
1.0	1.5	0	+ +GC
0.1	0	1.0	+ + +GC
0.5	0	1.0	+ + +GC
1.0	0	1.0	+ + +GC
0.1	0	1.5	+ + +GC
0.5	0	1.5	+ + +GC
1.0	0	1.5	+ + + GC

S: swelling; LG: light green; GC: green compact callus; – : no callus; + : less callus growth; + + : moderate callus growth; + + + : abundant callus growth

Table 2. Effect of growth regulators on shoot proliferation from cotyledon derived calli of *Piper nigrum*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate

Growth regulators (mg/l)			Percent of calli forming shoot	Number of shoot per culture
BA	NAA	IAA		
1.5	0.5	-	58.89	8.4
2.0	0.5	-	65.88	11.8
2.5	0.5	-	84.18	16.4
3.0	0.5	-	76.92	13.2
1.5	-	0.5	62.24	9.2
2.0	-	0.5	73.15	12.3
2.5	-	0.5	90.00	18.8
3.0	-	0.5	79.37	15.2

Using cytokinin only in the medium in later sub-culture could increase shoot production and plantlet formation, and shoots were elongated sufficiently with lower cytokinin concentration. Individual shoots from *in vitro* grown shoot clumps were excised and after trimming of basal leaves, they were transferred to rooting media containing various concentrations of IBA and IAA. IBA was found to be more effective in respect of root induction, and 90% shoots produced roots within 15-20 days of culture in media having 1.5 mg/l IBA and mean number of roots per shoot was 7.8 (Table 3). Rooted plantlets were transferred to polybags. More than 88% plants survived following a method of pretransplanation. *In vitro* regenerated plants showed vigorous and uniform growth (Fig. C) and no morphological abnormalities were observed. The present investigation provides a method that ensures a multiple shoot induction from cotyledon explants of black pepper via intermediate callus phase.

Table 3. Effect of auxins on root formation from *in vitro* grown shoot of *Piper nigrum*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate

Auxin	Concentration (mg/l)	Rooting (%)	Number of roots per shoot
IAA	0.2	—	—
	0.5	14.8	1.4
	1.0	21.4	2.5
	1.5	31.1	2.6
	2.0	26.5	1.6
IBA	0.2	35.2	3.4
	0.5	46.8	4.5
	1.0	63.4	6.6
	1.5	90.0	7.8
	2.0	58.8	5.8

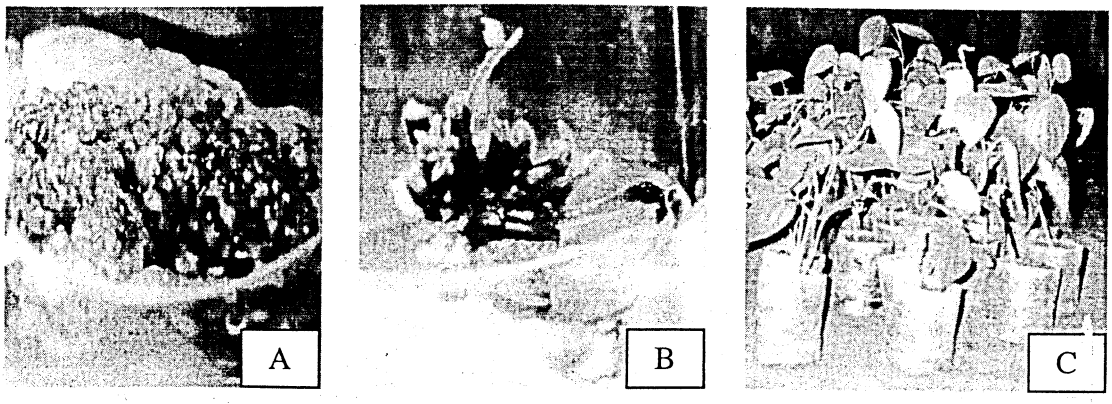

Fig. 1. Plant regeneration in black pepper. A. Induction of shoot buds after eight weeks of culture from cotyledon explant in MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l NAA. B. Proliferation of multiple shoots from *in vitro* grown explants in media MS + 2.5 mg/l BA+0.5 mg/l NAA. C. Potted plantlet eight weeks after transfer.

Plant regeneration from shoot tip (Philip *et al.* 1992), apical shoot and nodal segment (Rahman *et al.* 2000), explants have been reported in black pepper. However, in all these cases the regeneration of plants occurred from shoot primodia present in the explants (Bhat *et al.* 1992).

The findings of the present article report an effective protocol for *in vitro* plant regeneration in black pepper and the protocol may be used for the development of methods for alien gene transfer in black pepper.

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