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Establishment of a suitable regeneration protocol for rapid propagation of *Piper nigrum* L. through *in vitro* culture

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Abstract

An experiment was performed to establish a suitable regeneration protocol for the rapid propagation of *Piper nigrum* L. using nodal segments and leaf tissues as explants through direct and indirect organogenesis. After surface sterilization, several types of explant were inoculated onto gelled MS medium containing various concentrations and combinations of growth regulators for callus and direct shoot induction. The highest callus induction frequency was 92% and 84% in the case of leaf tissues and nodal segments, respectively, in gelled MS medium containing 2.0 mg/l 2,4-D. Multiple shoots (6.43±0.35 shoots per unit callus) were obtained when the calli from both explant types were cultured on MS medium containing 1.0 mg/l BAP. Nodal segments showed the best result (85%) in terms of direct shoot induction in MS medium supplemented with 1.5 mg/l BAP, where the highest number of shoots per explant was 5.03±0.69. The directly induced shoots were multiplied and elongated on MS medium containing 1.0 mg/l BAP and 0.5 mg/l IBA, the number and length of regenerated shoots per culture being 6.07±0.39 and 5.84±0.65 cm, respectively. The best response to root induction (86.67%) was observed when shoots were transferred to ½ strength of gelled MS medium supplemented with 1.5 mg/l IBA within 16-24 days, with 13.60±1.76 roots per shoot unit. The well-rooted shoots were successfully acclimated in a mixture of soil, sand, and compost (1:1:1) with a survival rate of 88%.

Key words: Direct and indirect regeneration, nodal segments, leaf explants, *Piper nigrum* L., *in vitro* culture.

INTRODUCTION

Piper nigrum L., also known as black pepper, is a perennial climbing vine of the Piperaceae family grown primarily for its fruit (Joseph *et al*., 1996). Dubbed the king of spices, it is one of the most famous spices in the world due to its trade in the world market (Srinivasan, 2007). Black pepper is grown as a crop throughout the tropics. With 36% of global production, Vietnam is currently the world's largest exporter and producer of black peppercorns (FAOSTAT, 2019). It is commonly known as ‘Golmorich’ in Bangladesh and is mainly grown in Moulvibazar and Sylhet for use as a spice and to make herbal medicine (Yusuf, 2009).

Due to the alkaloid piperine, black pepper has a distinct aroma and spicy taste. Piperine is found in black pepper fruit in amounts ranging from 2-9% (Gorgani *et al*., 2017). The fruits are used to make black, white, and green peppercorns, which are commonly used as a spice in cooking. It is also used to flavor processed foods, flavor meat products, as a preservative and in perfumery (Damanhouri & Ahmad, 2014). It is widely used to treat

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asthma, colon toxins, chronic indigestion, sinusitis, obesity, constipation, intermittent fever, fever, colic, stomach upset, cold extremities, and diarrhea in various traditional medicine systems (Ravindran, 2000; Ao *et al*., 1998). In addition, studies have shown that it has antibacterial properties (Dorman & Deans, 2000).

Black pepper is traditionally propagated by cutting to 2-6 nodes for field planting (Abbasi *et al*., 2010). It can also be propagated by seed, layering and grafting. However, black pepper is commonly attacked by fungal, bacterial, viral, and mycoplasma pathogens. Internal viral and mycoplasma infections are difficult to manage and spread via vegetative reproduction (Phillip *et al*., 1992). On the other hand, black pepper seed propagation takes a long time and is troublesome (Atal & Banga, 1962). Black pepper seed production is unpredictable, and its viability is retained for only about a week after harvest and is easily lost during storage when moisture levels are depleted (Atal & Banga, 1962; Ravindran *et al*., 2000; Chaudhury & Chandel, 1994). Therefore, conventional propagation techniques are not sufficient to meet the growing need for planting material.

Due to the difficulties with traditional methods, an efficient way to propagate black pepper faster is needed. In this regard, plant tissue culture is the most effective and reliable method to produce year-round disease-free, genetically stable, and identical black pepper progeny (Hu & Wang, 1983). Taking these facts into consideration, the main aim of this study was to establish an efficient protocol for the direct and indirect *in vitro* propagation of *Piper nigrum* L. for the large-scale production of good quality disease-free planting material.

MATERIALS AND METHODS

In the present study, healthy and disease-free plants of *Piper nigrum* L. thicket grown in the Botanical Garden of Jahangirnagar University were used as starting source for explants. Various explants such as nodal segments (2-3 cm) and young leaf tissue (1 cm^2) were used as the experimental materials. For surface sterilization, the explants were rinsed in distilled water with a few drops of liquid detergent for 10-15 minutes after washing under running tap water for 25-30 minutes. They were then thoroughly cleaned three to four times with distilled water to remove any detergent residue. The explants were then exposed to a 0.1-0.2% mercuric chloride ($HgCl_2$) solution in a laminar airflow booth for 1-7 minutes. The explants were rinsed three times with sterile distilled water to get rid of any remaining $HgCl_2$. To avoid the toxic effect of $HgCl_2$ on explant tissue, explants that had come into contact with $HgCl_2$ solution at each cut end were removed by cutting with a sterilized scalpel.

Nodal segments and leaf explants of manageable size were individually inoculated into culture flasks containing MS medium at various concentrations of 2,4-D (0.5-4.0 mg/l) for callus induction and proliferation. Nodal segments were individually inoculated onto MS medium for direct shoot induction with the appropriate concentrations of BAP (1.0-2.5 mg/l) alone or in combination with NAA (0.5 mg/l and 1.0 mg/l). All cultures grown in vitro were maintained at a constant temperature of 24 ±2°C and were illuminated on a

daily schedule of 16 hours light and 8 hours dark. The growth chamber had a light intensity of 3000 lux. For the induction and proliferation of shoots, nodal segments, and leaf explants derived healthy, whitish, friable calli were then minced into small pieces and placed on gelled MS medium containing various concentrations and combinations of cytokinins (BAP and Kn) and auxins (IBA and NAA). In order to increase the number of shoots, both directly and indirectly induced shoots were aseptically removed from the culture vessels and re-cultured on newly prepared medium containing the same or different combinations of hormones.

Regenerated shoots (4-5 cm long) obtained from the multiplication medium were excised aseptically and implanted individually onto newly prepared MS medium containing gelrite gelled ½ strength MS medium containing various concentrations and combinations of auxins (IBA and NAA) for root induction. The freshly transplanted cultures were kept in low light for three days before being kept in the light for root induction. The plantlets with sufficient roots in the test tube were then placed in a soil mixture of garden soil, sand and compost at a ratio of (1:1:1) for the hardening process. To prevent unexpected desiccation, the plants and their pots were then covered with transparent polyethylene bags. Water was sprayed on the inside of the polyethylene bags every eight hours to maintain a high level of humidity around the plantlets. The polyethylene envelopes were gradually perforated to expose the plantlets to their natural environment and were removed after 14 days. Finally, well-established plants were then transferred to the external normal environment.

RESULTS AND DISCUSSION

Various concentrations of BAP (1.0-2.0 mg/l) alone or in conjugation with NAA (0.5-1.0 mg/l) were used to test shoot regeneration efficiency of nodal segments for direct organogenesis. Direct shoot initiation from the nodal segments was observed in most of the media used, with the exception of the medium containing 1.0 mg/l BAP and 1.0 mg/l NAA (Table 1). With this combination, callus was induced instead of shoot formation. Among the media components used, gelled MS medium supplemented with 1.5 mg/l BAP proved to be the best for direct shoot induction (85%), where the shoot number per explant was 5.03±0.69 and the shoot length was 4.77±1.30 cm (Table 1; Figure 1). These results agree with those of Philip *et al*. (1992) in the case of *P. nigrum*; Bhat *et al*. (1995) in the case of *P. longum*, *P. betle* and *P. nigrum*; Ravindran *et al*. (2016) in the case of *P. longum*. The combined effect of BAP and NAA was also tested as part of this study, but yielded less significant results than using BAP alone. While in the case of *P. nigrum* (Khan *et al*., 2017 and Umadevi *et al*., 2015) BAP in combination with IAA is more effective than using BAP alone for direct shoot induction from the nodal explants.

MS medium with various concentrations of 2,4-D (0.5-4.0 mg/l) were tried for callus induction from the cut ends of the node segments and leaf tissues. All concentrations of 2,4-D tested, except 0.5 mg/l, showed variable degrees of callus induction with an optimum of 84% (nodal segments) and 92% (leaf tissue) callus induction frequency on MS medium at 2.0 mg/l 2,4-D (Table 2; Figures 2 and 3). Concentrations below and

above 2.0 mg/l 2,4-D reduced callus formation. Various reports have shown that MS medium containing 2,4-D is most effective for induction of callus and development, such as Subasinghe *et al*. (2004) and Hussain *et al*. (2011) in the case of *P. nigrum*; Delgado-Paredes *et al*. (2013) in the case of *P. cernuum* and *P. aduncum*; Santos *et al*. (2015) in the case of *P. permucronatum*. However, in this study, during the direct shoot induction experiment, it was found that gelled MS medium conjugated with 1.0 mg/l BAP and 1.0 mg/l NAA induced some calli from the nodal segments (Table 2). A similar promoting effect of BAP in combination with NAA or IAA on callus induction was also reported by Ahmad *et al*. (2013) in *P. nigrum* and Sathelly *et al*. (2016) in *P. longum*.

Table 1. Effect of different concentrations of BAP alone or in conjugation with NAA in gelled MS medium on direct shoot induction from *Piper nigrum* L. nodal segments

Plant growth regulators (mg/l)	Responding explants (%)	Number of shoot per explant (Mean ± SE*)	Shoot length (cm) (Mean ± SE*)
BAP			
1.0	45.00	2.26 ± 0.44	2.94 ± 0.86
1.5	85.00	5.03 ± 0.69	4.77 ± 1.30
2.0	64.00	3.51 ± 2.10	2.84 ± 1.73
BAP+NAA			
1.0+0.5	30.00	1.33 ± 0.78	2.05 ± 1.02
1.0+1.0	20.00	Callus formation	-
1.5+0.5	55.00	1.80 ± 0.81	1.92 ± 0.36
1.5+1.0	35.00	1.29 ± 1.57	1.70 ± 0.90
2.0+0.5	72.00	3.94 ± 2.09	4.07 ± 0.50
2.0+1.0	36.00	1.37 ± 1.02	1.86 ± 0.06

Values are means ± SE, (standard error of the mean) obtained from four independent experiments. At least 25 different cultures were maintained for each experiment.

Table 2. Effect of various concentrations of 2,4-D on callus induction from leaf explants and nodal segments of *Piper nigrum* L.

2,4-D (mg/l)	Callus induction frequency (%)		Callogenic response (*)
	Leaf tissue	Nodal segments	
0.5	-	-	-
1.0	52.00	44.00	+
1.5	72.00	64.00	++
2.0	**92.00**	**84.00**	**+++**
2.5	60.00	48.00	+
3.0	44.00	40.00	+
3.5	36.00	44.00	+
4.0	32.00	44.00	+
Nature of callus	Whitish in color and Friable in texture	Creamy in color and Granular in texture	

Callogenic response (*): No response (-); Poor (+); Good (++); Excellent (+++); 25 explants were inoculated in each concentration.

The obtained calli were then isolated and cultured for multiplication on the same medium composition. For indirect shoot induction, the proliferated calli were subcultured onto MS

medium supplemented with BAP (0.5–3.5 mg/l) alone or in combination with NAA (0.5–1.0 mg/l) (Table 3). Among the different BAP concentrations, the maximum percentage of responsive callus (92%) appeared on gelled MS medium containing 1.0 mg/l BAP (Figures 5, 6 and 7). At this concentration, the number of shoots regenerated per culture unit was 6.43±0.35 and the shoot length was 4.62±0.51. This was consistent with the results of Hussain *et al*. (2011) in the case of *P. nigrum*, who showed that shoot regeneration worked very well on MS medium supplemented with 0.5 mg/l BAP. It was also noted that as the BAP concentration increased above 1.0 mg/l, a sharp decrease in the percentage of responding callus was observed (Table 3). Similarly in *P. nigrum*, Hussain *et al*. (2011) and Thuyen *et al*. (2005); and in *P. barberi*, Anand & Rao (2000) found that with higher concentration the rate of organogenesis gradually decreased. As part of this study, the combined effect of BAP (1.0-2.5 mg/l) and NAA (0.5-1.0 mg/l) was also tested, which gives moderately good results when their lower concentrations are used (Table 3). A partially similar promoting effect of auxin, particularly IBA, with BAP on shoot induction of callus was also reported by Thuyen *et al*. (2005) and Ahmad *et al*. (2013) in *P. nigrum*.

Table 3. Effect of various concentrations of BAP alone or in combinations with NAA in gelled MS medium on shoot morphogenesis from induced callus of *Piper nigrum* L.

Plant growth regulators (mg/l)	Responsive callus (%)	Number of regenerated shoots per culture (Mean ± SE*)	Shoot length (cm) (Mean ± SE*)
0	0	-	-
BAP			
0.5	76.00	5.22 ± 1.02	3.45 ± 0.96
1.0	**92.00**	**6.43 ± 0.35**	**4.62 ± 0.51**
1.5	68.00	4.74 ± 0.60	3.38 ± 0.72
2.0	52.00	3.58 ± 0.12	2.88 ± 0.30
2.5	44.00	3.23 ± 1.07	2.56 ± 0.94
3.0	32.00	3.18 ± 0.55	2.31 ± 0.62
3.5	32.00	3.21 ± 0.31	2.06 ± 0.47
BAP+NAA			
1.0+0.5	65.00	3.71 ± 0.84	2.87 ± 0.65
1.0+1.0	45.00	3.23 ± 0.09	2.68 ± 0.52
1.5+0.5	70.00	4.06 ± 0.70	3.12 ± 0.88
1.5+1.0	50.00	3.54 ± 0.31	2.80 ± 0.39
2.0+0.5	55.00	3.62 ± 1.08	2.97 ± 0.75
2.0+1.0	30.00	2.81 ± 0.37	2.33 ± 0.70
2.5+0.5	40.00	2.90 ± 0.64	2.54 ± 0.67
2.5+1.0	20.00	2.55 ± 1.23	2.04 ± 0.92

Values are means ± SE (Standard error of the mean) obtained from four independent experiments. For every experiment, a minimum of 20 different cultures were maintained.

Directly regenerated shoots from the nodal segments were then transferred to MS medium supplemented with various concentrations and combinations of cytokinins (BAP and Kn) and auxins (IBA and NAA) to examine their efficiency in shoot proliferation. The best response in initiating multiple shoots (88%) was obtained in the medium containing 1.0

mg/l BAP and 0.5 mg/l IBA, where the number (6.07±0.39) and length (5.84±0.65 cm) of shoots per culture was maximum (Table 4; Figure 4). These findings confirm the findings of Philip *et al*. (1992) in the case of *P. nigrum*. They found that shoot growth and development was best on media containing BAP and IBA. In the present investigation medium supplemented with only BAP; BAP and Kn; BAP and NAA (Table 4) was found to be less effective in initiating multiple shoots and in their further growth. In the previous reports, BAP in combination with Kn was found to be better for promoting shoot proliferation in *P. betle* (Elahi *et al*. (2017) and in *P. longum* (Ravindran *et al*. (2016 and Sahelly *et al*. (2016) . Moreover, Thuyen *et al*. (2005) in case of *P. nigrum* reported that MS medium supplemented fortified with 5.0 mg/l BAP, 0.5 mg/l IBA and 0.5 mg/l Kn was the best medium for the growth and development of shoot.

Table 4. Effect of various concentrations of BAP alone or in conjugation with Kn in gelled MS medium on shoot multiplication of *Piper nigrum* L.

Plant growth regulators (mg/l)	Culture showed proliferation (%)	Number of regenerated shoots per culture (Mean ± SE*)	Shoot length (cm) (Mean ± SE*)
BAP			
1.0	50.00	3.84 ± 1.30	3.31 ± 0.83
1.5	65.00	4.27 ± 1.04	4.12 ± 0.66
2.0	45.00	3.40 ± 0.76	3.23 ± 0.62
2.5	30.00	2.00 ± 0.77	2.68 ± 0.80
BAP+Kn			
1.0+1.0	60.00	3.88 ± 2.02	3.56 ± 1.57
1.0+2.0	35.00	2.72 ± 0.55	3.13 ± 1.07
1.5+1.0	70.00	3.96 ± 1.90	3.68 ± 1.35
1.5+2.0	30.00	2.58 ± 0.50	2.79 ± 0.83
2.0+1.0	75.00	4.62 ± 0.83	4.90 ± 0.54
2.0+2.0	40.00	2.24 ± 0.36	2.65 ± 0.73
2.5+1.0	60.00	4.13 ± 2.03	4.08 ± 1.94
2.5+2.0	25.00	2.17 ± 0.60	2.50 ± 0.75
BAP+NAA			
1.0+0.2	52.00	3.76 ± 0.07	3.25 ± 0.12
1.0+0.5	64.00	4.58 ± 0.64	4.33 ± 0.90
2.0+0.2	48.00	3.61 ± 1.44	3.51 ± 0.80
2.0+0.5	56.00	3.74 ± 0.40	3.65 ± 0.79
BAP+IBA			
1.0+0.2	72.00	4.00 ± 0.56	4.34 ± 0.78
1.0+0.5	**88.00**	**6.07 ± 0.39**	**5.84 ± 0.65**
2.0+0.2	60.00	3.68 ± 0.28	3.89 ± 0.31
2.0+0.5	64.00	4.17 ± 0.62	3.96 ± 0.87

Values are means (± SE, Standard error of the mean) obtained from four independent experiments. For every experiment, a minimum of 20 different cultures were maintained.

The regenerated shoots were removed and placed in ½ strength MS medium containing various concentrations and combinations of auxins (IBA and NAA) for rooting. Roots

were initiated within 16-30 days of subculture for all treatments. However, MS medium containing 1.5 mg/l IBA showed the optimal response to root induction (Figure 8). At this concentration, 86.67% of the explants responded to root initiation within 16-24 days and the mean number of roots per shoot unit was recorded as 13.60±1.76 (Table 5). These results are consistent with those of Khan *et al*. (2017) and Hussain *et al*. (2011) who reported healthy root development from the base of 80-100% shoots cultured on ½ strength of MS medium supplemented with 1.5 mg/l IBA in case of *P. nigrum*.

Table 5. Effect of various concentrations and combinations of auxins in ½ strength of gelled MS medium on rooting of *in vitro* regenerated shoots of *Piper nigrum* L.

Auxins (mg/l)	Rooting (%)	Number of roots per unit shoot (Mean ± SE*)	Days to initiate roots	Root regeneration response (*)
IBA				
0.5	45.00	5.33 ± 2.37	16-26	+
1.0	56.00	7.40 ± 0.50	16-25	++
1.5	**86.67**	**13.60 ± 1.76**	**16-24**	**+++**
2.0	52.00	8.12 ± 1.54	16-24	++
NAA				
0.5	60.00	5.86 ± 0.66	18-26	+
1.0	76.00	10.06 ± 0.53	18-25	+++
1.5	43.33	6.44 ± 1.09	18-26	+
2.0	32.00	4.65 ± 0.72	18-25	+
IBA+NAA				
0.5+0.5	30.00	4.48 ± 0.77	18-28	+
0.5+1.0	35.00	4.63 ± 0.94	18-28	+
1.0+0.5	36.00	4.71 ± 0.97	18-28	+
1.0+1.0	32.00	4.85 ± 0.58	18-26	+
1.5+0.5	56.67	7.49 ± 0.29	16-28	++
1.5+1.0	43.33	6.17 ± 1.30	16-28	+
2.0+0.5	24.00	4.70 ± 2.11	18-28	+
2.0+1.0	32.00	4.85 ± 0.36	18-30	+

Root regeneration response (*): very poor (+); moderately good (++); satisfactory (+++); Values are means (± SE, Standard error of the mean) obtained from four independent experiments. For every experiment, a minimum of 24 different cultures were maintained.

After sufficient root development, the plantlets were removed from the culture medium and placed in pots containing soil, sand and compost in a 1:1:1 ratio (Figure 9). Three weeks after transplantation, when the regenerated plants were well established in the soil, they were transferred to larger pots for additional growth and development. Plantlets had a survival rate of 88%. Similarly, maximum survivability using the same soil mix was determined by Anand & Rao, (2000) in *P. barberi*; Hussain *et al*. (2011) and Khan *et al*., 2017 in *P. nigrum*; Ravindran *et al*. (2016) in *P. longum* and Elahi *et al*. (2017) in *P. betle*.

Figs. 1-9: *In vitro* regeneration of *P. nigrum* L. 1. Direct shoot induction after 30 days of inoculation in MS medium supplemented with 1.5 mg/l BAP. 2-3. Induction of callus from (2) nodal segment and (3) leaf tissue after 30 days of inoculation in MS medium supplemented with 2.0 mg/l 2,4-D. 4. Multiplication of nodal segments derived shoot in MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l IBA after 20 days of 2nd sub-culture. 5-7. Whitish friable callus turned into (5) deep green color within 15 days, (6) induce multiple shoot buds after 30 days and (7) produce multiple shoots after 45-50 days of inoculation in MS medium supplemented with 1.0 mg/l BAP. 8. *In vitro* root induction on half-strength of MS medium supplemented with 1.5 mg/l IBA after 25 days of culture. 9. Acclimatization of the regenerated plantlets in poly bags containing soil, sand and compost (1:1:1)

The current study can be used as a low-cost approach to black pepper (*Piper nigrum* L.) tissue culture by nodal segment and leaf tissue culture. The technique can be used for the commercial production and conservation of this export oriented important spice.

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