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IN VITRO REGENERATION OF BLACK PEPPER (*Piper nigrum* L.)

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Abstract

This investigation was done to determine how various plant growth regulators such as benzyl adenine (BA) and indole-3-butyric acid (IBA) affected the *in vitro* plant regeneration of the black pepper plant. The largest number of shoots was produced when the shoot tips and nodal segments of black pepper were utilized as explants and inoculated in Murashige Skoog (MS) media supplemented with 1.5 mg/L BA. The treatment with 2.0 mg/L BA at 8 weeks after induction (WAI) produced the most leaves (3.2). In the 2.0 mg/L treatment of IBA, the greatest number of roots was regenerated. The treatment 2.0 mg/L BA + 2.0 mg/L IBA produced the highest results for shoot induction, shoot length, and overall leaf production when the effects of both hormones were combined. The same treatment also produced the highest proportion of root induction and longest roots. Regenerated plantlets survive at a 46.7% rate in a shaded building and at a 57.1% rate in the open air under bright sunlight. In order to produce black pepper on a big scale, an effective approach for *in vitro* regeneration of black pepper has been established.

Keywords: Benzyl adenine, Indole-3-butyric acid, *In vitro* regeneration

Introduction

A flowering plant in the Piperaceae family known as black pepper (*Piper nigrum* L.) is grown for its fruit, sometimes known as a peppercorn. It is mostly used as a spice and seasoning after it is dried. The fruit has a solitary seed and measures approximately 5 mm (0.20 inch) in diameter when it is fresh and fully grown. Black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit), and white pepper (ripe fruit seeds) are the more accurate names for peppercorns and the ground pepper that is made from them (Harrison and Paul, 2016). Old English "pipor," Latin "piper," and Sanskrit "pippali," which means "long pepper," are the origins of the word pepper. People started referring to the unrelated new world chili pepper (genus *Capsicum*) when they used the word pepper in the 16th century. It is primarily referred to as "Golmorich" in Bangladesh. It is indigenous to the West Indies, South America, Indonesia, Malaysia, and India. However, it is also frequently grown in tropical areas. The 'King of Spices' is the name given to it (Srinivasan, 2007; Mathew *et al.*, 2001). Since ancient times, peppercorns have been ground, dried, and fried for flavor and as a traditional remedy. One of the most

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popular spices used in cuisines worldwide and one of the most traded spices in the world is black pepper. It is spicy because of the chemical molecule piperine, which differs from the capsaicin found in chili peppers in terms of heat. The scavenging of superoxide anion, hydrogen peroxide, and nitric oxide are just a few of the reactive oxygen and nitrogen species that it has been shown in studies to have antioxidant effects against. It has also been found that antioxidant enzymes improve *in vivo*. Through several pathways such as cytotoxicity, apoptosis, autophagy, and interference, *Piper nigrum* also demonstrated anticancer effects against numerous cell lines from the breast, colon, cervical, and prostate. It is the excellent source of manganese, iron, vitamin K, and good source of dietary fiber. In various areas of Bangladesh, particularly in hilly terrain, black pepper is grown. However, there are no statistics accessible in our nation on the area and black pepper production. Black pepper is used extensively in our nation each year for both culinary and medical purposes. The majority of them are imports. Cuttings, layering, and grafting are all methods for multiplying black pepper. The creation of recombinants during seed propagation frequently leads to genetic variety, but other techniques of propagating black pepper are slow and time-consuming. Therefore, it is necessary to develop effective techniques for the quick spread of black pepper. The fastest and most dependable approach for producing disease-free, genetically stable, and identical children under this situation is plant tissue culture (Hussain *et al.,* 2011). The enhancement of black pepper plants, the preservation of germplasm, and clonal propagation have all benefited greatly from tissue culture techniques (Sajc et al., 2000). If a method could be developed, it would enable quick clonal replication of planting materials in Bangladesh in a relatively short amount of time. Plants create signal molecules called phytohormones, or plant hormones, which are found in incredibly tiny amounts. All aspects of plant growth and development, including embryogenesis (Méndez Hernández *et al.,* 2019), the control of organ size, pathogen defence (Shigenaga *et al.,* 2016; Burger *et al.,* 2019), stress tolerance (Ku *et al.,* 2018; Ullah *et al.,* 2018), and reproductive development (Pierre-Jerome *et al.,* 2018) are governed by plant hormones. Depending on their chemical compositions, different hormones can be grouped into various classes. Each class of hormones can have a variety of chemical configurations, but they all share a common set of physiological functions. Abscisic acid, auxins, cytokinins, ethylene, and gibberellins were the first five major families of plant hormones to be identified through research (Thomas et al., 1979). Compounds called auxins have a favorable impact on cell growth, bud development, and root initiation. To promote root growth, auxins, particularly indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA), are frequently used. A class of substances known as cytokinins affect how cells divide and how shoots form. Taking into account the aforementioned facts, the current inquiry has been carried out to determine the effectiveness of various hormones with the ideal concentration of BA and IBA for *in vitro* testing.

Materials and Methods

Piper nigrum (black pepper) planting supplies were gathered from a variety of nurseries in Agargaon, Sher-e-Bangla Nagar, Dhaka 1207. In the current study, experimental materials included nodal segments and shoot tips. Black pepper plants were harvested for their shoots with young leaves. In order to use the shoot as an explant, the

superfluous leaves were taken off and the shoot was pruned to a size of 1-2 cm. The nodal segments and shoot tips of the explants were thoroughly cleaned under running water. Trix is soaked in with the explants for ten minutes. then repeatedly rinsed with diluted water. Explants were then submerged for 20 minutes in a 100 mg/L ascorbic acid solution. In the Laminar Air Flow Cabinet, the remaining tasks were completed. The explants were taken out of that solution after 20 minutes and repeatedly rinsed with distilled water then sterilized for 1 minute with 70% ethanol, followed by 1 minute of distilled water washing. Once more, the explants were sterilized for 5 minutes with a 0.2–0.5% $HgCl_2$ and Tween 20 solution. The explants were then cleaned at least three times with distilled water. The explants' final sizes ranged from 0.5 to 1 cm. The explants were finally prepared for careful insertion into the culture vessel.

Surface sterilized nodal explants (2 to 3 cm) were individually inoculated on liquid MS media (Fig. a) supplemented with various doses of BA (BA (0.5, 1.0, 1.5, and 2.0 mg/L) and control (0.0 mg/L)). For direct shoot induction, four levels of IBA (1.0, 1.5, 2.0, and 2.5 mg/L) were practiced with each level of BA (1.0, 1.5, 2.0, and 2.5 mg/L). Extracted induced axillary shoots were grown for root induction on four concentrations of IBA (0.5, 1.0, 1.5, and 2.0 mg/L) and control (0.0 mg/L) in an aseptic environment. The culture vials with fully formed plantlets were moved to normal room temperature after 2.5 months. The rooted plantlets were taken out of the culture vials the following two to three days, and the medium that was still attached to the roots was carefully rinsed away with tap water. Individual plantlets were transplanted into plastic containers filled with a 1:1:1 mixture of soil, sand, and cow dung. For seven days following transplantation, a moist, clear poly bag was placed over the plants and pot to avoid desiccation. The plantlets were housed in a shade house for 12 days to lessen unexpected shock. Plantlets were then moved to the field after 12 days.

Results and Discussions

Significant variations were observed among different concentrations of BA on days to shoot induction. Minimum 9.4 days were required in the treatment 1.5 mg/L BA (Fig. b). Legesse *et al.,* 2017; found the lowest response of number of shoots in BA 2.0 mg/L while the highest response was observed in medium supplemented with BA 5.0 mg/L in black pepper. The highest number of shoot was obtained (1.8, 2.6 and 3) at 3 WAI, 5 WAI and 8 WAI respectively at 1.5 mg/L (Table 1) Whereas shoot regeneration was not observed in control treatment at 3 WAI, 5 WAI but very incipient shoot found at 8 WAI. Soniya and Das (2002) reported that maximum number of shoot was recorded on MS medium supplemented in 2mg/L BA for *Piper nigrum*. It partially contradicts with the result. Legesse *et al.,* 2017; reported that 4mg/L BA was best for shoot proliferation of *Piper nigrum*. Shoot regeneration response is less in Black pepper. It might be the genetical ability of the spices crop. The treatment BA 2.0 mg/L (Fig. c) gave the maximum number of leaves (1.8, 2.0 and 3.2) at 3 WAI, 5 WAI and 8 WAI, respectively. No shoot was produced in controlled treatment at 3 WAI and 5 WAI. Lowest number of leaves (1.2) was found at control treatment at 8 WAI (Table 2).

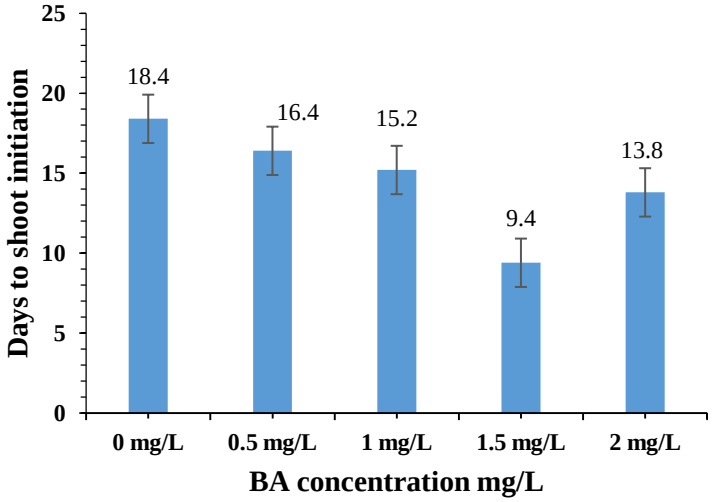


Fig. 1. Effects of BA on days to shoot induction in Black pepper

Table 1. Effects of different concentration of BA on number of shoot at different weeks after induction

(WAI)

BA mg/L	Number of shoot per plant		
	3 WAI	5 WAI	8 WAI
0	0 c	0 d	1 c
0.5	1b	1.2 cd	2 b
1.0	1b	1.6 bc	2 b
1.5	1.8 a	2.6 a	3 a
2.0	1.2 b	2 b	2 b
CV percent	28.28	27.03	15.81
LSD (0.05)	0.4	0.5	1

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

Table 2. Effects of different concentration of BA on number of leaf at weeks after induction (WAI)

BAmg/L	Number of leaf		
	3 WAI	5 WAI	8WAI
0	0 b	0b	1.2 c
0.5	1.0b	1.2 b	1.8b
1.0	1.0b	1.2b	2.2b
1.5	1.2 b	1.2 b	2.2b
2.0	1.8 a	2.0 a	3.2a
CV%	28.28	30.93	21.09
LSD(0.05)	0.4	0.5	0.6

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

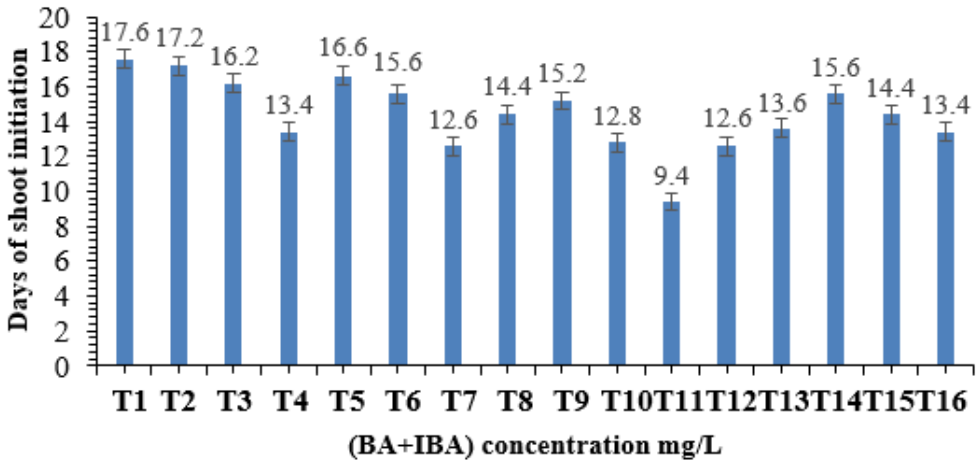
Legesse *et al.* (2017) reported the maximum number of leaves (3.16) on BA concentration on 3mg/L of BA, but the minimum number of leaves (1.62) was recorded on controlled treatment in Black pepper. Increasing the amount of cytokinins like BA up to 5 mg/L gave as the maximum number of leaves per shoot in *Piper nigrum* as reported by Soniya and Das (2002) which is partially similar with the result. There was significant influence of different concentrations of IBA on the number of roots per shoot. The treatment 2.0 mg/L (Fig. d) gave the highest number of root (2.0, 3.0 and 4.0) at 3 WAI, 5 WAI and 8 WAI (Table 3). Furthermore, indicated that IBA interacted significantly with the culture medium and the materials, having a strong influence for plantlet rooting. Significant variations were observed among the combined effect of different concentrations of BA and IBA on days to shoot induction. The minimum duration 9.4 days was obtained in BA 2.0 mg/L+ IBA 2.0 mg/L than rest of the treatments.

Table 3. Effect of different concentration of IBA on number of root at different weeks after induction

(WAI)

IBAmg/L	Number of roots per shoot		
	3WAI	5WAI	8WAI
0.5	1.2bc	1.8bc	2.2c
1.0	1.0 c	2.0bc	2.0 c
1.5	1.6 ab	2.2 b	3.0 b
2.0	2.0 a	3.0 a	4.0 a
CV%	24.38	21.08	7.99
LSD(0.05)	0.5	0.6	0.3

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.



*I= Standard error bar

Fig. 2. Effect of (BA+IBA) on days to shoot initiation in black pepper

Khan *et al.*, 2016; reported that in BAP 1.0 mg/L and IAA 1 mg/L supplemented medium 10 - 20 days were required for shoot initiation in black pepper. The highest number of shoot (1.8, 2.4 and 3.2) was noticed from the BA 2.0 mg/L + IBA 2.0 mg/L (Fig. e). (Monney *et al.*, 2016; reported that highest number of shoot 1.28 was found in BA 3.0 mg/L+ IBA 0.1 mg/L in *Cryptolepis sanguinolenta*. Which is contradicts with the result (Table 4). The highest length of shoot (1.91 cm, 3.23 cm and 5.10 cm) at 3 WAI, 5 WAI and 8 WAI, respectively was noticed from the BA 2.0 mg/L + IBA 2.0 mg/L (Table 5).

Table 4. Effects of BA and IBA on the number of shoot at different weeks after induction (WAI)

BA+IBA (mg/l)	Number of shoot		
	3 WAI	5 WAI	8 WAI
1+1	1.2 b	1.4 cd	1.6 ef
1+1.5	1 b	1.2 d	1.4 f
1+2	1.2 b	1.4 cd	2 cde
1+2.5	1.4 ab	1.4 cd	1.6 ef
1.5+1	1.4 ab	1.8 bc	2 cde
1.5+1.5	1.2 b	1.8 bc	1.8 def
1.5+2	1.4 ab	2 ab	2 cde
1.5+2.5	1.2 b	2 ab	2 cde
2+1	1.4 ab	2 ab	2.4 bc
2+1.5	1.4 ab	1.6 bcd	2 cde

BA+IBA (mg/l)	Number of shoot		
	3 WAI	5 WAI	8 WAI
2+2	1.8 a	2.4 a	3.2 a
2+2.5	1.2 b	1.6 bcd	2 cde
2.5+1	1.2 b	1.6 bcd	2.2 bcd
2.5+1.5	1.2 b	1.4 cd	2.2 bcd
2.5+2	1.2 b	1.6 bcd	2.2 bcd
2.5+2.5	1.4 b	1.8 bc	2.6 b
CV percent	36.49	27.72	19.05
LSD (0.05)	0.6	0.6	0.5

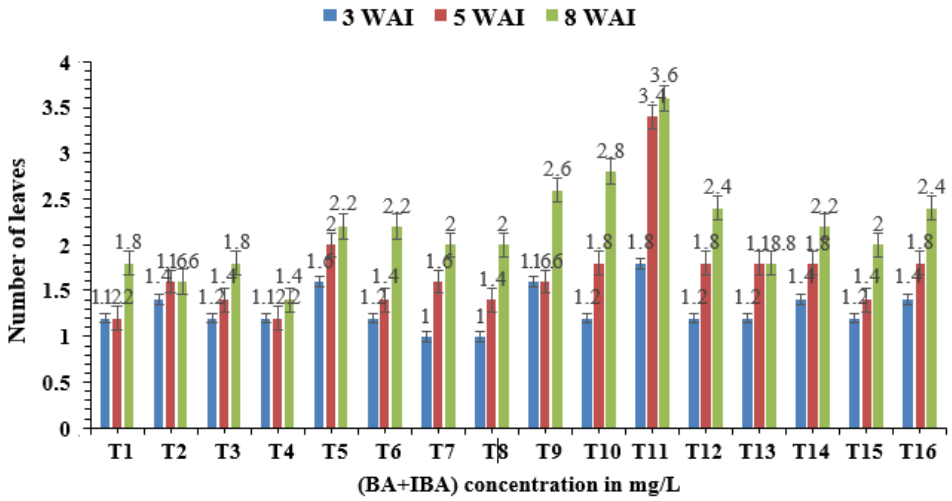
Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

Table 5. Effect of different concentration on BA and IBA on the length of shoot

BA+IBA (mg/L)	Length of shoot		
	3 WAI	5 WAI	8 WAI
1+1	1.36 jk	2.16 d	2.40 g
1+1.5	1.34 l	2.32 c	2.73 de
1+2	1.34	2.33 c	2.75 cde
1+2.5	1.37 j	2.34 c	2.72 e
1.5+1	1.71 e	2.53 b	2.83 bcd
1.5+1.5	1.74 d	2.52 b	2.83 bcd
1.5+2	1.75 c	2.54 b	2.83 bcd
1.5+2.5	1.75 cd	2.52 b	2.82 bcde
2+1	1.41 I	2.33 c	2.85 b
2+1.5	1.41 i	2.34 c	2.85 bc
2+2	1.90 a	2.71 a	3.23 a
2+2.5	1.85 b	2.32 c	2.86 b
2.5+1	1.50 g	2.14 de	2.50 fg
2.5+1.5	1.60f	2.16 d	2.53 f
2.5+2	1.43 h	2.15 de	2.53 f
2.5+2.5	1.35 kl	2.12 e	2.54 f
CV percent	0.66	1.35	3.02
LSD (0.05)	0.0129	0.0400	0.1045

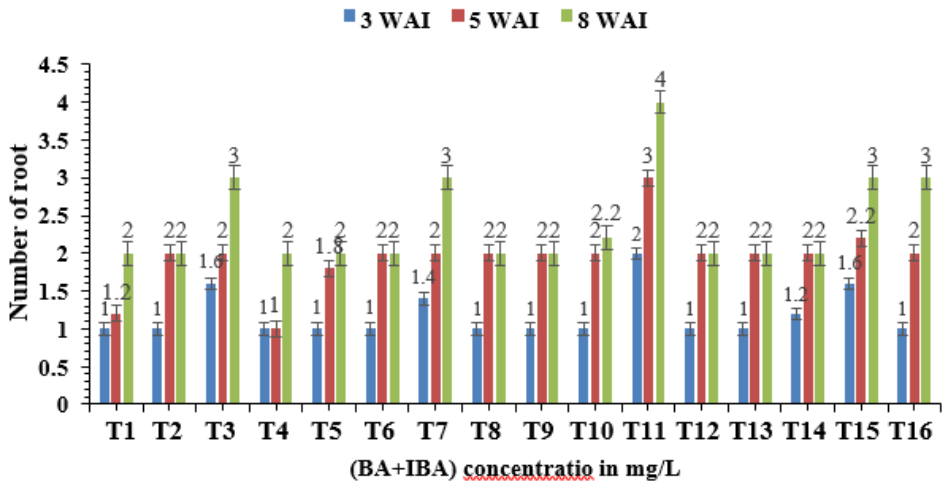
Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

The treatment BA 2.0 mg/L+ IBA 2.0 mg/L (Fig. f) gave the highest number of leaves (1.8, 3.4 and 3.6) at 3 WAI, 5 WAI and 8 WAI respectively. The treatment 2.0 mg/L BA + 2.0 mg/L IBA (Fig. g) gave the highest number of root (2.0,3.0 and 4.0).



*I=Standard error bar

Fig. 3. Combined effects of BA and IBA on number of leaves in black pepper



*I=Standard error bar

Fig. 4. Combined effects of BA and IBA on number of root in black pepper

After considerable number of shoots and roots were developed at 8 weeks of culture. The plantlets were removed from vial carefully without any root damage. In the growth cabinet and in the shade house, plants (Fig. h) were acclimatized and hardened before being transferred to the field conditions. At first 15 plants were transplanted and 7 were survived in shade condition (46.67%). Finally, in normal atmospheric condition 7 plants (Fig. i) were transplanted among them 4 survived and survival rate was 57.14%. Anand *and Rao,* 2000; reported that in natural condition 75% plantlets survived. observed 90% plantlets survival in soil in shade house.

Fig. a. Inoculation of explants in culture media

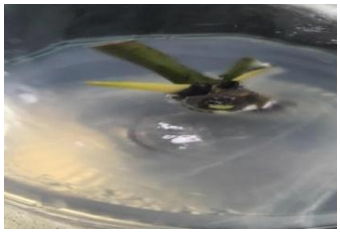


Fig. b. Number of shoot in MS medium supplemented with BA1.5mg/L

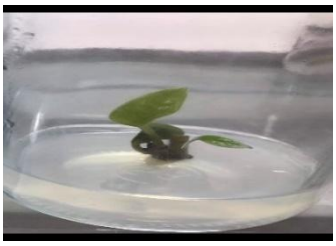


Fig. c. Number of leaves at 8 WAI in the treatment of 2.0mg/L BA

Fig. d. Root development in the treatment of 2.0mg/LIBA

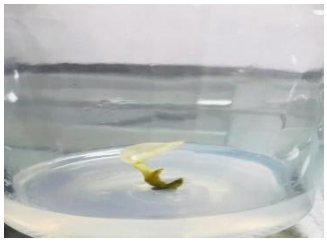


Fig. e. Number of shoot at 8 WAI in the treatment of BA2.0mg/L+IBA 2.0mg/L

Fig. f. Number of leaves at 8 WAI in the treatment of 2.0mg/LBA+ 2.0mg/LIBA

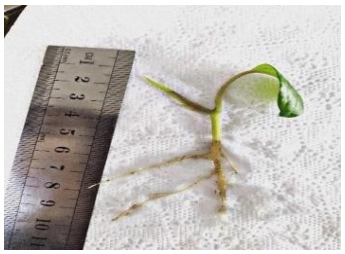


Fig. g. Number of root at 5 WAI in the treatment of 2.0mg/LBA+2.0mg/LIBA

Fig. h. Acclimatization of plantlets in the shade condition

Fig. i. Establishment of plantlet in natural condition

Conclusion

In the current study, a successful regeneration and multiplication procedure for *Piper nigrum in vitro* clonal propagation using nodal segments is described. For the growth of black pepper, other explants such meristems and root tips can be tested. Further study can be done with different concentrations and combinations of auxin and cytokinine group of hormones for the micropropagation of black pepper.

Conflicts of Interest

The authors declare no conflicts of interest regarding publication of this paper.

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