Effects of Plant Growth Regulators on Micropropagation of Black Pepper (Piper nigrum) in Commercial-Scales

ABSTRACT

Aim: Black pepper (*Piper nigrum*) is renowned as a popular spice used to flavor dishes around the world and is also an agricultural export commodity that brings high economic value to many countries. However, traditional propagation methods by seed, cuttings, layering, and grafting reveal many limitations such as the low multiplication coefficient, the low survival rate of seedlings, and especially when there is an outbreak of disease, the ability to control the disease source is exceedingly difficult, leading to the spread of disease. Therefore, an alternative method through apical meristem micropropagation has been applied to contribute to sustainable and disease free pepper cultivars. Propagation by apical shoot meristem gives high propagation efficiency, good and uniform seedling quality, and can be propagated in large numbers in a brief period.

Methodology: This study is conducted to contribute to optimizing the process of micropropagation of pepper plants to meet the needs of producing excellent quality seedlings, serving the Vietnamese pepper industry. The two steps in the micropropagation sequence studied in this project are shoot formation and bud cluster multiplication. In this study, we investigate the effects of BA (6-Benzylaminopurine) and NAA (α-Naphthalene acetic acid) in shoot formation. Regenerated shoots were then cultured in MS media supplemented with different combinations of Kinetin (6-Furfurylaminopurine) and NAA in bud cluster multiplication experiment.

Results: The results showed that the apical shoot meristem cultured on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L-1 NAA and 2.0 mg L-1 BA was the optimal medium for shoot formation rate (100%). Besides, MS culture medium supplemented with Kinetin 1.5 mg L-1 + NAA 0.5 mg L-1 produced superior results in bud cluster multiplication compared with other treatments (8, 20 buds) after 8 weeks (about 2 months).

Conclusion: BA, Kinetin and NAA give important rules on micropropagation of black pepper in commercial-scale

Keywords: Black pepper, bud cluster, micropropagation, shoot formation, tissue culture

1 INTRODUCTION

Black pepper (*Piper nigrum*) belongs to the family Piper, native to the Western Ghats (India), a genus of about 1,000 species, of which about 110 species are found in India and Asian countries. Black pepper is a popular spice used to flavor all sorts of dishes worldwide. It has been known as the 'King

of Spices' [1, 2] with its intense aroma and spicy taste. Black pepper is also an important traditional medicine because it has the compound Piperine, a pungent alkaloid [3] and tiny amounts of safrol, pinene, sabinene, limonene, caryophyllene and linalool compounds. It has been studied and used to treat asthma, chronic indigestion, obesity, sinuses, nasal congestion, fever, pain abdomen, stomach disease and diarrhea [4].

Black pepper was introduced to Vietnam when the French growing them in Vietnam in the 17th century. At the end of the 19th century, black pepper became a type of product grown in Phu Quoc, Ha Tien (Kien Giang), and Hon Chong. Until the beginning of the 20th century, it was grown popularly on the land of basalt soil in the Southeastern and Central regions. Black pepper now become the main export agricultural product of Vietnam which brings high economic value. Therefore, the right and sustainable development of pepper trees has great economic significance, contributing to the economic growth of the country in general, the economy of the region, and the locality, as well as contributing to the eradication of hunger, reducing poverty, and improving the lives of farmers. However, in recent years, the massive increase in pepper growing areas has led to diseased pepper plants, including viral diseases.

The results of the investigation about viral diseases in pepper in some provinces in the Southeast region showed that 42-93% of pepper gardens had symptoms of virus infection expressed on leaves and plants such as flowerpots, leaf creasing, curling leaf margin, dwarf tree deformity [5, 6]. The cause also comes from the traditional propagation methods of the people. Black pepper can be traditional propagated by seed, cuttings, layering and grafting [7]. The seed propagation of pepper often leads to genetic variation due to the formation of recombinants. While other traditional propagation methods of pepper are not only slow and time-wasting, but also extremely easy to lead to the spread of diseases and diseases, affecting the quality of seedlings [8]. Therefore, effective methods for faster propagation of black pepper must be introduced.

Up-to-date, plant tissue culture is the most efficient and reliable method for the mass and rapid production of disease-free, genetically stable, and identical varieties of black pepper [9]. The biotechnology of tissue culture over the past years has achieved many outstanding achievements in many areas such as micropropagation from plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions [10]. Tissue culture techniques have made a significant contribution in improving the quality and quantity of seedlings, genetic conservation, and improvement of crops in general, including black pepper [4, 11, 12]. In the world, there have been many successful in vitro studies on pepper plants that have been recorded as cultured from shoot tips [4, 13, 14, 15]; culture of protocorm from leaf callus [4, 14]; phylogenetic studies from callus [16]. However, so far, apical shoot meristem propagation has been the method for high propagation efficiency, good and uniform seedling quality, and can be propagated in large numbers in a brief period [17].

Moreover, a suitable hormone regimen combined with a proper basal medium is paramount in the success of plant tissue culture. The current in vitro propagation method[29] is usually done with

different cytokinin such as BAP, zeatin, kinetin and TDZ (thidiazuron) [17, 6]. Still, BAP is believed to be the optimal growth regulator (PGRs)[30] in the industry due to its affordable price and good efficacy in promoting shoot multiplication in vitro. Therefore, there are very few studies reporting on the effect of Kinetin on the shoot multiplication of pepper plants because of the excessive cost. Although, kinetin is also a plant cytokinin that promotes cell division, commonly used in plant tissue culture to stimulate the callus (as with auxin) and regeneration of shoot tissues from callus (with lower concentration). Especially the process of regeneration of shoots from the apical shoot meristem, currently this process has been studied, but the samples after being sterile stimulated to shoot formation only reached 66% - 86% [17].

This study was conducted to evaluate the effects of BAP and NAA on the ability to form shoot; and Kinetin and NAA on the ability to create and develop bud clusters of black pepper to increase propagation efficiency in commercial-scale

2 MATERIALS AND METHODS

2.1 Materials

Vinh Linh black pepper variety was chosen as material for this research, provided by the Pepper Research and Development Center. The explants used for this thesis are apical meristems of healthy black pepper plants grown in the greenhouse at the Western Highlands Agriculture and Forestry Science Institute, DakLak, Vietnam.

MS basic nutrient medium (Murashige and Skoog, 1962) [18] supplemented with 10% coconut water, 20 g L^{-1} sucrose, and 8 g L^{-1} agar adjusted to pH = 5.8-6 before autoclaving . Media was supplemented with plant growth regulators: BAP (6-Benzylaminopurin), NAA (α-Naphthalenacetic acid), Kinetin (N^6-furfuryladenine)

Culture conditions: In vitro culture conditions was set up 16 hours of light/ 8 hours of darkness, light intensity 37.04 µmol/s/m^2, temperature 26 ± 2^{o}C

2.2 Methods

2.2.1 Strilization of explants

The apical shoots sterilization and excision procedure was as method of Mai et al [17]. Pre–sterilized apical shoots cuttings were taken and cut into segments of 4–5 mm. The explants were first rinsed with running tap water and treated with diluted soap water for 5 minutes. Then, the samples were washed a second time with tap water to remove all traces of detergent and further treated with 0.2% mercury chloride for 10 minutes and 0.3% nano silver solution for 30 minutes in safety cabinet. Finally, the explants were washed with sterilized distilled water three times to remove all traces of mercuric chloride and nano silver. Each explant was inoculated into MS medium, and after two weeks, uncontaminated samples were used as a source of materials for further experiments.

2.2.2 Effect of plant growth regulators on performance of shoot via shoot apical meristem culture of black pepper.

2.2.2.1 Experiment 1: Effect of BAP concentrations on performance of shoot via shoot apical meristem culture of black pepper: Uncontaminated and alive apical shoots (free from fungal and bacterial infections, green samples) prepared previously will be used in this experiment. Each apical shoot was cut and inoculated into the MS medium supplemented with different BAP (0.0-0.5-1.0-1.5-2 mg L^{-1}). Evaluation of shoot length (cm), number of shoots per sample, shoot induction rate (%), and shoot characteristics

2.2.2.2 Experiment 2: Effect of different combinations of BA and NAA concentrations on performance of shoot via shoot apical meristem culture of black pepper: Uncontaminated and alive apical shoots (free from fungal and bacterial infections, green samples) prepared previously will be used in this experiment. Each apical shoot was cut and inoculated into the MS medium supplemented with different combinations of BAP (2 mg L^{-1}) and NAA (0.0-0.1-0.3-0.5-0.7 mg L^{-1}). Evaluation of shoot length (cm), number of shoots per sample, shoot induction rate (%), and shoot characteristics

2.2.3 Effect of plant growth regulators on performance and multiplication of bud clusters of the black pepper.

2.2.3.1 Experiment 3: Effect of different Kinetin concentrations on performance and multiplication of bud clusters of the black pepper: Shoots regenerated in experiments 1 and 2 (free from fungal and bacterial infections, green samples) were cut into segments of 2–3mm each and inoculated in the MS medium supplemented with different concentrations of Kinetin (0.0-0.1-0.3-0.5-0.7 mg L^{-1}) for bud cluster mutiplication. Evaluation of shoot length (cm), number of shoots per explant, number of leaves per shoot, number of nodes per shoot, and shoot characteristics

2.2.3.2 Experiment 4: Effect of different combinations of Kinetin and NAA concentrations on performance and multiplication of bud clusters of the black pepper: Shoots regenerated in experiments 1 and 2 (free from fungal and bacterial infections, green samples) were cut into segments of 2–3mm each and inoculated in the MS medium supplemented with different combinations of Kinetin (1.5 mg L^{-1}) and NAA (0.0-0.1-0.3-0.5-0.7 mg L^{-1}) for bud cluster multiplication. Evaluation of shoot length (cm), number of shoots per explant, number of leaves per shoot, number of nodes per shoot, and shoot characteristics

2.3 Data collection and analysis

The experiment was carried out in a completely randomized factorial design (CRD). Each treatment was carried out 4 replication, each replication was culture 10 bottle (350 ml), one bottle was cutured 1 cluster. Data were collected after 8-weeks. Data were statistically analyzed using statistical software (IBM SPSS® v.20) for Oneway ANOVA analysis with Duncan's test at $\alpha = 0.05$

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3. RESULT

3.1 Effect of BAP concentrations on performance of shoot via shoot apical meristem culture of black pepper

In this experiment, the effects of different treatments of BAP were studied on shoot formation from the shoot apical meristem of black pepper. The use of different BAP levels in the MS medium showed significant differences (P<0,05) in relation to shoot induction rate, average multiplication coefficient, and average shoot length (Table 1 and Figure 1, 2).

The effect of different concentrations of BA on average shoot induction rate showed that 2.0 mg L^{-1} BAP significantly produced the highest average shoot induction rate (65%) as compared to the rest of the experiments (Table 1 and Figure 1, 2). However, only 2.0 mg L^{-1} BAP showed a significant difference, while the rest of the experiments did not show a significant difference. Similarly, increasing concentrations of BAP from 0.0 to 2.0 mg L^{-1} showed a significant positive effect (P<0.05) noted on average shoot length (cm), and number of shoots per explant. The significantly highest average shoot length (3.10 cm) and the significantly highest average multiplication coefficient (2.20 shoots/explant) was also recorded in the medium supplemented with 2.0 mg L^{-1} BAP compared to the lowest in medium without hormones (Figure 1 A,B), despite there being no statistically significant difference between 1.0 mg L^{-1} and 1.5 mg L^{-1} BAP in average shoot length, and between the control and 0.5 mg L^{-1} BAP in average multiplication coefficient .

To summarize, analyzed data showed a significant positive effect of BA on shoot induction rate and shoot development. The most suitable concentration for inducing and shoot development is the medium MS supplemented with 2.0 mg L^{-1} BAP.

Table 1. Effect of BAP concentrations on shoot formation from the shoot apical meristem of black pepper

BAP (mg L-1)	Shoot length (cm)	Shoot induction rate (%)	No. of Shoots per explant
0.0	1.53±0.07 e	20.00±0.00 c	1.00±0.00
0.5	1.93±0.06 d	30.00±6.67 bc	1.10±0.07 d
1.0	2.25±0.07 c	40.00±0.00 b	1.35±0.06 c
1.5	2.70±0.05 b	45.00±5.77 b	1.65±0.06 b
2.0	3.10±0.08 a	65.00±5.77 a	2.20±0.09 a

Different letter designations (a–b) in the same column indicate a significant difference at the 95% confidence level. The values represent mean ± standard deviation

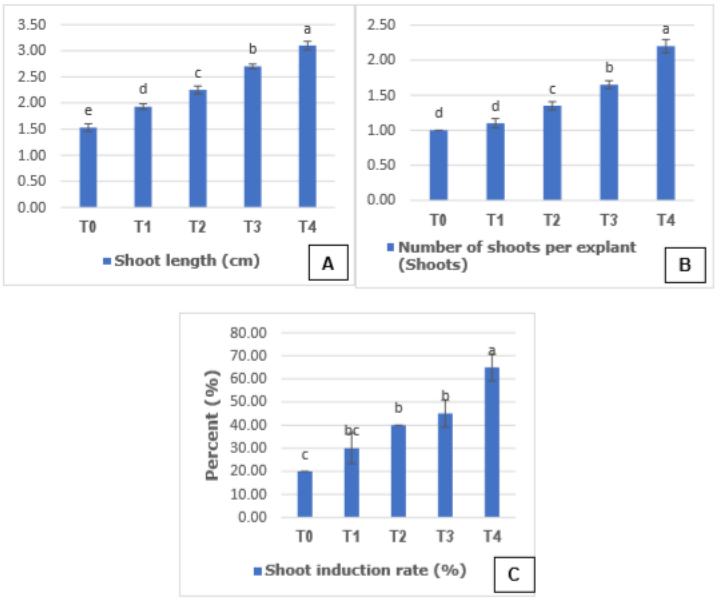

Figure 1. Effect of different treatments of BAP on shoot formation from the shoot apical meristem of black pepper after 8 weeks (about 2 months). (A) Shoot length; (B) Number of shoots per explant; (C) Shoot induction rate. In each bar chart, treatments that are ascribed with different letters are significantly different at P ≤ 0.05. The bars are means of 4 replications with standard error (±SE)

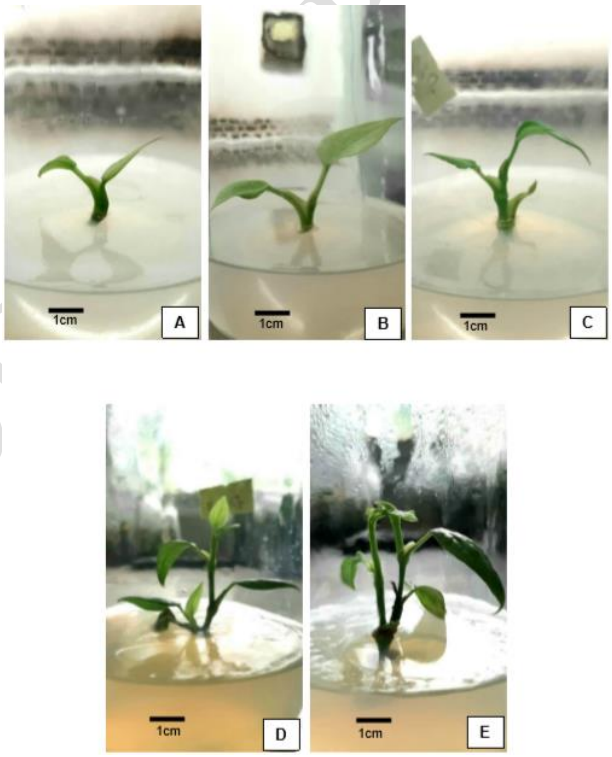

Figure 2. Representative shoots formation based on the effect of different treatments of BAP after 8 weeks (about 2 months). (A) MS medium.

(B) MS medium supplemented with 0.5 mg L^{-1} BAP.

(C) MS medium supplemented with 1.0 mg L^{-1} BAP.

(D) MS medium supplemented with 1.5 mg L^{-1} BAP.

(E) MS medium supplemented with 2.0 mg L^{-1} BAP.

3.2 Effect of different combinations of BAP and NAA concentrations on performance of shoot via shoot apical meristem culture of black pepper

In this experiment, the effects of different combinations of BAP and NAA concentrations was studied on shoot formation from the shoot apical meristem of black pepper. The addition of NAA enhanced the shoot induction rate and increased the shoot length and multiplication coefficient (Table 2 and Figure 3, 4).

Table 2. Effect of combinations of BAP and NAA concentrations on shoot formation from the shoot apical meristem of black pepper.

Concentration of PGRs (mg L^{-1})		Shoot length (cm)	Shoot induction rate (%)	No. shoots per explant
BAP	NAA			
2.0	0.0	3.10±0.08 c	65.00±5.77 b	2.20±0.09 c
2.0	0.1	3.35±0.07 c	65.00±5.77 b	2.40±0.09 bc
2.0	0.3	4.23±0.07 b	70.00±11.55b	2.60±0.00 b
2.0	0.5	5.73±0.09 a	100.00±0.00 a	3.05±0.11 a
2.0	0.7	2.30±0.09 d	35.00±5.77 c	1.80±0.09 d

PGS (plant growth regulators)

On MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, shoot induction rate (100%), average shoot length (5.73 cm) and average multiplication coefficient (3.05 shoots/explant) were reported to be the highest significant difference compared with the rest treatments. Shoot induction rate was significantly higher on the medium of the combination of BAP 2.0 mg L^{-1} and 0.5 mg L^{-1} NAA (100%) than that of without NAA (65%). The combination of BAP and NAA also showed a positive effect on average shoot length and average multiplication coefficient. However, non-significant differences were noted in using 0.1 mg L^{-1} , 0.3 mg L^{-1} NAA and the control treatment regarding average shoot induction rate, average shoot length, and average multiplication coefficient (Figure 3, 4). In contrast, the highest concentrations of NAA (0.7 mg L^{-1}) showed detrimental effects on average shoot induction rate, average shoot length, and average multiplication coefficient; for example, NAA at 0.7 mg L^{-1} produced a significantly lowest shoot induction rate (35%), lowest shoot length (2.30 cm), and lowest multiplication coefficient (1.80 shoots/explant) (Figure 3, 4).

In general, the combination of 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA is the most suitable for shoot formation in 2 experiments 1 and 2.

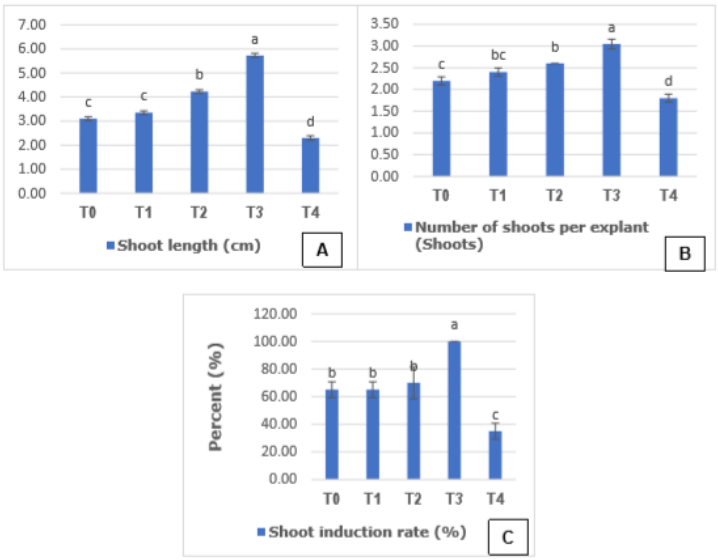

Figure 3. Effect of different combinations of BAP and NAA concentrations on shoot formation from the shoot apical meristem of black pepper after 8 weeks (about 2 months). (A) Shoot length; (B) Number of shoots per explant; (C) Shoot induction rate. In each bar chart, treatments that are ascribed with different letters are significantly different at $P \leq 0.05$. The bars are means of 4 replications with standard error (±SE).

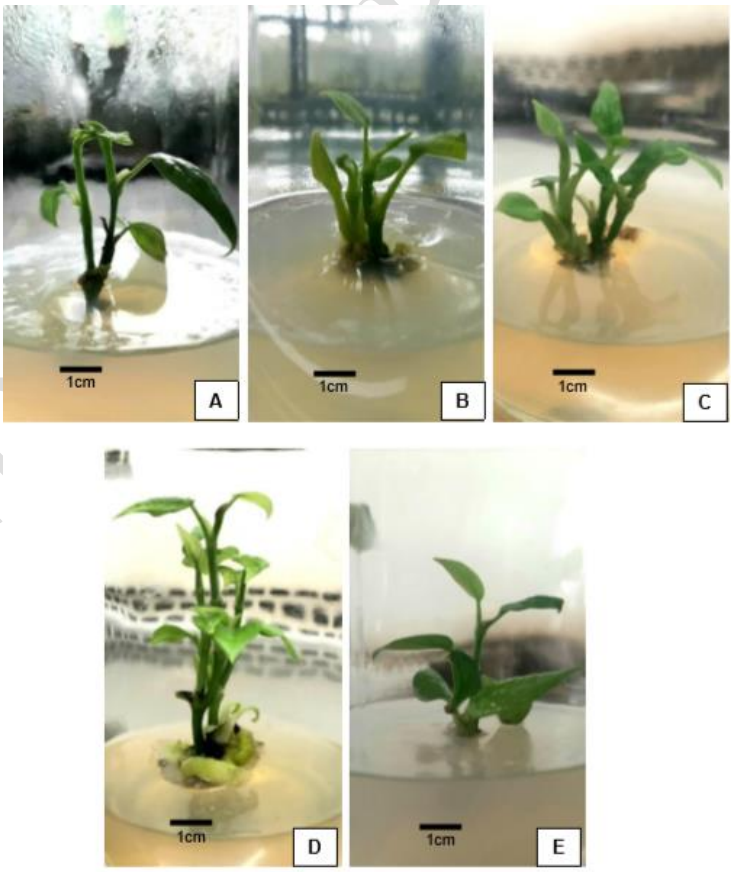

Figure 4. Representative shoots formation based on the effect of different combinations of BAP and NAA concentrations after 8 weeks (about 2 months). (A) MS medium supplemented with 2.0 mg L^{-1} BAP.

(B) MS medium supplemented with 2.0 mg L−1 BAP and 0.1 mg L^{-1} NAA.

(C) MS medium supplemented with 2.0 mg L−1 BAP and 0.3 mg L^{-1} NAA.

(D) MS medium supplemented with 2.0 mg L−1 BAP and 0.5 mg L^{-1} NAA.

(E) MS medium supplemented with 2.0 mg L−1 BAP and 0.7 mg L^{-1} NAA.

3.3 Effect of different Kinetin concentrations on performance and multiplication of bud clusters of the black pepper.

This experiment aimed to evaluate the optimization treatment for the high multiplication coefficient of shoots. The responses of shoots formation in the bud cluster related to the different treatments of Kinetin are presented in Table 3 and Figure 5, 6.

Table 3. Effect of Kinetin concentrations on bud clusters formation of black pepper

Kinetin (mg L-1)	Shoot length (cm)	No. of Shoots per explant	No. of Leaves per shoot	No. of Nodes per shoot
0.0	2.25±0.01 d	1.00±0.00 e	2.65±0.06 d	2.65±0.06 d
0.5	3.53±0.06 c	1.80±0.09 d	3.35±0.11 c	3.35±0.15 c
1.0	3.75±0.07 c	2.35±0.15 c	3.50±0.07 c	3.60±0.09 bc
1.5	5.33±0.07 a	3.45±0.11 a	5.45±0.11 a	4.45±0.06 a
2.0	4.93±0.12 b	2.75±0.11 b	4.60±0.13 b	3.80±0.16 b

Data presented in Table 3 and Figure 5, 6 indicates that the significantly highest bud clusters multiplication and formation of healthy shoots (P<0,05) were obtained on MS medium supplemented with 1.5 mg L^{-1} Kinetin. The addition of Kinetin in the multiplication medium at 1.5 mg L^{-1} induced the highest average shoot length of 5.33 cm, highest average multiplication coefficient (3.45 shoots/explant), highest number of leaves per shoot (5.45), and highest number of nodes per shoot (4.45) in 8 weeks (Figure 5, 6). The significantly lowest formation of shoots (P<0,05) in the bud cluster was recorded on the MS medium without any hormone (Table 3, Figure 5, 6). In detail, the control treatment showed the least changed in average shoot length (2.25 cm), average multiplication coefficient (1.00 shoot/explant), the number of leaves per shoot (2.65), and the number of nodes per shoot (2.65). The MS medium at 2.0 mg L^{-1} Kinetin ranked third with the average shoot length of 6.03 cm, 6.55 shoots/explant, 4.75 leaves/shoot, and 4.70 nodes/shoot within the same period of culture (Table 3, Figure 5).

The result also indicated no significant difference (P>0.05) between 0.5 mg L^{-1} and 1.0 mg L^{-1} of Kinetin. Overall, the analyzed data dedicated that the gradual increase in shoots formation in the bud cluster that positively correlates with increasing Kinetin concentration (ranges from 0 to 1.5 mg L^{-1}). The same trend was also presented in the shoot characteristic of clusters between 0 and 1.5 mg L^{-1} of Kinetin. However, when the concentration of Kinetin was 2.0 mg. L^{-1}, there was inhibition of shoot formation causing the decreased number of shoots per explant and the yellow or wilted leaves in the

explant (Table 3, Figure 6). This means that Kinetin is no longer effective in bud cluster formation at high concentrations (Table 3).

Further noted that the high-coefficient bud multiplication and formation of healthy shoots (P<0,05) was acquired on MS medium supplemented with 1.5 mg L^{-1} Kinetin is superior to the rest of the experiment 3 (Table 3, Figure 5). Therefore, 1.5 mg L^{-1} Kinetin were used for subsequent experiment.

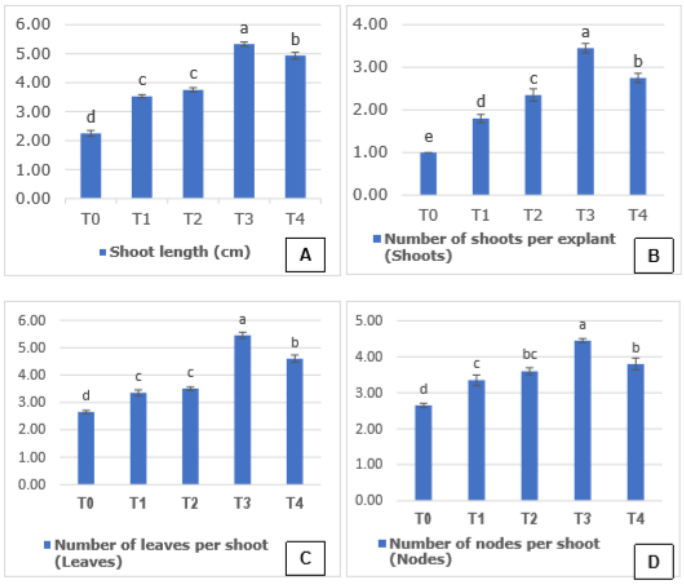

Figure 5. Effect of different treatments of Kinetin on bud clusters formation of black pepper after 8 weeks (about 2 months). (A) Shoot length; (B) Number of shoots per explant; (C) Number of leaves per shoot; (D) Number of nodes per shoot. In each bar chart, treatments that are ascribed with different letters are significantly different at P ≤ 0.05. The bars are means of 4 replications with standard error (±SE).

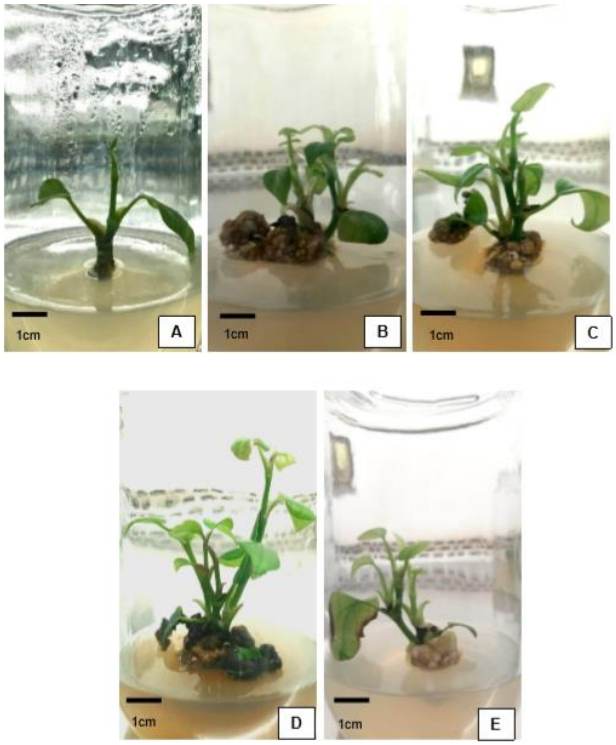

Figure 6. Representative shoots cluster based on the effect of different treatments of Kinetin after 8 weeks (about 2 months). (A) MS medium.

(B) MS medium supplemented with 0.5 mg L^{-1} Kinetin.

(C) MS medium supplemented with 1.0 mg L^{-1} Kinetin.

(D) MS medium supplemented with 1.5 mg L^{-1} Kinetin.

(E) MS medium supplemented with 2.0 mg L^{-1} Kinetin.

3.4 Effect of different combinations of Kinetin and NAA concentrations on performance and multiplication of bud clusters of the black pepper

This experiment aimed to evaluate the optimization treatment for the high multiplication coefficient of shoots. The responses of shoots formation in the bud cluster related to the different combinations of Kinetin and NAA concentrations are presented in Table 4 and Figure 7, 8.

Table 4. Effect of combinations of Kinetin and NAA concentrations on bud clusters formation of black pepper

Concentration of PGRs (mg. L-1)		Shoot length (cm)	No. of Shoots per explant	No. of Leaves per shoot	No. of Nodes per shoot
Kinetin	NAA				
1.5	0.0	5.33±0.07 c	3.45±0.11 e	5.45±0.11 b	4.45±0.06 c
1.5	0.1	4.13±0.07 e	4.05±0.06 d	4.30±0.07 d	4.65±0.06 c
1.5	0.3	5.00±0.07 d	5.10±0.15 c	5.05±0.15 c	5.10±0.07 b
1.5	0.5	6.50±0.05 a	8.20±0.09 a	6.35±0.06 a	6.20±0.09 a
1.5	0.7	6.03±0.07 b	6.55±0.06 b	4.75±0.06 c	4.70±0.07 c

Data presented in Table 4 and Figure 7, 8 indicates that the combination of Kinetin and NAA significantly produced a higher shoot number as compared to those without NAA hormones. The shoot number per explant increased significantly ($P<0.05$) by increasing the NAA concentration from

0.1 to 0.5 mg L^{-1} (Figures 7 and 8). The significantly highest average multiplication coefficient (8.20 shoots/explant) were obtained on MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.5 mg L-1 NAA as compared to the lowest in medium without NAA hormones (3.45 shoots/explant). Besides, the average shoot length (6.50 cm), average number of leaves per shoot (6.35), and average number of nodes per shoot (6.20) also obtained on the medium with a combination of 1.5 mg L-1 Kinetin and 0.5 mg L^{-1} NAA showed the highest significant differences compared to with control and other NAA supplementation treatments. However, average shoot length values obtained in the MS medium using 0.1 mg L-1 (4.13 cm) and 0.3 mg L^{-1} NAA (5.00 cm) were lower than those in the control (5.33 cm), and only 0.7 mg L^{-1} NAA has a higher mean response length value than the control. In contrast, average number of leaves per shoot and average nodes per shoot obtained in the remaining treatments showed non- significant differences.

Overall, the analyzed data dedicated that increasing the concentration of NAA (range from 0 to 0.5 mg. L^{-1}) had a significant positive effect ($P<0.05$) noted on number of shoots per explant (Table 4 and Figure 7, 8). Still, they showed a significant negative effect ($P<0.05$) noted on shoot length (cm), number of shoots per explant, number of leaves per shoot, and number of nodes per shoot in the medium at 0.7 mg L-1 NAA (Table 4 and Figure 7, 8).

To summarized, the high-coefficient bud multiplication and formation of healthy shoots ($P<0,05$) was acquired on MS medium supplemented 1.5 mg L-1 Kinetin and 0.5 mg L^{-1} NAA is superior to the rest of the treatments in 2 experiments 3 and 4.

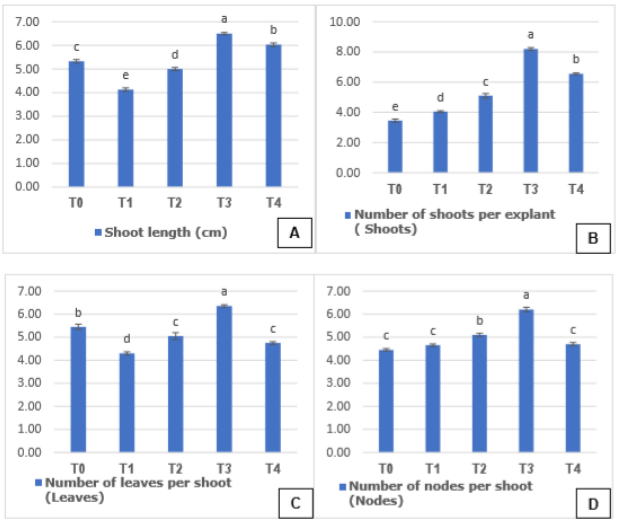

Figure 7. Effect of different combinations of Kinetin and NAA concentrations on bud clusters formation of black pepper after 8 weeks (about 2 months). (A) Shoot length; (B) Number of shoots per explant; (C) Number of leaves per shoot; (D) Number of nodes per shoot. In each bar chart, treatments that are ascribed with different letters are significantly different at $P \leq 0.05$. The bars are means of 4 replications with standard error (±SE).

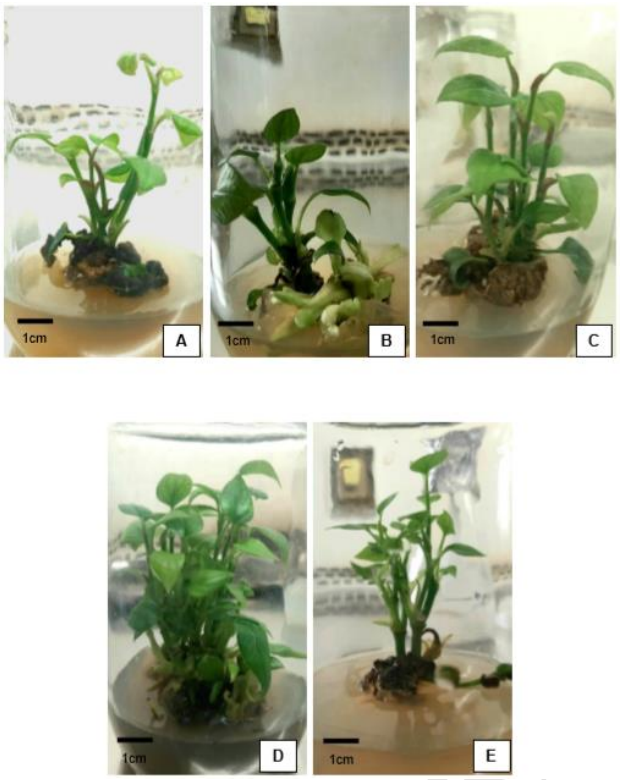

Figure 8. Representative shoots cluster based on the effect of different combinations of Kinetin and NAA concentrations after 8 weeks (about 2 months).

(A) MS medium supplemented with 1.5 mg L^{-1} Kinetin.

(B) MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.1 mg L^{-1} NAA.

(C) MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.3 mg L^{-1} NAA.

(D) MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.5 mg L^{-1} NAA.

(E) MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.7 mg L^{-1} NAA.

4. DISCUSSION

4.1 Effect of plant growth regulators on performance of shoot via shoot apical meristem culture of black pepper.

In experiment 1, the use of different BAP levels in the MS medium showed significant differences (P<0,05) in relation to shoot induction rate, shoot number per explant, and shoot length (Table 1 and Figure 1, 2). Earlier studies have proved that cytokinin added to the medium are considered essential in the stimulation of shoot formation and multiplication in vitro [19]. The main effect of cytokinin in tissue culture is to promote cell division and regulate morphogenesis [20].

Therefore, the addition of BAP in the MS medium at 2.0 mg $L^{-1}1$ induced the highest shoot induction rate (65%), also highest shoot length (3.10 cm) and highest multiplication coefficient (2.20 shoots/explant) (Figure 1, 2). In contrast, the different concentrations of BAP ranging from 0.5–1.5 mg L^{-1} on MS medium showed no significant difference (P>0.05) compared to the control treatment because cytokinin at low concentrations stimulates axillary buds' growth, while high concentrations induce shoot formation [20]. Besides, the activity of BAP for fast multiplication of different cultivars of

pepper nigrum has been reported in several studies. For example, Legesse, et al [21] showed that MS medium supplemented with 4 mg L^{-1} BAP was effective for shoot multiplication in shoot-tip explants of black pepper; while Hussain, et al [22] reported that the shoot initiation was poor with 2.5 and 3.0 mg L^{-1} BAP which indicate that higher the concentration of BA lowers the rate of organogenesis of black pepper.

Results of these experiments supported this study; the concentration of BAP higher than 2 mg L^{-1} was not effective for shoot induction of black pepper. However, the results still showed a significant positive effect ($P<0.05$) noted on shoot induction rate (%), shoot length (cm), and number of shoots per explant when increasing concentrations of BA from 0.0 to 2.0 mg L^{-1}.

Cytokinin is needed for the induction of shoot formation but inhibits shoot elongation. The combination of auxin and cytokinin promoting better shoot differentiation when used alone was also reported in the study of Zhao, et al [23]. Gaspar, et al [24] suggested that the experiments in which shoot formation usually gave high results when using high concentrations of cytokinin and low to moderate concentrations of auxins. The effectiveness of using BAP with auxins such as NAA, IAA and IBA, which improved the shoot induction rate and promoted shoot length (cm), and multiplication coefficient of black pepper were well reported [21, 17, 6]. Therefore, in experiment 2, the most optimize of cytokinin (BAP) concentration (2 mg L^{-1}) recorded in experiment 1 was combined with auxin (NAA) at low concentration (0.1; 0.3; 0.5; 0.7 mg L^{-1}) to evaluate the ability to induce shoot formation and increase shoot length of black pepper.

In experiment 2, the significantly highest shoot formation rate (100.00±0.00%)were obtained on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. The results are significantly better compared to the use of BAP alone in MS media (65%) and the result conducted by Mai, et al [17] when they achieved 86.67% shoot induction rate at 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} IBA. The longest shoot was also recorded in MS media supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (5.73 cm), followed by 4.23 cm in 2.0 mg L^{-1} BAP and 0.3 mg L^{-1} NAA, 3.35 cm in 2.0 mg L^{-1} BAP and 0.1 mg L^{-1}NAA, and the lowest result of 2.30 cm was observed in media with 2.0 mg L^{-1} BAP and 0.7 mg L^{-1} NAA. The same trend is presented in the number of shoots per explant. The shoot response to auxins is represented by a curve, of which the peak or maximum response was reached before inhibition effect took place [25]. Therefore, it was expected that the increase in concentration of NAA resulted in the reduction of new shoots and shoot length.

The point where the stimulation of NAA was turned into inhibition in this experiment was 0.7 mg L^{-1} NAA (Table 2 and Figure 3, 4). This result was quite similar compared to the study of Mai, et al [17], who reported that when increasing the concentration of IBA in the culture medium to 0.6 mg L^{-1}, the shoot induction rate and shoot length both decreased. In general, the results showed a significant positive effect ($P<0.05$) noted on shoot induction rate (%), shoot length (cm), and number of shoots per explant when increasing concentrations of NAA from 0.0 to 0.5 mg. L^{-1}. Still, they showed no significant difference ($P>0.05$) among treatments using low concentration of NAA (0.1 and 0.3 mg. L^{-1}) and the control treatment.

Thus, these experiments showed that MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA has the potential to be an improved procedure applied to induction of shoot formation of black pepper.

4.2 Effect of plant growth regulators on performance and multiplication of bud clusters of the black pepper

BA is believed to be the optimal plant growth regulator in the industry due to its affordable price and good efficacy in promoting shoot multiplication in vitro. Although, kinetin is also a plant cytokinin that promotes cell division, commonly used in plant tissue culture to stimulate the callus (as with auxin) and regeneration of shoot tissues from callus (with lower concentration) [24].

In experiment 3, Kinetin promoted the formation of shoot clusters from generated shoots in vitro after 8 weeks of culture (Table 3 and Figure 5, 6). On medium with individual kinetin, the highest multiplication coefficient (3.45 shoots/explant) was found on 1.5 mg. L^{-1} kinetin medium with an average shoot length of 5.33 cm, average number of leaves per shoot of 5.45, and average number of nodes per shoot of 4.45 (Table 3 and Figure 5, 6). However, when the kinetin concentration was at 2.0 mg. L^{-1}, the number of shoots per explant, average shoot length, number of leaves per shoot, and nodes per shoot decreased.

The addition of kinetin and NAA to the culture medium showed obvious significant differences in effect on bud cluster multiplication. The combination of kinetin and NAA at all concentrations increased multiplication coefficient and were higher than the control treatment (Table 4 and Figure 7, 8). The analyzed results showed that under the influence of kinetin and NAA, after 8 weeks of culture, the shoots developed well.

In particular, the MS medium supplemented with kinetin 1.5 mg L^{-1} + NAA 0.5 mg L^{-1} stimulated the highest multiplication coefficient (8.20±0.09 shoots/explant) with an average shoot length of 6.50 cm, average number of leaves per shoot of 6.35, and average number of nodes per shoot of 6.20. The results are significantly better compared to the utilization of individual kinetin (control treatment) and the study of Mai, et al [17], who claimed that the highest multiplication coefficient (7.73 shoots/explant) were reported when the culture media supplemented with BA at 1.0 mg L^{-1} and 0.1 ml L^{-1} coconut water.

The results also showed that Kinetin's effect is slightly better than that of BA. The addition of NAA at concentrations of 0.1 mg L^{-1} and 0.3 mg L^{-1} contributed to a significant increase in the average number of shoots per explant, however, decrease in average shoot length. There could be a synergistic effect of kinetin and NAA, wherein, Kinetin promotes cell division and proliferation while NAA enhances cell elongation and division [26, 27]. Therefore, at low concentrations of NAA, the effect was insufficient to induce faster growth, leading to a shorter average shoot length compared to the control treatment. Furthermore, NAA concentrations at 0.7 mg L^{-1} inhibited the growth of explants, resulting in significant reduction in average multiplication coefficient, and average shoot length. This

result was quite similar compared to the study of Thuyen, et al [28], who reported that using high concentrations of NAA (0.5 - 1 mg L^{-1}) will produce more callus mass, inhibiting shoot growth.

Thus, the results in this study were evidence demonstrating that MS medium supplemented with 1.5 mg L^{-1} Kinetin and 0.5 mg L^{-1} NAA has become an improved protocol that could be applied for bud cluster multiplication.

5 CONCLUSION

In conclusion, this study uncovered that the MS medium supplemented with 2.0 mg L^{-1} 1 BAP + 0.5 mg L^{-1} NAA gave promising results in relation to induce shoot formation (100%)) and producing healthy and long multiple shoots. The combination of 1.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA in MS medium was also considered as the best protocol for bud cluster multiplication. However, more research should be conducted based on these results to re-examine the result and optimize the protocol. It is important to obtain significant protocol for micro propagation of black pepper with maximum regeneration and minimum growth regulator concentrations to be able to generate a healthy, diseasefree, and affordable cultivar source, easily access to more farmers in the future.

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