IN VITRO PROPAGATION OF TWO COMMERCIALLY IMPORTANT INDONESIAN BANANA CULTIVARS, 'CAVENDISH' AND 'BARANGAN': THE EFFECT OF VARIOUS BIOTIN CONCENTRATIONS

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Abstract. The study aimed to investigate the effect of biotin on *in vitro* shoot and root proliferation on 'Cavendish' and 'Barangan' banana explants. Sucker of 'Barangan' and 'Cavendish' bananas were used as planting materials. Five levels of biotin (0.5, 1.0, 1.5, 2.0 and 2.5 mg·L^{-1}) and a control were tested on both banana cultivars. A completely randomized design with four replicates was used in this trial. There were four cultures in each experimental unit. Observed variables were the occurrence of culture browning, shoot formation and root formation. An ANOVA followed by an LSD test was employed in statistical data analysis. The results showed that both 'Barangan' and 'Cavendish' cultures experienced browning from first week of initiation in preconditioning medium. Browning was reduced when explants were subcultured onto new fresh media, and regenerated shoots within 2 weeks. Enriching culture media with biotin was proven to be beneficial for regenerating shoots and roots in both cultivars. In both 'Barangan' and 'Cavendish', applying biotin up to 1.5 mg·L^{-1} encouraged shoot formation. However, this shoot formation declined when 2.0 mg·L^{-1} or more biotin present in the culture medium. Meanwhile, in root formation, the application of biotin up to 2.0 mg·L^{-1} produced the highest number of explants growing roots in 'Barangan', while in 'Cavendish' the highest number of explants growing roots was obtained in biotin application of 1.5 mg·L^{-1}. There was no root formation in 'Cavendish' explants without biotin. The study showed that enrichment of the culture media with biotin benefited the *in vitro* growth and development of both 'Barangan' and 'Cavendish' bananas. However, the 'Barangan' responded better than the 'Cavendish'.

Keywords: *in vitro* culture; micropropagation; *Musa acuminata*; tissue culture; vitamins.

INTRODUCTION

'Barangan' and 'Cavendish' are types of banana popular in Indonesia because of their sweet and fresh taste, so they can be commercially developed. These cultivars are rich in nutrients including carbohydrates, fat, protein, starch, cellulose, and tannin. They also have a high content of vitamins (A, B1, B2, B6, B12, C, and D) and minerals (Ca, Na, K, Mg, P, Zn and Fe) [16].

The high market demand for 'Barangan' and 'Cavendish' bananas has resulted in the need to expand the banana plantation area. Meanwhile, large-scale banana cultivation requires a large supply of seeds whose origins are clear, uniform in growth, healthy, and are free from pest and disease attacks. These are the main limiting factors in the intensification and extensification of banana cultivation [68].

Conventional vegetative propagation of banana takes time, the number of seeds produced is limited, and it opens up opportunities for the spread of pathogens. In addition, the non-uniformity of the progeny produced could increase production costs. Therefore, a propagation technology that provides many healthy banana seeds in a relatively short time is much needed. The appropriate modern technology to solve this problem is by the use plant tissue culture or micropropagation technique [14, 45, 71]. Plant tissue culture or micropropagation is the technique for isolating plant parts such as tissues, organs, or embryos, then culturing them in a sterile artificial medium to regenerate and differentiate into complete plants [74].

Banana tissue culture, currently widely known, uses shoots obtained from field-grown seedlings of approximately 1 to 1.5 m high as an explant source. The shoots contain bud eyes (growing points) which will grow when apical dominance is removed. Apical dominance inhibits plant vegetative growth, both root and stem growth. Therefore, banana suckers are the most appropriate source of explants for propagation through tissue culture techniques [1, 24, 29, 73].

The success of tissue culture for plant propagation depends on many factors, one of which is medium composition. The medium provides a well-balanced composition of essential nutrients and growth regulators for the growth and development of various plant tissues. Other important components in plant tissue culture medium are vitamins, nitrogenous substances required in small amounts to act as catalysts that increase metabolism and differentiation of plant cells [2]. Various media formulations thereof show vast differences in vitamin composition such as B5 [22], LS [37], WPM [40], MS [43], Nitsch and Nitsch [46], VW [64], and White [66].

Under an *in vitro* system, vitamins in plants are produced in sub-optimum quantities, so they are must be added externally to meet cell needs [4]. Among the available vitamins, biotin is the one that is also commonly used in plant tissue culture technology [3]. Biotin, which is also known as vitamin H or B7 [48], plays an important role in carboxylation reactions as a cofactor for the carboxylase enzyme., decarboxylase, and transcarboxylase [5]. The biosynthesis pathways of biotin utilize the transfer of sulfur from cysteine to cofactor precursors [6].

The use of biotin in tissue culture was reported by Yelnititis [67] on ramin (*Gonystylus bancanus*) explants and by Samarina *et al.* [55] on *Gerbera jamesonii* tissue culture. Al-Khayri [4] used the

vitamins biotin and thiamine to induce somatic embryogenesis of date palm (*Phoenix dactylifera*). Biotin has been used in banana to establish embryogenic callus and somatic embryogenesis [17, 23, 30, 35]. Biotin has also been successful in the Plant Tissue Culture Laboratory of the West Tanjung Jabung Food Crops and Horticulture Service, Jambi Province. It has become part of the Technical Instructions for the "Development of Banana Saplings Seeds through Tissue Culture Techniques".

Although *in vitro* regeneration systems have been established for banana [14, 19, 28, 33, 39, 41, 58, 59, 61, 63], the role of biotin in plant regeneration has not been defined. The objective of this study was to investigate the effect of biotin on the *in vitro* development of shoots and roots from sucker explants of 'Barangan' and 'Cavendish' bananas and obtain the best biotin concentration to support the growth of explants, both in 'Barangan' and 'Cavendish' bananas.

MATERIALS AND METHODS

Plant materials

This study was conducted at the Plant Biotechnology Laboratory, Faculty of Agriculture, University of Jambi, Indonesia. Plant materials used were 'Barangan' and 'Cavendish' banana suckers obtained from 3 - 4 months old field-grown plants. Suckers were cleaned by washing under running tap water to remove soil traces and other unwanted materials. All leaf sheaths were peeled completely to expose the suckers, and naked suckers were soaked in 1% (v/v) sodium hypochlorite (NaOCl) solution for about 30 minutes followed by rinsing three times with sterile water and left dry on sterile tissue paper.

Culture medium

The medium used was MS composition [43] supplemented with myoinositol (100 $mg \cdot L^{-1}$), nicotinic acid (0.5 $mg \cdot L^{-1}$), pyridoxine-HCl (0.5 $mg \cdot L^{-1}$), thiamine-HCl (0.1 $mg \cdot L^{-1}$), glycine (2 $mg \cdot L^{-1}$), and sucrose (30 $mg \cdot L^{-1}$). Culture medium also supplemented with biotin according to the treatment (0.0 $mg \cdot L^{-1}$, 0.5 $mg \cdot L^{-1}$, 1.0 $mg \cdot L^{-1}$, 1.5 $mg \cdot L^{-1}$, 2.0 $mg \cdot L^{-1}$, and 2.5 $mg \cdot L^{-1}$). The pH of the medium was adjusted to 5.8 ± 0.2 using either 0.1 N NaOH or 0.1 N HCl prior to being solidified with 0.8% (w/v) agar.

Media with different biotin concentrations were heated on a hot plate while stirring with magnetic stirrer to dissolve the agar. Immediately after the agar was completely dissolved the media were poured into culture flasks of 10 mL each, and autoclaved for 15 minutes at a temperature of 121 °C and 1.06 $kg \cdot cm^{-2}$ pressure [7, 13]. Culture flasks containing medium were kept in the stock room at 25 °C before use.

Explant preparation

Culture materials used in this experiment were prepared based on the previously described protocol by Zulkarnain *et al.* [73]. Explants were prepared by excising sterile suckers to approximately 1x1x1 cm. For about one month, explants were cultured on preconditioning medium (MS basal composition without biotin and growth regulator). This was aimed at evaluating culture development, especially related to the occurrence of browning and contamination, and the cause of contamination. The protocol for preparing 'Barangan' and 'Cavendish' banana explants from suckers of field-grown plants is summarized in Figure 1.

Following one month in preconditioned culture, explants were transferred to new MS media supplemented with Benzylamino Purine 1.0 $mg \cdot L^{-1}$ and biotin at different concentrations. On both 'Barangan' and 'Cavendish' bananas biotin was tested at 0.0 $mg \cdot L^{-1}$, 0.5 $mg \cdot L^{-1}$, 1.0 $mg \cdot L^{-1}$, 1.5 $mg \cdot L^{-1}$, 2.0 $mg \cdot L^{-1}$ and 2.5 $mg \cdot L^{-1}$. One explant was cultured in each culture flask.

Culture maintenance

The cultures were maintained in a culture room with a temperature of 25 ± 1 °C and photoperiod of 16 hours per day. The light source came from a white, fluorescent light from tube fluorescent lamps (6500 K of color temperature) installed on the culture racks. This lighting produced an intensity of approximately 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic active radiation (PAR) [8, 9].

Experimental design

The trial used a completely randomized design with four replicates, with 24 experimental units for each cultivar. Each experimental unit consisted of 4 cultures, with 192 culture flasks for both cultivars.

Observations were made on culture browning (the formation of phenolic compounds), shoot formation and development (number of shoot-forming explants and time of shoot emergence), and root formation and development (number of root-forming explants and time of root emergence). The detection of browning was made by visual observation. The cultures were observed for any signs of discoloration from a slight tint to a dark, solid-looking mass around the explants.

Data analysis

Analysis of Variance (ANOVA) by using the Microsoft Excel Spreadsheet application [42] was employed in data analysis, followed by the Least Significant Difference test [10] to see the effect of different levels of biotin on the variables.

RESULTS

Culture browning

Culture browning occurred from the first week of culture initiation in preconditioning medium, on both 'Barangan' and 'Cavendish' cultivars. Browning started to occur from the wounded surface of the explants and then spread to the culture medium. After four weeks in a preconditioning medium, explants were transferred to a new fresh medium with various

concentrations of biotin according to treatments. Although browning occurred in both explants and media, subculture on fresh media proved that browning stopped and explants regenerated shoots within 2 weeks of subculture, both in 'Barangan' and 'Cavendish' (Fig. 2).

Figure 1. The protocol for explant preparation and planting in tissue culture of 'Barangan' and 'Cavendish' bananas. A = banana suckers obtained from field-grown plants as source of explants; B = leaf sheath are peeled exposing the suckers; C = clean suckers; D = suckers are sterilized in 70% alcohol; E = suckers are cut into cube-shaped explants sizing approximately 1 cm^3; F = explants are cultured on media supplemented with biotin; G = cultures are maintained on culture racks in culture room.

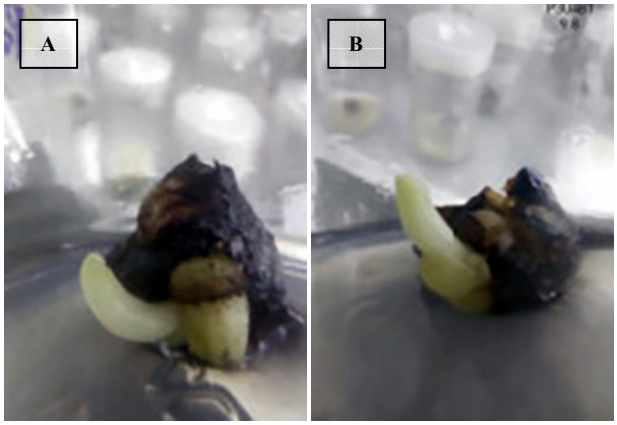

Figure 2. Shoots proliferated from browning sucker explants of 'Barangan' (A) and 'Cavendish' (B) following subculture into new fresh media (2 weeks after subculture).

Shoot Formation

Statistical analysis demonstrated that the two banana cultivars responded differently to aplying biotin. In 'Barangan, analysis of variance showed that the effect of biotin on the percentage of shoot-forming explants was insignificant (*P-value* = 0.16). The number of shoot-regenerating explants ranged from 58.33% (0.5 mg·L^{-1} biotin) to 100.00% (1.5 mg·L^{-1} biotin). In 'Cavendish', the application of biotin also showed no significant effect on the number of shoot-forming explants (*P-value* = 0.51). The number of shoot-forming explants ranged from 50.0% (2.0 mg·L^{-1} biotin) to 83.34% (2.5 mg·L^{-1} biotin). The effect of biotin application on the percentage of shoot-forming explants in both cultivars is presented in Figure 3.

The time of shoot emergence in 'Barangan' explants was also not significantly affected by biotin (*P-value* = 0.57). The shortest time for shoot emergence (24.88 days after subculture) was found on explants cultured on medium with 2.0 mg·L^{-1} biotin. In comparison, the longest time for shoot emergence (39.17 days after subculture) was shown on explants grown on medium with 1.0 mg·L^{-1} biotin. On the other hand, the time of shoot emergence in 'Cavendish' explants was significantly affected by the presence of biotin (*P-value* = 0.00). The shortest time for shoot emergence (14.67 days after subculture) in 'Cavendish' was found on explants grown on media with 2.5 mg·L^{-1} biotin. However, this was not significantly different from those explants cultured on media with 2.0 mg·L^{-1} biotin or 1.5 mg·L^{-1} biotin (17.25 and 18.59 days after subculture, subsequently). The longest time for shoot emergence in 'Cavendish' was found on explants cultured on media without biotin (36.04 days after subculture) (Fig. 4).

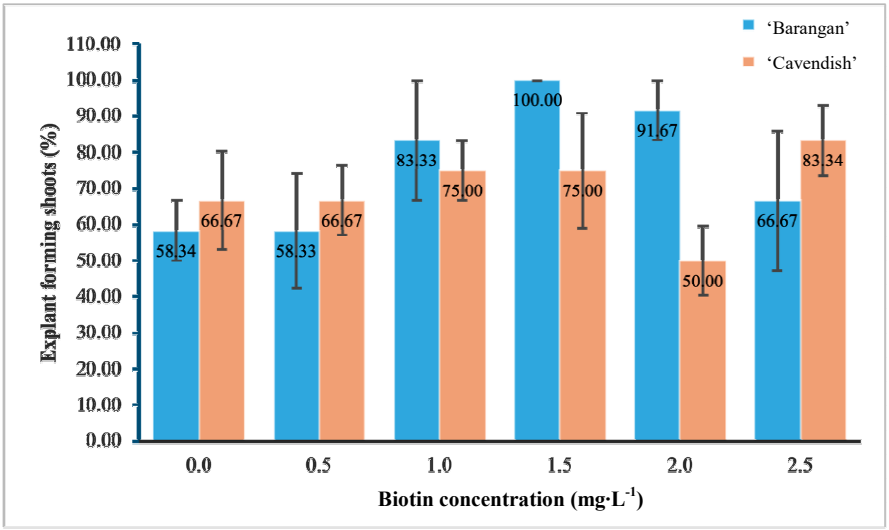

Figure 3. The effect of various concentrations of biotin on the number of shoot-forming explants in tissue culture of 'Barangan' ($LSD_{0.05}$=39.15) and 'Cavendish' ($LSD_{0.05}$=35.98) bananas (error bars indicate Standard Errors).

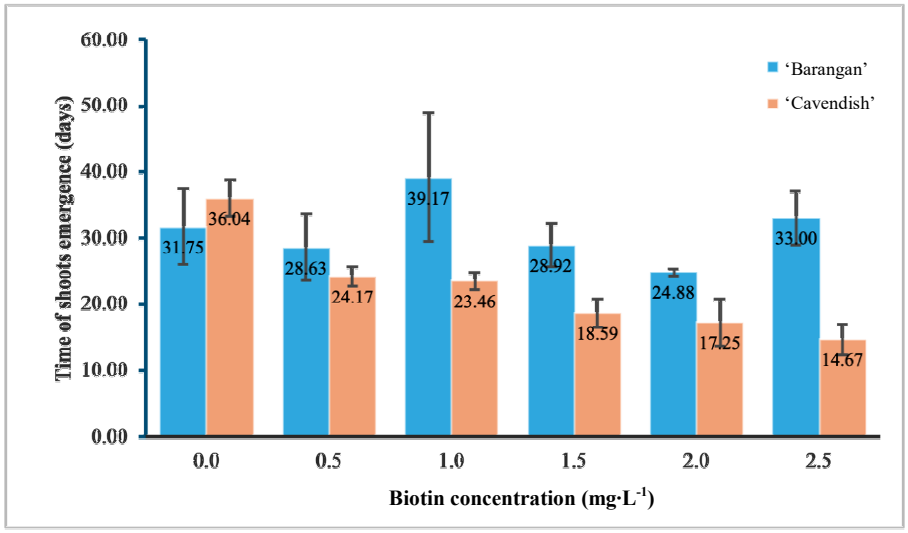

Figure 4. The effect of different concentrations of biotin on the time of shoots emergence (days after subculture) from sucker explants in tissue culture of 'Barangan' ($LSD_{0.05}$=16.24) and 'Cavendish' ($LSD_{0.05}$=7.09) bananas (error bars indicate Standard Errors).

Explant browning did not inhibit shoot or root formation and subsequent growth. Despite browning, the explants still showed signs of being living tissue. They could regenerate shoots (Fig. 5 and 6) and roots (Fig. 9 and 10) on 'Barangan' and 'Cavendish'. The shoots appeared healthy, green or whitish green, and emerged from brown or dark brown explants. Likewise, healthy roots were formed on shoots that grew from brown explants.

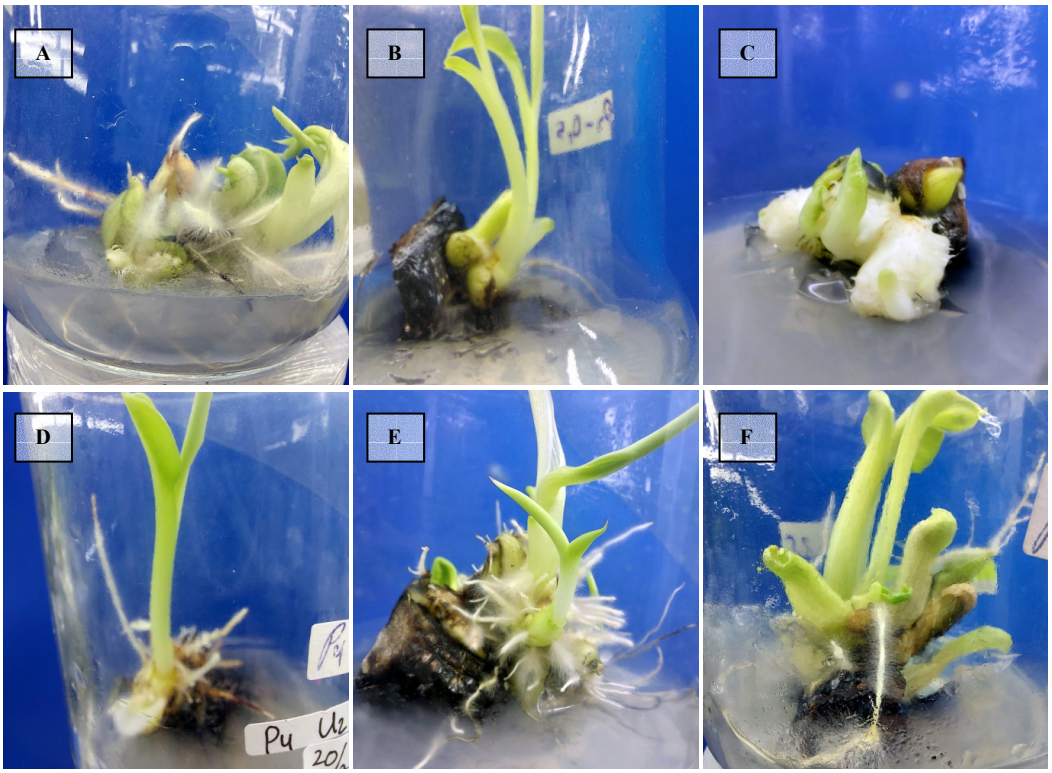

Figure 5. The effect of different concentrations of biotin on *in vitro* shoot growth of 'Barangan' banana (60 days after subculture) (A=0.0 mg·L^{-1}. B=0.5 mg·L^{-1}. C=1.0 mg·L^{-1}. D=1.5 mg·L^{-1}. E=2.0 mg·L^{-1}. F=2.5 mg·L^{-1}).

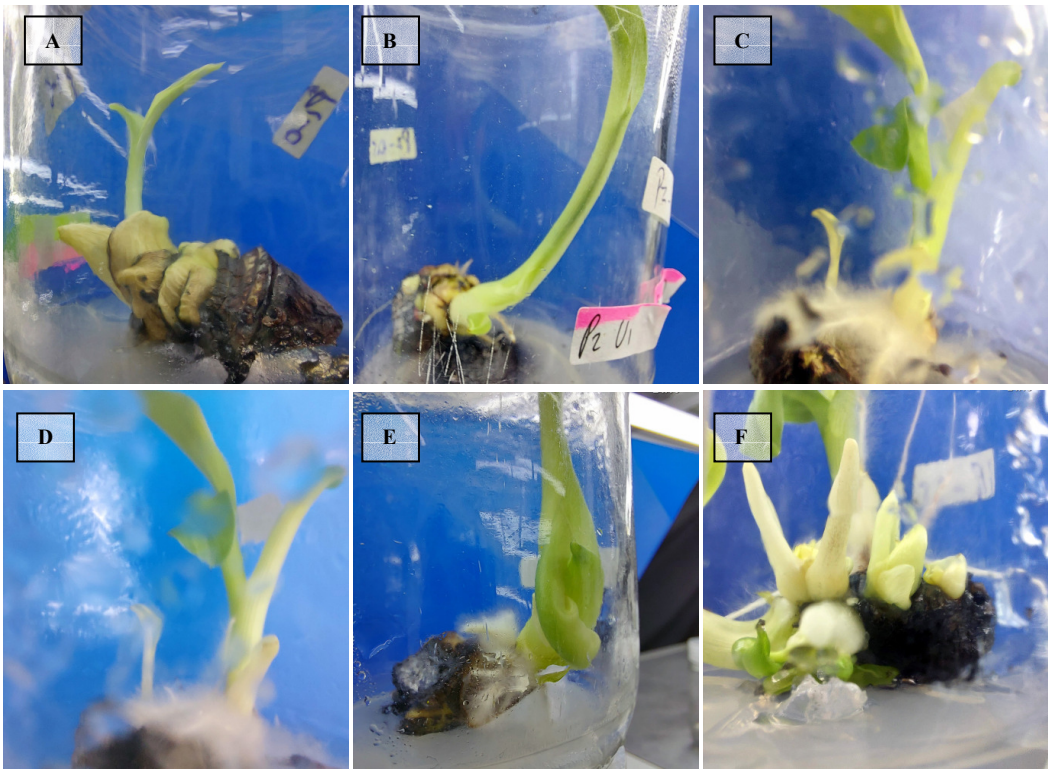

Figure 6. The effect of different concentrations of biotin on *in vitro* shoot growth of 'Cavendish' banana (60 days after subculture) (A=0.0 mg·L^{-1}. B=0.5 mg·L^{-1}. C=1.0 mg·L^{-1}. D=1.5 mg·L^{-1}. E=2.0 mg·L^{-1}. F=2.5 mg·L^{-1}).

Root Formation

The ANOVA showed that the application of biotin did not significantly affect root formation in either 'Barangan' (*P-value* = 0.39) or 'Cavendish' (*P-value* = 0.13). The number of root-forming explants in 'Barangan' ranged from 33.33% (0.5 mg·L^{-1} biotin) to 83.34% (2.0 mg·L^{-1} biotin). Meanwhile, in 'Cavendish', the number of root-forming explants ranged from 16.67% (1.0 mg·L^{-1} biotin) to 50.00% (1.5 mg·L^{-1} biotin). The effect of different biotin concentrations on the number of root-forming explants in 'Barangan' and 'Cavendish' sucker explants is presented in Fig. 7.

Biotin application also did not show any significant effect on the time of root emergence either in 'Barangan' (*P-value* = 0.39) or 'Cavendish' (*P-value* = 0.50). In 'Barangan', the shortest time for root formation was found on the explants cultured on 2.5 mg·L^{-1} biotin (39.63 days after subculture). The longest time was on media supplemented with 0.5 mg·L^{-1} biotin (56.67 days after subculture). Meanwhile, in 'Cavendish' the shortest time for root formation was demonstrated by explants grown on media with 2.5 mg·L^{-1} biotin (31.75 days after subculture) and the longest time was on media supplemented with 1.5 mg·L^{-1} biotin (54.50 days after subculture). The effect of different concentrations of biotin on the time of root emergence on 'Barangan' and 'Cavendish' sucker explants is presented in Fig. 8.

DISCUSSION

Biotin, known as vitamin H or vitamin B7, is one of the tissue culture media components. However, few studies have been reported on investigating its effect. Some reports claimed that biotin is crucial for organogenesis and growth. However, others have not found any important role of biotin in *in vitro* plant

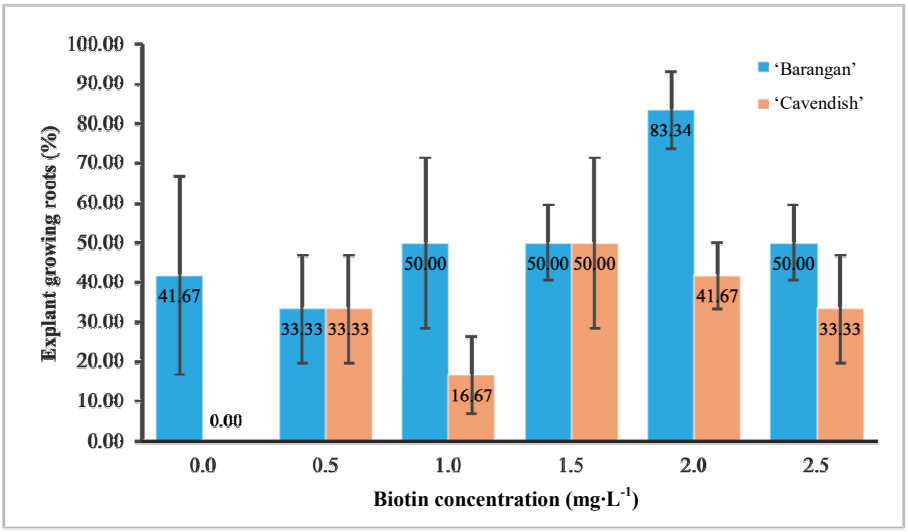

Figure 7. The effect of different concentrations of biotin on the number of explants forming roots in tissue culture of 'Barangan' ($LSD_{0.05}$=16.26) and 'Cavendish' ($LSD_{0.05}$=21.07) bananas (error bars indicate Standard Errors). Note: There was no root formation in 'Cavendish' when cultured in a medium without biotin.

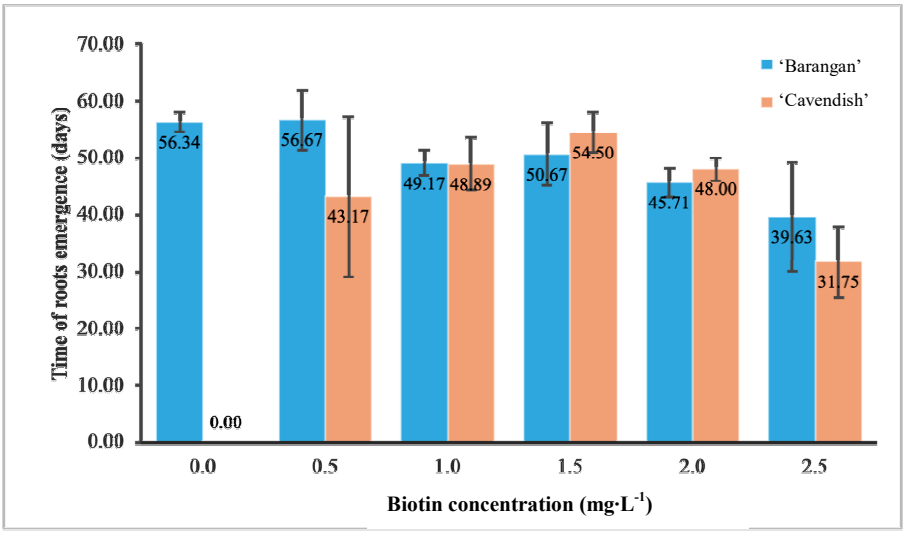

Figure 8. The effect of different concentrations of biotin on the time of roots emergence (days after subculture) from sucker explants in tissue culture of 'Barangan' and 'Cavendish' bananas (error bars indicate Standard Errors). Note: there was no root formation in 'Cavendish' when cultured on medium without biotin.

propagation. However, the use of biotin in plant tissue culture has been studied by many researchers [2, 55, 62]. Biotin was effective for callus initiation, growth, and somatic embryogenesis [4, 18] in date palm tissue culture. This vitamin encouraged the growth of protocorm-like bodies in *Phaleonopsis* Silky Moon orchid [62].

Browning is a common phenomenon in tissue culture with explants isolated from gummy tissues such as banana suckers due to the release of phenolic compounds [27, 34, 49, 50]. The release of phenolic compounds is a natural tissue response to oxidation due

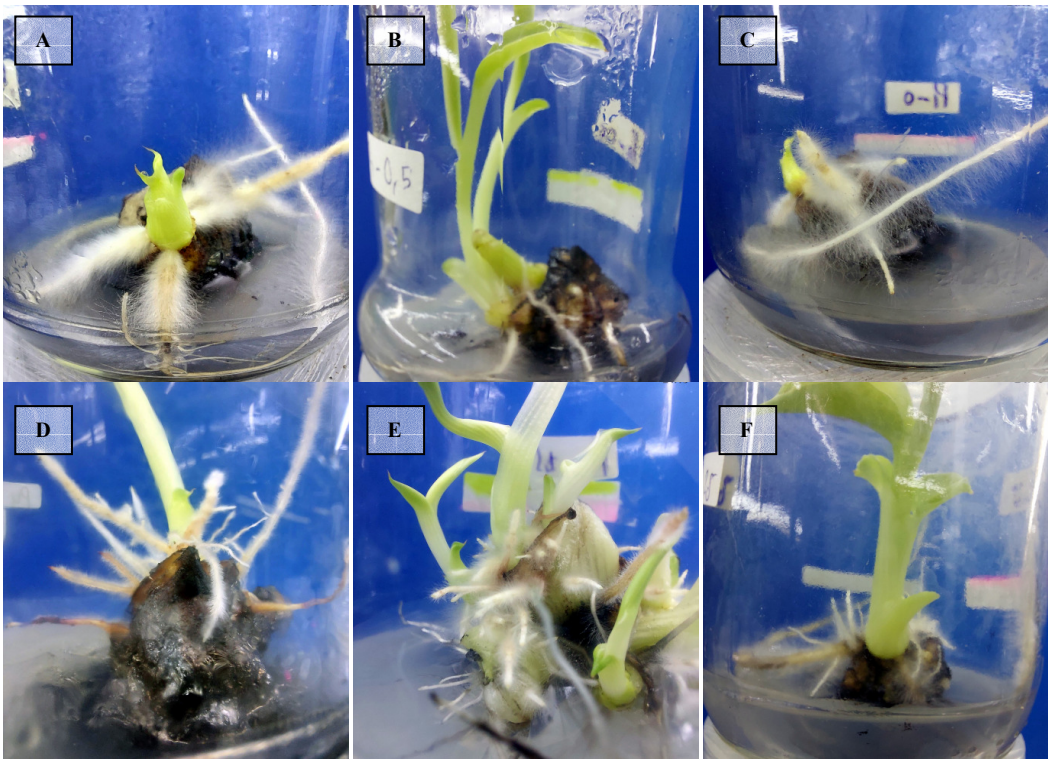

Figure 9. The effect of different concentrations of biotin on *in vitro* root growth of 'Barangan' banana (60 days after subculture) (A=0.0 mg·L^{-1}. B=0.5 mg·L^{-1}. C=1.0 mg·L^{-1}. D=1.5 mg·L^{-1}. E=2.0 mg·L^{-1}. F=2.5 mg·L^{-1}).

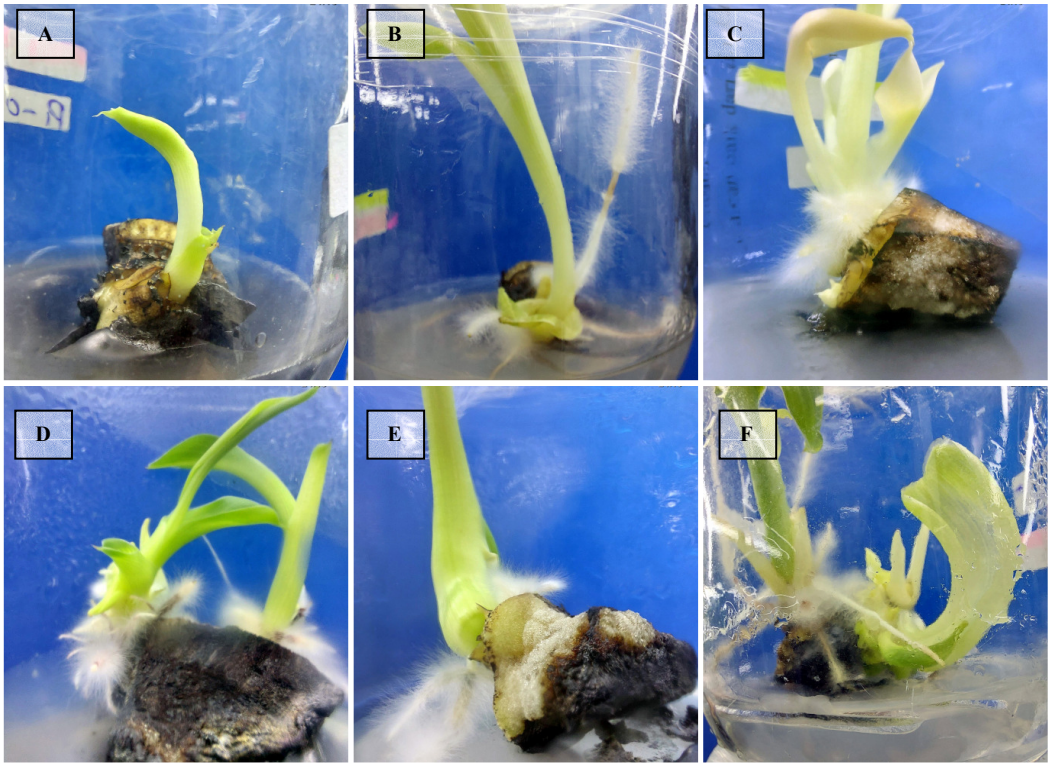

Figure 10. The effect of different concentrations of biotin on *in vitro* root growth of 'Cavendish' banana (60 days after subculture) (A=0.0 mg·L^{-1}. B=0.5 mg·L^{-1}. C=1.0 mg·L^{-1}. D=1.5 mg·L^{-1}. E=2.0 mg·L^{-1}. F=2.5 mg·L^{-1}).

to injury during isolation [20]. These compounds could be toxic to plant tissues, suppressing explant growth and development [53] and even causing explant death [25].

The occurrence of culture browning in most tissue culture systems is mainly due to the enzymatic reactions [12, 15, 70]. Three enzymes are involved in tissue browning: polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase [31, 44, 70]. The browning occurs when such enzymes catalyze various reactions to remove reactive oxygen, heal injured plant tissues [69], and produce melanin [20], a dark pigment that causes the explants to turn brown. This exudate forms a barrier around the cultured tissue inhibiting nutrient absorption [36, 60]. Furthermore, these materials dissolve into the media and causing media browning.

After transfer to new fresh media, explants continue to grow, and no browning was observed. The media remained clear for 60 days of culture. This proves that subculture is a recommended method to overcome browning in banana tissue culture. In accordance with this, Permadi *et al.* [50] suggested that quickly subculturing to a fresh medium could protect explants from the detrimental effects of browning.

Shirazi *et al.* [56] suggested that culture browning could be reduced by subculturing explants onto fresh media. These subcultures can be conducted routinely every week, once a week [38]. Frequent transfer of explants onto new media could prevent explant browning [51] since the cut surface of the explant sealed up and phenolic leaching stopped [65]. Subculture may also avoid infiltrating harmful by-products of phenol oxidation into explant tissues.

Evaluation of explant growth due to biotin application showed the effect of this vitamin on shoot growth. It was found that biotin increased the number of shoot-growing explants and accelerated shoot proliferation on 'Barangan' and 'Cavendish'. The best results in 'Barangan' were obtained when 2.0 $mg \cdot L^{-1}$ were added which resulted in 91.67% explants forming shoots (Figure 3) and shortest time for shoot proliferation (24.88 days after culture initiation) (Fig. 4). Meanwhile, the best shoot proliferation in 'Cavendish' was obtained when biotin was used at 2.5 $mg \cdot L^{-1}$ which resulted in 83.34% shoot-forming explants (Fig. 3) and the fastest shoot proliferation (14.67 days after culture initiation) (Fig. 4).

In addition to playing an important catalytic role as a cofactor in various enzymatic reactions [12, 15, 70] as previously mentioned, biotin regulates gene expression in plants [11]. Concerning regulatory action, some authors also reported the effects of biotin on callus growth and somatic embryogenesis [4, 18]. Samarina *et al.* [55] reported that adding of 1 - 3 $mg \cdot L^{-1}$ biotin in MS media could increase the length of shoots in *Gerbera jamesonii* up to 6 cm.

It was noted that biotin application in tissue culture of 'Barangan' and 'Cavendish' banana sucker explants also increased the number of root-forming explants, as well as accelerated the emergence of roots on 'Barangan' and 'Cavendish'. The best results in 'Barangan' were obtained when 2.0 $mg \cdot L^{-1}$ were added which resulted in 83.34% root-forming explants (Figure 7) and the shortest time for root proliferation (47.51 days after culture initiation) (Figure 4). While in 'Cavendish' the best results were obtained at biotin of 1.5 $mg \cdot L^{-1}$ (50% root-forming explants) but the shortest time for root emergence (54.50 days after culture initiation).

Figures 5 and 6 indicate that biotin was crucial for root proliferation in 'Cavendish'. There was no root formation when biotin was removed from the culture medium. However, root proliferation in 'Barangan' was not biotin-dependent. All explants cultured on all levels of biotin produced roots with different amounts and speeds.

Biotin supplementation has been linked to changes in the expression of genes [11] involved in cell cycle and cell expansion, affecting how shoots and roots grow and develop [54]. Biotin can affect cellular and genetic processes in cell specification, initiation, and elongation of shoots and roots [55].

Our data indicated that biotin had a promotive effect on shoot and root growth, as evidenced by the number of shoot- and root-forming explants. Biotin promoted the growth of *in vitro* shoots and roots in banana planlets due to meristematic cell differentiation [54]. Therefore, biotin can affect different cellular and genetic processes in the development of shoots and roots. The number of shoot- and root-forming explants increased in a manner that was directly related to biotin level in the culture medium. This effect that has been widely reported in cases of *in vitro* culture of bananas [17, 23, 30, 32, 72].

Since biotin is a cofactor of carboxylases, such as acetyl CoA carboxylase, propionyl CoA carboxylase, and pyruvate carboxylase [57], the presence of biotin in the medium may raise mitochondrial activity by increasing carboxylase activity. In plants, mitochondria produce reactive oxygen species (ROS) [47], which can act like signaling molecules, modulate gene expression, and lead to the development of shoots and roots. Foreman *et al.* [21] and Potocký *et al.* [52] demonstrated that ROS molecules are necessary for the growth. In addition, it has been determined that the production and accumulation of ROS regulate calcium homeostasis by modulating of Ca^{2+} permeable channels [26]. Therefore, biotin could also regulate calcium homeostasis in plants and modify the *in vitro* growth of explants.

It can be concluded that the enrichment of culture media with biotin is beneficial for the growth and development of sucker explants of 'Barangan' and 'Cavendish' bananas. However, 'Barangan' showed a better response than 'Cavendish'. This can be seen from shoots and roots regeneration in the 'Barangan' explants which is higher than in 'Cavendish'. However, the time needed by the explants to regenerate shoots and roots was relatively longer than 'Cavendish'.

In both 'Barangan' and 'Cavendish', applying biotin up to 1.5 $mg \cdot L^{-1}$ encouraged shoot formation. However, this shoot formation declined when 2.0 $mg \cdot L^{-1}$ or more biotin was added to the culture medium. Meanwhile, in root formation, the application of biotin up to 2.0 $mg \cdot L^{-1}$ resulted in the highest number of 'Barangan' explants growing roots, while in the 'Cavendish', the highest root formation was obtained in the biotin application of 1.5 $mg \cdot L^{-1}$.

Therefore, it is clear that enrichment of culture media with biotin vitamins is very beneficial in supporting efforts to propagate agricultural plants using tissue culture techniques. Our results showed its beneficial effect on the growth and development of banana sucker explants, which is why further investigations of biotin action are needed.

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Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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