Growth Response of Siames Orange (*Citrus nobilis* L.) Explants on MS Media with the Addition of BAP and NAA in Vitro

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Abstract

The cultivation of Siamese orange (*Citrus nobilis* L.) in Kampar Regency has the main obstacle, namely the attack of diplodia disease caused by *Botryodiplodia theobromae*. This disease causes plant death and decreased citrus production. In vitro culture techniques can be carried out as an effort to produce disease-free seeds through the use of meristems as explant, in vitro culture will speed up the multiplication process and produce lots of seeds. This research aims to investigate the effect of interaction BAP NAA on MS medium on growth of Siamese orange explant. The study was carried out at the Biotechnology Laboratory, Faculty of Agriculture, Riau University and arranged to two-factor factorial randomized group design, which is BAP with four levels concentration 0, 1, 3, and 5 ppm and NAA four levels concentrations 0, 0.1, 0.2, and 0.3 ppm with three replications. The results showed that the treatment without BAP and 0.1 ppm NAA had the best effect on plantlet formation as indicated by the root emergence time of 17 DAP (days after planting) and 1 ppm BAP and 0.1 ppm NAA treatment had the best effect on callus formation. Siamese orange (*Citrus nobilis* L.).

Keywords: Auxin, cytokinine, in vitro, MS medium, Siamese orange.

Introduction

Citrus plants are one of the fruit commodities that have high economic value because they contain high vitamins and antioxidants that are beneficial for health so that their development needs attention. The majority of oranges grown in Indonesia are Siam oranges, which account for 70% of all oranges cultivated (Hanif (8)). Siamese oranges from Kampar are well-known in Riau Province. This Siamese orange (*Citrus nobilis* Lour.) was first cultivated in Kuok Village, Kampar Regency. According to Fauzana *et al.* (7), Kuok Village is also the centre of Siamese orange production in Riau Province.

The main obstacle to citrus cultivation in Kampar Regency is the presence of pests and diseases that have an impact on the diversion of land for the cultivation of other crops. Diplodia disease caused by the fungus *Botryodiplodia theobromae* is one of the diseases that attack the stems of citrus plants. This fungal attack causes plants to die so that citrus production decreases (Dewi *et al*. (4). Diplodia disease that attacks residents' Siamese orange plantations in Kampar Regency also has an impact on reducing the area of land planted with Siamese oranges (Sihombing, (24)). Conventional plant propagation generally requires a long time and technical constraints in the field to obtain a large number of seeds, therefore, in vitro culture techniques are an option in an effort to provide seeds of a plant (Basri, (3)). In vitro culture techniques can be used to eliminate systemic diseases in a plant so that disease-free seedlings are obtained, besides that in vitro culture can accelerate the propagation process so as to produce a lot of seedlings in a relatively fast time.

In vitro culture is a technique to grow cells, tissues, and plant organs in the laboratory on an artificial medium containing complete nutrients aseptically (sterile) in order to regenerate into whole plants (Dwiyani, (5)). The provision of chayote seedlings from Kampar in vitro can be done through seed cultivation contained in the fruit. Citrus seeds have polyembryonic properties, namely the formation of zygotic embryos and a number of adventitious embryos in one seed. Adventitious embryos from these nuselar cells can produce plants that are genetically uniform in accordance with their parents and free from disease if propagated vegetatively through in vitro culture techniques that are treated with the addition of nutrients and growth regulators. The groups of growth regulators that are very important in in vitro culture are auxins and cytokinins. The auxin groups that are often used include IAA, IBA, 2.4-D, and NAA, while the cytokinin groups are BA, BAP, Zeatin and Kinetin. This study uses BAP and NAA as growth regulators, according to Hendaryono and Wijayani (10) among auxin group growth regulators, NAA has the advantage that it is not easily decomposed by enzymes released by cells or heating in the sterilisation process. Aziz *et al.* (2) stated that BAP is the most active growth regulators in the process of cell division, propagation and in vitro shoot formation in several types of plants. According to Rionaldi (21) NAA at certain concentrations serves to spur this process. The addition of NAA and BAP is expected to support the growth of citrus subcultures

so that they grow immediately and will spur the formation of buds and roots.

Research on the addition of growth regulators NAA and BAP on the multiplication of shoots of Kanci citrus shoots (*Citrus* sp.) in vitro has been done by Rahmi (20) and found that BAP treatment at a concentration of 2.5 mg.l^{-1} is the best treatment for the percentage of explants that experience multiplication and when shoots appear faster. Research on the addition of BAP and NAA to Murashige and Skoog (MS) media for the growth of citrus plant explants in vitro until now has never been done.

This study aims to determine the effect of the interaction of BAP and NAA on MS media on the growth of citrus explants and to get the best combination of BAP and NAA in supporting the growth of citrus explants.

Material and Methods

This research was conducted from November 2021 – February 2022 at the Biotechnology Laboratory of the Faculty of Agriculture, Riau University, Bina Widya Campus km 12.5, Simpang Baru Village, Tampan District, Pekanbaru. This research is a factorial experiment, which is arranged according to a group randomised design. The first factor was BAP with four levels of treatment: 0 ppm, 1 ppm, 3 ppm and 5 ppm. The second factor is NAA with four treatment levels: 0 ppm; 0.1 ppm, 0.2 ppm and 0.3 ppm so that 16 treatment combinations were obtained and repeated three times. Data from variance analysis were further, tested with Duncan's multiple range test at the 5% level. Observation data were transformed using the formula $\sqrt{y}$ + 0.5 and analysed using statistical analysis system (SAS) software except for the observation of the percentage of living explants.

The implementation of this research was carried out by planting explants in LAFC. The explants planted were adventitious buds derived from nuselar embryos from in vitro sprouts of Kampar-origin Siam oranges aged 2 weeks after planting. Shoots that do not experience contamination are then cut along 3 cm and transferred from pre-treatment media (MS_0) to treatment media (MS with the addition of BAP and NAA according to treatment) by planting one explant per bottle using sterile tweezers, and incubated in the culture room and then made observations. The parameters observed consisted of: root emergence time (DAP) by counting the number of days required for the emergence of roots approximately 0.5 cm long, the number of roots (fruit) was counted at the end of the study (60 DAP) and what was counted were roots that already had a length of 0.5 cm, root length (cm) measured at the end of the study using a crossbar, shoot height increase (cm) measured at the end of the study using a crossbar, increase in the number of leaves (blade) calculated at the end of the study, and the percentage of living explants (%) calculated using the formula: $\frac{\text{number of living explants}}{\text{total number of explants}}$ x 100%

Results and Discussion

The root emergence time (DAP)

The results of the analysis of variance showed that the provision of BAP factors had a significant effect while the provision

of NAA factors and the interaction of the two had no significant effect on the time of emergence of roots of adventitious shoot explants of siamese orange plants. The results of Duncan's multiple range test at the 5% level are presented in Table 1.

Table 1 shows that the treatment of 0 ppm BAP was able to grow roots while those given BAP were not able to grow roots, presumably the concentration of BAP given was too high, thus inhibiting root growth. In accordance with the opinion of Fathurrahman (6) which states that too high a concentration of BAP can be an obstacle to nutrients absorbed by explants to form new individuals. NAA treatment alone is able to grow roots that are not significantly different between treatments.

Table 1. Average time to root emergence (DAP, day after planting) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP concentration (ppm)	NAA (ppm)				Average
	0	0.1	0.2	0.3	
0	31.83 a	17.00 b	18.00 b	21.00 b	21.95 A
1	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
3	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
5	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
Average	7.95 A	4.25 A	4.50 A	5.25 A	

Numbers in the same row and column followed by the same lowercase letter, numbers in the same row or column followed by the same uppercase letter are not significantly different according to Duncan's multiple range test at the 5% level.
Notes. Zero (0.00) indicates no root formation in the explants.

In accordance with the results of research by Tyas *et al*. (28) that the provision of exogenous auxin (NAA) can accelerate root induction in pamelo leaf explants. Rahman *et al*. (19) added that the addition of exogenous auxin to the culture media can stimulate an increase in root initiation and faster root formation. In addition, the buds used as explants also contain auxin hormones that can stimulate cell division in the process of root formation initiation.

Table 1 also shows that in the provision of other treatment combinations cannot give rise to roots in conjoined orange explants, but able to grow callus (Figure 1). The growing callus is characterised by swelling at the bottom of the explant. This swelling indicates that the explants have responded to the treatment given. The formation of callus is caused by the balance of auxin and cytokinin in the treatment given auxin and cytokinin and the stimulation of wounds that cause the balance in the cell wall to change direction, some protoplasts flow outward so that callus begins to form. According to Yelnitis (30), callus induction begins with the elongation of the wounded part, due to the balance of the ratio of growth regulator in the explant with growth regulator in the growth medium so as to spur cell division and enlargement in the injured hypocotyl. Arianto *et al*. (1) also stated that the wound caused by the slice at the base of the explant will cause growth regulators in the growing medium to diffuse into the plant tissue. Growth regulators that have been

absorbed will then stimulate cell division, especially the cells at the base of the explants.

Adventitious buds also appeared on some explants that formed callus, namely when 1 ppm BAP without NAA, 3 ppm BAP without NAA, 5 ppm BAP without NAA, 3 ppm BAP and 0.1 ppm NAA, 5 ppm BAP and 0.1 ppm NAA, and 3 ppm BAP and 0.3 ppm NAA were applied (Figure 2).

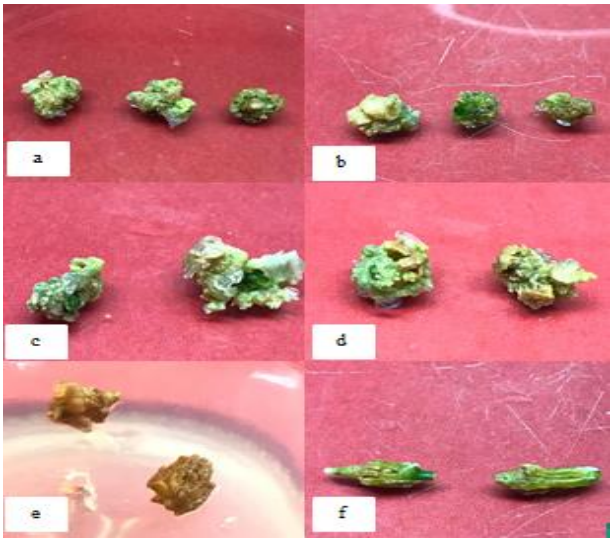

Figure 1. Callus is formed when the concentration of auxin is relatively balanced with cytokinin. Description: a) Treatment of 1 ppm BAP and 0.3 ppm NAA, b) Treatment of 5 ppm BAP without NAA, c) Treatment of 1 ppm BAP and 0.1 ppm NAA, d) Treatment of 1 ppm BAP and 0.2 ppm NAA, e) Treatment of 5 ppm BAP and 0.2 ppm NAA, f) Treatment of 5 ppm BAP and 0.3 ppm NAA.

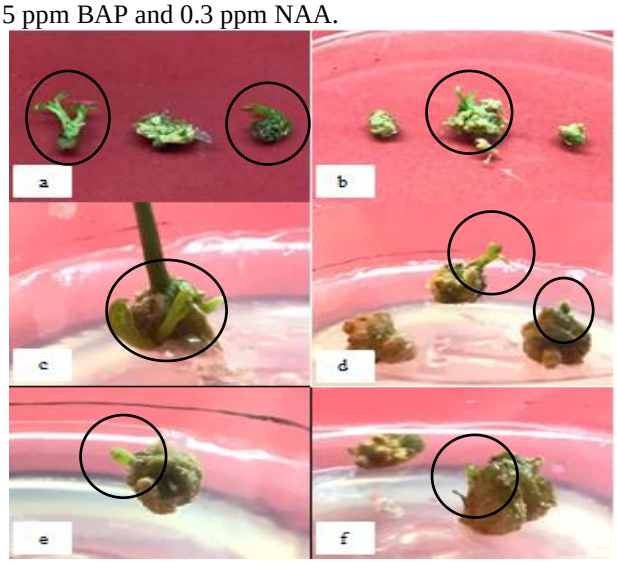

Figure 2. Adventitious shoots that grow on Siamese orange (*Citrus nobilis* L.) explants aged 60 DAP on MS media. Description: a) Treatment of 1 ppm BAP without NAA, b) Treatment of 3 ppm BAP and 0.1 ppm NAA, c) Treatment of 3 ppm BAP and 0.3 ppm NAA, d) Treatment of 3 ppm BAP without NAA, e) Treatment of 3 ppm BAP and 0.2 ppm NAA, f) Treatment of 5 ppm BAP and 0.1 ppm NAA.

At the end of the study, there were green-coloured protrusions on the callus that had formed (Figure 2). These protrusions are adventitious buds that will grow into new shoots. The most clearly formed adventitious buds were in the treatment of 1 ppm BAP without NAA. The formation of adventitious buds is thought to be due to

the influence of the addition of higher cytokinin into the culture medium. In accordance with the statement of Kartiman *et al*. (11) that BAP functions in cell division, encourages shoot proliferation, and differentiation of adventitious buds from callus. The results by Sepdian (23) also showed that BAP has the best ability to induce buds in stevia plants compared to kinetin and TDZ.

Number of root

The results of the analysis of variance showed that the interaction of BAP and NAA and the NAA factor had no significant effect, while the BAP factor had a significant effect on the number of roots of adventitious shoot explants of siamese orange plants. The results of Duncan's multiple range test at the 5% level on the number of roots can be seen in Table 2. Table 2 shows that the treatment without BAP was able to grow roots, whereas the treatment with BAP was not able to grow roots. This is because the concentration of exogenous auxin given is higher than BAP so that it leads to root initiation. In accordance with the opinion of Pradhan *et al*. (17) which states that the growth of plantlet roots is strongly influenced by the presence of relatively high auxin growth regulator. Marlin (12) states that root formation in in vitro culture requires auxin without cytokinin or cytokinin in low concentrations. Increasing the concentration of auxin to a certain extent in this study can increase the number of roots in explants even though statistical analysis is not significantly different. This is probably because the endogenous auxin in the explants is optimal to stimulate the division and elongation of root cells. The concentration of auxin increased to 0.2 ppm was able to produce the most number of roots, but the increase in the provision of 0.3 ppm NAA decreased the number of roots formed. In accordance with the results of research by Putri *et al*. (18) which showed that the higher the concentration of NAA, the lower the average value of the number of roots because the increase in NAA concentration can inhibit root growth. The addition of BAP with various concentrations was not able to grow roots. It is suspected that the concentration of BAP given is too high so that it inhibits root growth.

Based on Figure 3, it can be seen that the treatment of 0.2 ppm NAA without BAP was able to grow more roots than other treatments. This shows that the explants planted in the treatment media without BAP are able to grow roots.

Table 2. Average number of roots (fruit) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP concentration (ppm)	NAA (ppm)				Average
	0	0.1	0.2	0.3	
0	1.11 b	3.66 a	5.58 a	4.77 a	3.78 A
1	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
3	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
5	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
Average	0.27 A	0.97 A	1.39 A	1.19 A	

Numbers in the same row and column followed by the same lowercase letter, numbers in the same row or column followed by the same uppercase letter are not significantly different according to Duncan's multiple range test at the 5% level.
Notes. Zero (0.00) indicates no root formation in the explants.

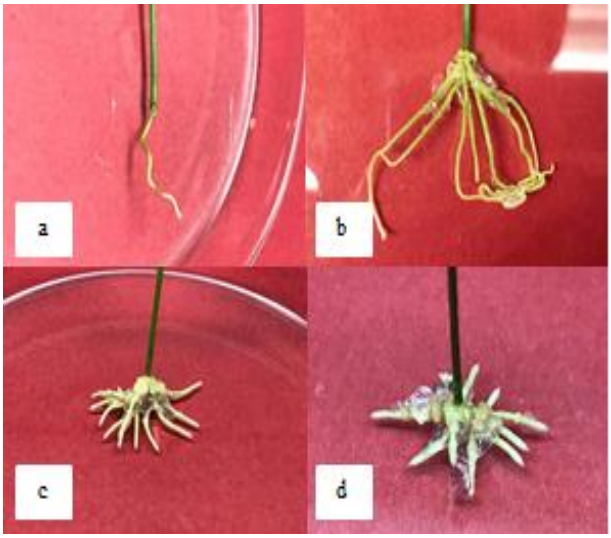

Figure 3. Number of roots that grow on the explants of Siamese orange (*Citrus nobilis* L.) aged 60 DAP on MS media. Description: a) Treatment without BAP and NAA, b) Treatment without BAP and 0.1 ppm NAA, c) Treatment without BAP and 0.2 ppm NAA, d) Treatment without BAP and 0.3 ppm NAA.

Root length (cm)

The results of the analysis of variance showed that the interaction of BAP and NAA and the NAA factor had no significant effect, while the BAP factor had a significant effect on the length of the roots of adventitious shoot explants of siamese orange plants. The results of Duncan's multiple range test at the 5% level of root length can be seen in Table 3.

Table 3 shows that the root length in the treatment without BAP and without NAA tends to produce the longest average root length, but when increasing the concentration of NAA in the treatment without BAP, there is a tendency to decrease the root length produced. Increasing the concentration of BAP inhibits the formation of plantlet roots. Marlin (12) states that at higher levels of auxin than cytokinin, tissue morphogenesis will lead to root formation. Hartati *et al.* (9) stated that root formation in plantlets is regulated by a higher ratio of auxin than cytokinin. High cytokinin concentrations will usually inhibit the formation or growth of plantlet roots.

Table 3. Average root length (cm) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP concentration (ppm)	NAA (ppm)				Average
	0	0.1	0.2	0.3	
0	3.00 a	2.54 a	0.93 a	0.84 a	1.83 A
1	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B

3	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
5	0.00 c	0.00 c	0.00 c	0.00 c	0.00 B
Average	0.75 A	0.63 A	0.23 A	0.21 A	

Numbers in the same row and column followed by the same lowercase letter, and numbers in the same row or column followed by the same uppercase letter are not significantly different according to Duncan's multiple range test at the 5% level. Notes. The number zero (0.00) indicates no root formation in explants.

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Based on Figure 4, it can be seen that there are differences in the length of roots that grow in the treatment without growth regulator, 0.1 ppm NAA without BAP, 0.2 ppm NAA without BAP, and 0.3 ppm NAA without BAP. Treatments that were not given additional growth regulator into the media showed the longest roots. This is thought to be because the endogenous auxin hormone content in the explants is high enough so that without being given growth regulator the roots are able to grow well. Siron (25) states that endogenous hormones are sufficient for plant growth so that the addition of BAP and NAA from outside does not provide an increase in plant growth.

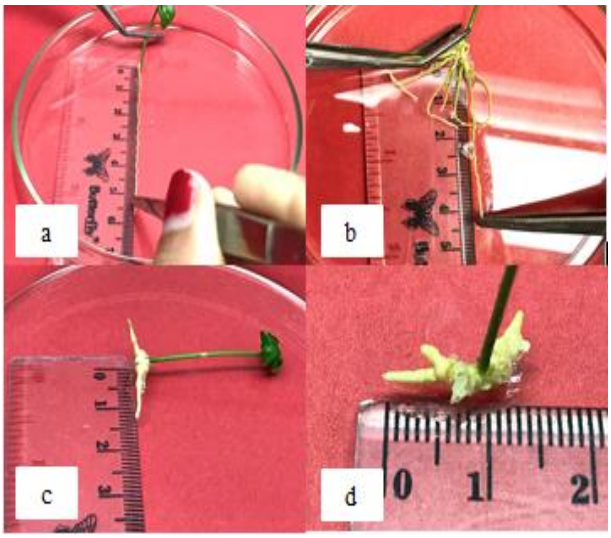

Figure 4. The length of the roots that grow on the explants of siam orange (*Citrus nobilis* L.) aged 60 DAP on MS media. Description: a) Treatment without BAP and NAA, b) Treatment without BAP and 0.1 ppm NAA, c) Treatment without BAP and 0.2 ppm NAA, d) Treatment without BAP and 0.3 ppm NAA.

Shoot height increment (cm)

The results of the analysis of variance showed that the provision of BAP factors and the interaction of BAP and NAA had a significant effect while the provision of NAA factors had no significant effect on the increase in shoot height of adventitious shoot explants of siamese orange plants.

The results of Duncan's 5% multiple range test on shoot height increment can be seen in Table 4.

Based on the data in Table 4, the shoot height increase in the treatment of 1 ppm BAP and 0.3 ppm NAA is significantly different from the shoot height increase in the treatment without BAP and 0.2 ppm NAA, without BAP and 0.3 ppm NAA and 5 ppm BAP and 0.2 ppm NAA and is not significantly different from the shoot height increase in other treatments. The most shoot height increase was shown by the treatment of 1 ppm BAP and 0.3 ppm NAA which was 0.92 cm. This is thought to be due to the concentration of cytokinin given is higher than auxin, so it can spur the growth of shoots on the explants. In accordance with the opinion of Mashud (13) which states that BAP is a cytokinin that functions to stimulate shoot growth, affects cell metabolism, and functions as a driver of physiological processes that depend on the concentration given. Widiastoety (29) added that stem elongation occurs due to the process of division, elongation and enlargement of new cells that occur in the apical meristem and stem internode, which causes the plant to increase in height. Rustikawati et al. (22) also stated that BAP plays a role in cell division, and when combined with a little auxin can also cause cell display, and one of the effects is expressed by an increase in shoot height.

Table 4. Average shoot height increase (cm) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP concentration (ppm)	NAA (ppm)				Average
	0	0.1	0.ar	0.3	
0	0.42 abc	0.46 abc	0.10 c	0.11 c	0.27 B
1	0.75 ab	0.61 abc	0.49 abc	0.92 a	0.69 A
3	0.47 abc	0.48 abc	0.56 abc	0.46 abc	0.49 AB
5	0.42 abc	0.39 abc	0.26 bc	0.44 abc	0.37 B
Average	0.51 A	0.48 A	0.35 A	0.48 A	

Numbers in the same row and column followed by the same lowercase letter, and numbers in the same row or column followed by the same uppercase letter are not significantly different according to Duncan's multiple range test at the 5% level. Notes. The number zero (0.00) indicates no root formation in explants

Increase number of leaves (strands)

The results of the analysis of variance showed that the provision of BAP factors and the interaction of BAP and NAA had a significant effect while the provision of NAA factors had no significant effect on the number of leaves of adventitious shoot explants of siamese orange plants. The results of Duncan's multiple range test at the 5% level on the increase in the number of leaves can be seen in Table 5.

Based on Table 5, the number of leaves in the treatment of 1 ppm BAP and 0.1 ppm NAA is significantly different from the number of leaves in the treatment without growth regulator, 3 ppm BAP without NAA, 5 ppm BAP without NAA,

0.1 ppm NAA without BAP, 5 ppm BAP and 0.1 ppm NAA, 0.2 ppm NAA without BAP, 3 ppm BAP and 0.2 ppm NAA, 5 ppm BAP and 0.2 ppm NAA, and 0.3 ppm NAA without BAP and not significantly

different from the number of leaves in other treatments. The highest number of leaves was obtained in the treatment of 1 ppm BAP and 0.1 ppm NAA, which was 2.69 leaves. Triningsih *et al*. (27) suggested that the provision of BAP into in vitro media can have a good effect on the speed of leaf emergence. This is in accordance with the opinion of Nurhanis (14) which states that the provision of cytokinin at a higher concentration than auxin in in vitro culture will stimulate the growth of shoots and leaves.

Table 5. Average increase in the number of leaves (strands) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP concentration (ppm)	NAA (ppm)				Average
	0	0.1	0.2	0.3	
0	0.41 bcd	0.66 de	0.08 e	0.16 e	0.58 C
1	2.33 ab	2.69 a	2.02 abc	2.25 abc	2.32 A
3	1.58 bcd	1.91 abc	1.50 bcd	1.83 abc	1.70 B
5	1.22 cd	1.72 bcd	1.50 bcd	2.08 abc	1.63 B
Average	1.63 A	1.74 A	1.27 A	1.58 A	

Numbers in the same row and column followed by the same lowercase letter, and numbers in the same row or column followed by the same uppercase letter are not significantly different according to Duncan's multiple range test at the 5% level.

Percentage of living explants (%)

Data from the observation of the parameters of the percentage of living explants (%) in Siamese orange explants at the age of 60 DAP. The average percentage of live explants from various treatments of BAP and NAA can be seen in Table 6.

Table 6 shows that the average percentage of live explants of Siamese orange plants in all treatments tends to be high, which is above 50% (75 – 100%). Living explants are explants that are able to survive and do not experience browning or contamination by microorganisms. This is thought to be because the explants used for this study are the result of subculture so that the explants are sterile. High percentage of living explants indicates that the method of sterilisation and planting of explants used in this study is appropriate, besides that the explants have high vitality and are able to carry out continuous growth so that the explants can survive in the given medium. In accordance with the opinion of Sundari (26) which states that the ability of explant life in vitro culture will depend on the explant itself, while the durability of the explant to stay alive is influenced by the type and composition of the medium used.

Explants that do not live in in vitro culture are caused by contamination by microorganism, namely fungi. According to Oratmangun (15) contamination can occur due to microorganisms entering the media culture bottles or tools that are less sterile, dirty work and culture rooms, carelessness when planting and can come from both internal and external explants. The appearance of fungal contamination in this study is thought to be due to culture bottles or tools that are less sterile.

Contamination caused by fungi is characterised by white spots on the media and around the explants and continues to spread covering the surface of the media. According to Orlikowska *et al*. (16), the presence of microbes whose growth dominates the explants causes competition for nutrients in the media, limited available oxygen, and increased explant mortality.

Table 5. Average increase in the number of leaves (strands) of citrus explants aged 60 DAP on the application of BAP and NAA. Data were transformed using $\sqrt{y} + 0.5$

BAP Concentration (ppm)	NAA Concentration (ppm)	Percentage of living explants
0	0	100
0	0.1	75.00
0	0.2	91.67
0	0.3	91.67
1	0	83.33
1	0.1	83.33
1	0.2	75.00
1	0.3	83.33
3	0	100
3	0.1	100
3	0.2	91.67
3	0.3	91.67
5	0	91.67
5	0.1	91.67
5	0.2	91.67
5	0.3	100

Conclusion

Based on the results of the study, it can be concluded that the interaction of BAP and NAA has a significant effect on the increase in shoot height and the increase in the number of leaves, but has no significant effect on the time of root emergence, number of roots, root length, and percentage of living explants. The treatment of giving no BAP and 0.1 ppm NAA gives effect to the formation of plantlets because it is able to grow roots at 17 DAP and the combination of 1 ppm BAP and 0.1 ppm NAA gives effect to the formation of callus of siamese orange plants (*Citrus nobilis* L.).

Conflict of interest

The authors declare no conflict of interest.

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