Sampah | Gmail - S | LoAAbstr | 2981Mur | Gmail - IC | Gmail - [I | Gmail - [I | MURNID | (10) Wha

smujo.id/nb/authorDashboard/submission/11280

Notifications

[nb] Editor Decision

2022-08-13 03:19 AM

MURNI DWIATI, AGUS HERY SUSANTO, IMAN BUDISANTOSO:

We have reached a decision regarding your submission to Nusantara Bioscience, "Somatic embryogenesis of the selected intergeneric hybrid between Phalaenopsis 2166 and Vanda 'Saint Valentine': Application of NAA and TDZ".

Our decision is to: Accept Submission

Nusantara Bioscience

11280Article Text....doc | 11280Article Text....doc | 2981 Murni Dwiati.pdf | Gmail - [ICSARD2....html | Show all

4:03 PM 8/13/2022

Sampah | Gmail - S | LoA Abstr | 2981 Mur | Gmail - IC | Gmail - [I | Gmail - [I | MURNID | (10) Wha

smujo.id/nb/authorDashboard/submission/11280

Notifications

[nb] Editor Decision

2022-06-22 07:29 AM

Murni Dwiati, Agus Hery, Iman:

We have reached a decision regarding your submission to Nusantara Bioscience, "Somatic embryogenesis of the selected intergeneric hybrid between Phalaenopsis 2166 and Vanda 'Saint Valentine': Application of NAA and TDZ".

Our decision is: Revisions Required

Reviewer C:

Recommendation: Revisions Required

11280-Article Text....doc | 11280-Article Text....doc | 2981 Murni Dwiati.pdf | Gmail - [ICSARD2....html | Show all

4:01 PM 8/13/2022

smujo.id/nb/authorDashboard/submission/11280

Messages

Note	From
Dear Author(s), Pls, find attached file for an uncorrected proof (Copyedited file). The revised manuscript (Corrected proof) is awaited. Do not worry about layout changes due to revision; our staff will fix it again. Note: Kindly TURN ON track changes when you make improvements. dewinurpratiwi, SomaticMurni Dwiati.doc	dewinurpratiwi 2022-08-11 08:08 AM
Dear Editor(s), Thank you for sending us the uncorrected proof version of our manuscript. We have checked it carefully and it seems that no improvement is required. So, we think that it could be subjected to further process for publication. Thank you for assistance. Sincerely yours, Murni Dwiati and team	murnidwi 2022-08-11 08:53 PM

Add Message

11280-Article Text....doc | 11280-Article Text....doc | 2981 Murni Dwiati.pdf | Gmail - [ICSARD2...html | Show all

3:58 PM 8/13/2022

Somatic embryogenesis of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine': Application of NAA and TDZ

Abstract. An intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' has successfully produced a hybrid seedling with several characters of potentially developing into plant individuals with flowers of better performance. Therefore, callus of the selected hybrid should be developed into ~~plbs~~ PLBs by means of *in*~~-~~ *vitro* culture technique employing somatic embryogenesis supported by the application of growth regulators. This study aims to unveil the effect of NAA and TDZ in stimulating the formation of embryogenic calli of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine'. The experiment was arranged in a factorial Randomized Complete Block Design (RCBD) involving two factors, i.e. types of growth regulators and the levels of concentrations of each substance. It was found that the combination of NAA and TDZ had significant effect on the growth of the embryogenic calli. The combination of NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} resulted ~~in~~ the calli that potentially differentiate into ~~plbs~~PLBs. This finding indicates that NAA and TDZ should be applied appropriately to stimulate somatic embryogenesis in the intergeneric hybrid.

Formatted: Font: Italic

Formatted: Font: Italic

Key words: Intergeneric hybrid; NAA; *Phalaenopsis* 2166; TDZ; *Vanda* 'Saint Valentine'

Running title: Somatic embryogenesis of intergeneric hybrid

INTRODUCTION

The members of Family Orchidaceae are mostly known as ornamental plant species due to their distinctive characteristics of flowers. However, overexploitation and alteration in land-use have caused some orchid species vulnerable to extinction. For instance, all of the 115 identified orchid species from Mount Ungaran, Central Java, Indonesia, are listed in Appendix II of the CITES and four of them are even listed in the IUCN Red List (Kurniawan et al., 2021). On the other hands, some wild orchid species can be potentially used as parental lineages to produce hybrid varieties of desirable better performances including flower colour, shape, and resistance (Li et al., 2021).

An intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' has resulted in several hybrid seedlings which have been characterized, both phenotypically and molecularly. Based on the leaf shape, edge, and colour, the intergeneric hybrids were likely to resemble *Phalaenopsis* 2166 as the female parent, although some variations of leaf shape and colour were also observed. In general, it could be said that maternal inheritance of the phenotypic characters in the intergeneric hybridization occurred. Hence, it is reasonable that the hybrid seedlings showed the best growth when grown in New Phalaenopsis (NP) medium (Dwiati et al., 2020a). Then, molecular characterization by the use of *ndhE* partial gene revealed that 11 of 14 hybrids obtained had the same sequences of *ndhE* partial gene as that of *Phalaenopsis* 2166. The sequences have now been registered in NCBI database with accession number MH646649. The other three hybrids, i.e. F1.9, F1.11, and F1.14, showed slightly different *ndhE* sequences from that of *Phalaenopsis* 2166, and they have also been registered in NCBI database as MH646651. One of the three hybrids, i.e. F1.14, has somewhat spotted reddish-purple leaf that is predictable to produce conspicuously attractive flowers thus potentially to be developed into a large number of plant individuals (Dwiati et al., 2020b; Dwiati & Susanto, 2021).

To develop the promising hybrid, an in vitro culture technique should be employed, by which the hybrid calli are grown using ½ MS media enriched with NAA and TDZ. In this case, NAA is used for stimulating callus formation, while TDZ is intended to promote the propagation of the embryogenic callus (Mayer et al., 2010; Gantait & Sinniah, 2012). Some other previous studies on the stimulation of somatic embyogenesis by the use of TDZ alone or in combination with NAA have been reported, such as those in *Phalaenopsis amabilis* (Mose et al., 2017), *Dendrobium aqueum* (Parthibhan et al., 2018), commercial *Phalaenopsis* hybrids (Zanello & Cardoso, 2019), and *Phaphiopedilum niveum* (Soonthornkalump et al., 2019). Therefore, the objective of this study is to demonstrate the effect of NAA and TDZ application on the stimulation of somatic embryogenesis of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* ‘Saint Valentine’. Once the somatic embryos of the hybrid are produced, they could be developed further into plbs.

MATERIALS AND METHODS

Experimental design

The study was conducted as an experiment arranged in a factorial Randomized Complete Block Design (RCBD) using two factors, i.e. types of growth regulators (NAA and TDZ) and their respective levels of concentrations. The NAA concentrations consisted of 0 mgL^{-1}, 0.5 mgL^{-1}, 1.0 mgL^{-1}, while those of TDZ comprised 0 mgL^{-1}, 1.0 mgL^{-1}, 1.5 mgL^{-1}, 2.0 mg L^{-1}. Each of the 12 treatment combinations thus made was given three replications resulting in a total of 36 experimental units.

Procedures

Preparation of media

Aquadest of 1.500 mL was prepared in a beaker glass, after which 50 mL stock A, solution containing macro-elements (½ MS), was added. Then, 1 MS micro-elements and vitamins consisting of 1 mL stock B solution, 1 mL stock C solution, 0.5 mL stock D solution, and 1 mL stock E solution, were subsequently added and homogenized. This mixture was then supplemented with 2gL^{-1} peptone, 150 mLL^{-1} coconut water, 150 mLL^{-1} alkaline water, 75 mgL^{-1} vitamin C, 0.50 mgL^{-1} PVP, 0.25 mgL^{-1} Na panthothenat, 0.25 mgL^{-1} pyridoxine HCl, 2 gL^{-1} active charcoal, and 20 gL^{-1} sucrose. NAA and TDZ solutions were applied to the media corresponding to the respective treatment. pH of individual treatment was adjusted to 5.2 by dripping NaOH or HCl as necessary. Each medium was added with 1.2 g agar and sterilized in an autoclave at 121°C; 0.15MPa for 20 minutes. The media were shaked gently for homogenity and were cooled at room temperature prior to pouring onto Petri dishes.

Comment [WU1]: There isn't necessary to name the steps of adding of different stocks. you can only refer to the kind and volume of the culture medium and the additive materials in the main protocol.

Planting of leaf explants

The leaves of the selected hybrid were washed under running water, air-dried, and put into sterile bottles. These were then added with sterile-distilled water and Tween-20 of three drops, after which the leaves were rinsed using sterile aquadest until the foams were totally removed. Then, the leaves were sterilized using 70% ethanol for 5 minutes, followed by $HgCl_2$ for 5 minutes and rinsed three times with sterile-distilled water. The leaves were put into a sterile Petri dish lined with filter paper, where they were cut into 0.5 x 0.5 cm pieces which served as explants. These were then planted onto aseptic media in the previously prepared Petri dishes corresponding to the respective treatment. Each medium was filled with two explants and put on the culture rack placed in the dark at temperature of 22°C and air humidity of 90%. The callus growth was observed daily. Since 30^{th} day after incubation, the explants were subjected to light exposure for 12 hours and dark exposure for 12 hours alternately until they were 108 days old when the scutelar phase started.

Parameters

The parameters that were examined comprised the date of callus formation, number of embryogenic calli formed (%), thickness of calli formed (mm), callus diameter (mm), and callus colour and consistency. All parameters were examined weekly since the date of explant incubation until the calli were 21 days old. Meanwhile, the development of calli was still examined 108 days after explant incubation.

Data analysis

The quantitative data obtained were analyzed using ANOVA. When significant effect of treatments was observed, further analysis was performed using Duncan Multiple Range Test (DMRT). Meanwhile, descriptive analysis was applied to the qualitative data.

RESULTS AND DISCUSSION

Callus formation

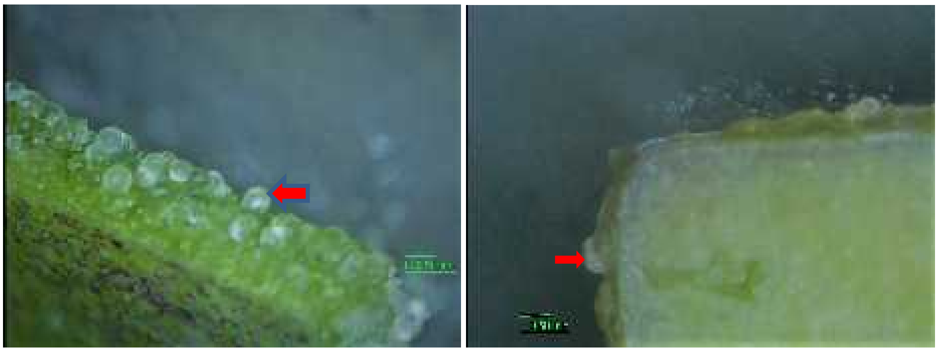
Callus formation had been observed since the third day of explant incubation, showing callus characteristics of sufficiently friable, green, and compact. These would grow into maximum at approximately three to four weeks, depending on the treatment applied. It can be seen in Table 1 that some treatment combinations of NAA and TDZ resulted in 100% of callus formation.

Table 1. Calli formed in the dark condition at 14th day after explant incubation

Treatment		Callus colour	Callus consistensy	Callus formation (%)	Embryogenic callus (%)	Callus thickness (mm)	Callus diameter (mm)
NAA (mgL^{-1})	TDZ (mgL^{-1})						
0.0	0.0	light green	sticky	50	50	0.565 i	0.305 b
0.0	1.0	light green	sticky	60	70	0.698 e	0.279 b
0.0	1.5	light green	sticky	65	70	0.669 f	0.306 b
0.0	2.0	light green	sticky	65	80	0.639 g	0.317 ab
0.5	0.0	light green	less friable	70	90	0.591 h	0.320 ab
0.5	1.0	light green	friable	100	90	0.763 b	0.312 ab
0.5	1.5	light green	friable	100	90	0.759 bc	0.302 ab
0.5	2.0	fresh green	friable	100	90	0.730 d	0.303 b
1.0	0.0	fresh green	friable	90	80	0.740 cd	0.291 b
1.0	1.0	dark green	friable	95	90	0.706 e	0.323 ab
1.0	1.5	dark green	friable	80	80	0.842 a	0.289 b
1.0	2.0	dark green	friable	75	85	0.663 f	0.355 a

Note: numbers followed by the same letter show non-significant difference after DMRT at α 0.05

It was found in this study that callus formation of 100 percent were obtained in the combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 2.0 mgL^{-1}. It seemed likely that NAA of 0.5 mgL^{-1} was the optimum concentration for promoting callus formation. In addition, the embryonic callus thus produced was 90 percent (Table 1). In addition, it was also shown from the table that a combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} at 14th day was the most optimum treatment in producing callus thickness and callus cell diameter, i.e. 0.763 mm and 0.312 mm respectively. Light green and friable calli were obtained.



A B

Figure 1. Development of embryogenic calli of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' (A) diameter of callus cells with NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} at 16th day; (B) globular phase obtained at 21st day

The development stage of the selected intergeneric hybrid callus began when the callus cells had reached their maximum size. At the 16th day the sufficiently friable calli started to enlarge, gradually reaching their maximum size (Figure 1A). Then, the callus cells would enlarge, followed by initial globular phase, which was indicated by the division of callus cells. The next stage, i.e. last globular phase, was characterized by the formation of several new cells inside the previous big callus cell as shown in Figure 1B. This was found at 21st day.

Callus development

It was shown in Table 2 and Figure 2A that the combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} resulted in light green and friable calli forming dome-like protuberances of 50 percent at 21st day. Here the explants had started to be subjected to light for an hour, and at 25th day the light condition was prolonged for six hours. Then, at 28th day callus cells under the treatment began to differentiate into coleoptelar (Figure 2B).

Formatted Table

Comment [WU2]: Was there a statistically significant difference between the treatments?

Formatted: Superscript

Comment [WU3]: It is better to write the letters in superscript form

Comment [WU4]: But it isn't like callus cell, it seems like Somatic embryos formed directly from the wounded region of a leaf. Microscopic incision is required for this case.
You can see: HUEI-LAN KUO, JEN-TSUNG CHEN, AND WEI-CHIN CHANG (2005) EFFICIENT PLANT REGENERATION THROUGH DIRECT SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF PHALAENOPSIS 'LITTLE STEVE',
In Vitro Cell. Dev. Biol.—Plant 41:453–456
And the other article about this subject

Comment [WU5]: 16th or 14th based on teable

Table 2. Calli formed in the dark condition at 21st day after explant incubation

Treatment		Callus colour	Callus consistency	Further developing phase	Callus undergoing further developing phase (%)
NAA (mgL^{-1})	TDZ (mgL^{-1})				
0.0	0.0	whitish green	sticky	callus not develop	0
0.0	1.0	light green	compact	initial globular	30
0.0	1.5	light green	compact	tree-like	20
0.0	2.0	light green	compact	initial globular	40
0.5	0.0	light green	friable	dome-like	40
0.5	1.0	light green	friable	dome-like	50
0.5	1.5	light green	friable	dome-like	30
0,5	2.0	fresh green	friable	tree-like	30
1.0	0.0	fresh green	friable	globular	35
1.0	1.0	dark green	friable	dome-like	40
1.0	1.5	dark green	friable	last globular	50
1.0	2.0	dark green	friable	globular	35

Globular phase was observed to occur in all treatments, except in control (NAA 0 mgL^{-1} and TDZ 0 mgL^{-1}). On the other hands, some treatments, i.e. the combination of NAA 0.5 mgL^{-1} and TDZ 0 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1}; 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1}; NAA 1.0 mgL^{-1} and TDZ 1.0 mg L^{-1} had even begun to form dome-like calli (Figure 2A).

Figure 2. Further development of embryogenic calli of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*'Saint Valentine' (A) callus form dome-like protuberance with NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} at 28th day; (B) initial coleoptelar phase with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} at 49th day

Comment [WU6]: Orchid plants are a group of monocotyledonous plants, but, these plants produce an organ called protocorm in both direct and indirect embryogenesis, which is completely different from this picture.

At 49th day the callus got into the development stage forming initial coleoptelars which were characterized by the occurrence of protuberances in some parts of cell side (Figure 2B). These would then develop into scutelar phase (Figure 3 B).

A B

Comment [WU7]: These images are similar and repetitive

Comment [WU8]: The image is duplicate

Figure 3. The next development of embryogenic calli of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' (A) last coleoptelar phase with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} under light condition for an hour at 70th day; (B) initial scutelar phase with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} under 12 hour-light and 12 hour-dark condition at 108th day

Discussion

Similar to callus formation observed in our study, calli of intergeneric hybrids between *Aranda* Wan Chark Kuan 'Blue' and *Vanda coerulea* appeared from the incision scar relatively fast since the third day of explant incubation (Gantait & Sinniah, 2012). In addition, 100% of callus formation from leaf explants of *Phalaenopsis amabilis* (L.) Blume using New Phalaenopsis (NP) enriched with TDZ of 3 mgL^{-1} was observed (Mose et al., 2017). TDZ can be used in replace of cytokinin, so that it can be applied as growth regulator along with auxin in an *in- vitro* culture media (Kou et al., 2016). Basically, variation in the application of growth regulators, especially auxin and cytokinin, in an *in- vitro* ~~in vitro~~ culture media could affect somatic embryogenesis (Guo et al., 2011; Moradi et al., 2017).

Promoting the growth of embryogenic callus is important in that the calli can undergo the next stages of development. Embryonic calli are those developed by means of somatic embryogenesis. Another two types of calli are known, i.e. proliferative and senescene calli. Both can not develop further through somatic embryogenesis, while even the latter will die (Shen et al., 2018). A combination of NAA 0.046 mgL^{-1} and TDZ 3.0 mgL^{-1} was needed ~~To~~ to promote embryogenic callus of *Dimorphorchis lowii*, ~~a combination of NAA 0.046 mgL^{-1} and TDZ 3.0 mgL^{-1} was needed~~. When NAA was applied in excess, it would just inhibit callus growth, because NAA was stable and could not be translocated easily in comparison to the other auxins, such as IAA and IBA. To reduce the high level of NAA in callus, it is conjugated with other substances available in the explant (Jainol & Gansau, 2017). The excessive NAA can be inactivated enzymatically in a conjugation with glucose to form glucosyl ester (Soonthornkalump et al., 2019). In general, plant growth regulators are very important to induce callus formation and it was found that coconut water merely had no effect on the callus induction from leaf explants in *Coelogyne cristata* (Naing et al., 2011). Half-strength Murashige and Skoog (½ MS) medium enriched with NAA 0.01 mgL^{-1} and BAP 0.05 mgL^{-1} was reported as the best one to induce somatic embryogenesis from embryonic calli of *Vanda tricolor* Lindl var. Pallida (Hardjo et al., 2021).

Since 30th day after incubation, the explants of the selected intergeneric hybrid of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine' were subjected 12 hour-exposure to light and another 12 hours in the dark. As a comparison, the explants of *Phalaenopsis* Classic spotted pink started to form last globular structure at 23rd day and the embryos began to form coleoptelar (Pereira et al., 2019). Induction of callus cells in orchids, which belong to monocotyledon, will go through somatic embryogenesis processes involving pre-embryo, globular, coleoptelar, and scutelar phases. Meanwhile, the somatic embryogenesis in dicotyledons consists of pre-embryo, globular, heart-shaped, torpedo-shaped, cotyledonary-shaped phases (Parthibhan et al., 2018; Zhao et al., 2017; Méndez-Hernández et al., 2019).

Somatic embryogenesis in *Phalaenopsis amabilis* was reported to begin at 8th week when grown in NP media supplemented with TDZ. The most rapid somatic embryogenesis was obtained with TDZ 3.0 mgL^{-1} at 11th day using leaf explants, while the slowest one was found with TDZ 3.0 mgL^{-1} using stem explants (Mose et al., 2017). In this study we found that somatic embryogenesis of the intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' began at 21st day in the modified ½ MS medium. Meanwhile, *Vanda tricolor* shoots produced from somatic embryogenesis showed the best development when subcultured in the New Dogashima (ND) medium without application of any plant growth regulator (Ashihah et al., 2022).

The fastest plbs induction in the intergeneric hybrids between *Aranda* Wan Chark Kuan 'Blue' and *Vanda coerulea* using leaf explants was observed in the treatment of TDZ 1.5 mgL^{-1} (Gantait & Sinniah, 2012). Some other studies showed that TDZ was proved very effective in inducing somatic embryogenesis in several orchid species, such as *Renanthera* Tom Thumb 'Qilin' (Wu et al., 2012), *Phalaenopsis amabilis* (Mose et al., 2017), and *Paphiopedilum niveum* (Soonthornkalump et al., 2019).

Regeneration of somatic embryogenesis in orchids began at 15th to 30th day in the concentration of TDZ ranging between 0.001 and 5 mgL^{-1} (Shen et al., 2018). Reduced auxin in the last development stage of somatic embryogenesis was needed, especially for stimulating plb proliferation and differentiation (Yang & Zhang, 2010). TDZ concentration and its interaction with light spectra were found highly determining direct somatic embryogenesis in *Phalaenopsis* orchids. The concentration of 3mgL^{-1} in interaction with red and far red light spectra was the efficient treatment to induce direct somatic embryogenesis in the orchids without somaclonal variation (Boldaji et al., 2021).

Pre-embryo is a further development of callus, which is characterized by two bipolar centres of meristems. These structures will develop into root and stem meristem respectively (Seth et al., 2017; Shen et al., 2018). Histological examination shows that callus resulting from somatic embryogenesis will develop sequentially into plbs, which consist of some meristimatic tissues undergoing further development to form roots, stem, and leaves (Sherif et al., 2018).

Some factors have direct effects on the somatic embryogenesis of orchids. They are genotypes, growth regulators, and media (Campos et al., 2017; Zanello & Cardoso, 2019). TDZ as growth regulator should be applied not exceed 3 mgL^{-1}. The high level of TDZ (3 to 5 mgL^{-1}) will inhibit cytokinin oxidase. This enzyme stimulates accumulation of endogenous purine-based cytokinin (Soonthornkalump et al., 2019). Meanwhile, ½ MS is the most common media used, in which N is in form of nitrate (NH_4NO_3) in a sufficiently high concentration, i.e. 1.7 mgL^{-1}. In addition, KNO_3 of 1.9 mgL^{-1} is also contained in ½MS media. Most plants absorb N in form of nitrate. Both NH_4NO_3 and KNO_3 can be used to stimulate somatic embryogenesis (Zanello & Cardoso, 2019). NP was reported as the media with N in form of nitrate suitable for

Formatted: Font: Italic

Formatted: Font: Italic

Comment [WU9]: For this concept it is necessary to refer to the reference related to the embryonic stages of orchids.

Phalaenopsis amabilis. This media contained NH_4NO_3 of 82 mgL^{-1}, KNO_3 of 424 mgL-^{1}, $Ca(NO_3)_2.4H_2O$ of 443.04 mgL^{-1}, and $Mg(NO_3)_2.6H_2O$ of 256.4 mgL^{-1} (Mose et al., 2017). Nitrogen, in form of either potassium nitrate or calcium nitrate, is very good to stimulate somatic embryogenesis, while that in form of ammonium nitrate less stimulate somatic embryogenesis. Nevertheless, explants in the absence of ammonium nitrate in the growth media will fail to undergo somatic embryogenesis (Méndez-Hernández et al., 2019).

The presence of TDZ in the culture media of *Oncidium flexuosum* without light would stimulate regeneration of plbs. Pre-embryos having no chlorophyll were formed in the dark condition, so that somatic embryogenesis occurred in the absence of chlorophyl. After treatment with no growth regulator and incubation in the light condition, embryos would be green initiating to form plbs (Zanello & Cardoso, 2019). It was proved that in the early stages of plb formation, characteristics of somatic embryonic callus similar to zygotic embryo development were observed, indicating that plbs were truly somatic embryos of orchids (Lee et al., 2013).

Somatic embryogenesis could result from proliferation of young plbs that were cultured in the MVW media containing NAA 0.1 mgL^{-1}. The increasing accumulation of endogenous auxin through the application of exogenous auxin in the early stages of somatic embryogenesis was needed. In the next stages of development reduced level of auxin enabled rapid proliferation and differentiation of meristems, which in turn would stimulate the emergence of shoots. Then, the plantlets thus produced were moved into MVW media without growth regulator (Soonthornkalump et al., 2019).

In conclusion, our present study found that ½ MS medium supplemented with the combination of NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} produced calli of the intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' that showed high potential of differentiating into plbs. This indicates that somatic embryogenesis in the selected hybrid could be stimulated by proper application of both NAA and TDZ.

Comment [WU10]: It is necessary to replace the duplicate images with an image that represents this

ACKNOWLEDGEMENTS

The authors would like to express their gratefulness to the Institute of Research and Community Service of Universitas Jenderal Soedirman for funding this project with the scheme of Applied Research Grand financial year 2019 with director decree number 158/UN23/14/PN01.00/2019. Appreciation is also addressed to Supriyono for laboratory work assistance.

REFERENCES

Ashihah FR, Rineksane IA, Astuti A. 2022. New Dogashima medium as subculture medium improve the growth of *Vanda tricolor* shoots from embryogenesis. IOP Conf. Series: Earth Environ. Sci. 985(1). https://doi.org/10.1088/1755-1315/985/1/012006.

Boldaji HN, Dylami SD, Aliniaeifard S, Norouzi M. 2021. Efficient method for direct embryogenesis in *Phalaenopsis* orchid. Int. J. Hort. Sci. Technol. 9(2): 37–50. https://doi.org/10.22059/ijhst.2020.296696.339.

Campos NA, Panis B, Carpentier SC. 2017. Somatic embryogenesis in coffee: the evolution of biotechnology and the integration of omics technologies offer great opportunities. Frontiers Plant Sci. 8: 1–12. https://doi.org/10.3389/fpls.2017.01460.

Dwiati M, Hardiyati T, Susanto AH, Chasanah T, Palupi D. 2020a. Growth medium for intergeneric hybrids between *Phalaenopsis* 2166 and *Vanda* "Saint Valentine." IOP Conf. Series: Earth Environ. Sci. 593(1). https://doi.org/10.1088/1755-1315/593/1/012023.

Dwiati M, Susanto AH. 2021. Intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* "Saint Valentine": characterization of parents using *ndhE* cpDNA partial sequence. J. Trop. Biodiv. Biotechnol. 6(3): 1–6. https://doi.org/10.22146/JTBB.65658.

Dwiati M, Susanto AH, Prayoga L. 2020b. Intergeneric hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine' showing maternal inheritance: genetic analysis based on *ndhE* partial gene. Biodiversitas 21(11): 5138–5145. https://doi.org/10.13057/biodiv/d211119.

Gantait S, Sinniah UR. 2012. Rapid micropropagation of monopodial orchid hybrid (*Aranda* Wan Chark Kuan "Blue" × *Vanda coerulea* Grifft. ex. Lindl.) through direct induction of protocorm-like bodies from leaf segments. Plant Growth Regulation 68(2): 129–140. https://doi.org/10.1007/s10725-012-9698-y.

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH. 2011. Thidiazuron: a multi-dimensional plant growth regulator. African J. Biotechnol. 10(45): 8984–9000. https://doi.org/10.5897/ajb11.636.

Hardjo PH, Savitri WD, Bagus I, Artadana M, Emantoko S, Putra D, Parac EP, Jan A. 2021. Callus-mediated somatic embryogenesis and plant regeneration in *Vanda tricolor* Lindl. var. Pallida. Jordan J. Biol. Sci. 14(5): 933–937.

Jainol JE, Gansau JA. 2017. Embryogenic callus induction from leaf tip explants and protocorm-like body formation and shoot proliferation of *Dimorphorchis lowii*: Borneon endemic orchid. Agrivita 39(1): 1–10. https://doi.org/10.17503/agrivita.v39i1.895.

Kou Y, Yuan C, Zhao Q, Liu G, Nie J, Ma Z, Cheng C, Teixeira da Silva JA, Zhao L. 2016. Thidiazuron triggers morphogenesis in *Rosa canina* L. protocorm-like bodies by changing incipient cell fate. Frontiers Plant Sci. 7: 1–13. https://doi.org/10.3389/fpls.2016.00557.

Kurniawan FH, Nazar L, Anjarwati R, Sasono HD, Rahayuningsih M. 2021. Orchids of Mount Ungaran (Indonesia) compiled from a decade of data collections between 2010 and 2021. Nusantara Biosci. 13(2): 238–252. https://doi.org/10.13057/nusbiosci/n130214.

Lee YI, Hsu ST, Yeung EC. 2013. Orchid protocorm-like bodies are somatic embryos. Amer. J. Bot. 100(11): 2121–2131. https://doi.org/10.3732/ajb.1300193.

Li C, Dong N, Zhao Y, Wu S, Liu Z, Zhai J. 2021. A review for the breeding of orchids: current achievements and prospects. Hort. Plant J. 7(5): 380–392. https://doi.org/10.1016/j.hpj.2021.02.006.

Mayer JLS, Stancato GC, Appezzato-Da-Glória B. 2010. Direct regeneration of protocorm-like bodies (PLBs) from leaf apices of *Oncidium flexuosum* Sims (Orchidaceae). Plant Cell Tissue Organ Cult. 103(3): 411–416. https://doi.org/10.1007/s11240-010-9782-9.

Méndez-Hernández HA, Ledezma-Rodríguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A, Avilez-Montalvo J, De-La-Peña C, Loyola-Vargas VM. 2019. Signaling overview of plant somatic embryogenesis. Frontiers Plant Sci. 10: 1–15. https://doi.org/10.3389/fpls.2019.00077.

Moradi S, Dianati Daylami S, Arab M, Vahdati K. 2017. Direct somatic embryogenesis in *Epipactis veratrifolia*, a temperate terrestrial orchid. J. Hort. Sci. Biotechnol. 92(1): 88–97. https://doi.org/10.1080/14620316.2016.1228434.

Mose W, Indrianto A, Purwantoro A, Semiarti E. 2017. The influence of thidiazuron on direct somatic embryo formation from various types of explant in *Phalaenopsis amabilis* (L.) Blume Orchid. Hayati J. Biosci. 24(4): 201–205. https://doi.org/10.1016/j.hjb.2017.11.005.

Naing AH, Chung JD, Lim KB. 2011. Plant regeneration through indirect somatic embryogenesis in *Coelogyne cristata* orchid. Amer. J. Plant Sci. 02(02): 262–267. https://doi.org/10.4236/ajps.2011.22028.

Parthibhan S, Rao MV, Teixeira da Silva, JA, Senthil Kumar T. 2018. Somatic embryogenesis from stem thin cell layers of *Dendrobium aqueum*. Biologia Plantarum 62(3): 439–450. https://doi.org/10.1007/s10535-018-0769-4.

Pereira JAF, Ferreira LT, De Morais MB, Ulisses C. 2019. Somatic embryos from *Phalaenopsis* classic spotted pink (Orchidaceae) protocorms. Ciencia Rural 49(7): 1 - 5. https://doi.org/10.1590/0103-8478cr20180822.

Seth S, Rath SC, Rout GR, Panigrahi J. 2017. Somatic embryogenesis in *Abutilon indicum* (L.) sweet and assessment of genetic homogeneity using SCoT markers. Plant Biosystems 151(4): 704–714. https://doi.org/10.1080/11263504.2016.1211193.

Shen HJ, Chen JT, Chung HH, Chang WC. 2018. Plant regeneration via direct somatic embryogenesis from leaf explants of *Tolumnia* Louise Elmore 'Elsa.' Bot. Stud. 59(1): 1 - 7. https://doi.org/10.1186/s40529-018-0220-3.

Sherif NA, Franklin Benjamin JH, Senthil Kumar T, Rao MV. 2018. Somatic embryogenesis, acclimatization and genetic homogeneity assessment of regenerated plantlets of *Anoectochilus elatus* Lindl., an endangered terrestrial jewel orchid. Plant Cell Tissue Organ Cult. 132(2): 303–316. https://doi.org/10.1007/s11240-017-1330-4.

Soonthornkalump S, Nakkanong K, Meesawat U. 2019. In vitro cloning via direct somatic embryogenesis and genetic stability assessment of *Paphiopedilum niveum* (Rchb.f.) Stein: the endangered Venus's slipper orchid. In Vitro Cell. Dev. Biol.-Plant 55(3): 265–276. https://doi.org/10.1007/s11627-019-09981-7.

Wu K, Zeng S, Teixeira da Silva JA, Chen Z, Zhang J, Yang Y, Duan J. 2012. Efficient regeneration of *Renanthera* Tom Thumb "Qilin" from leaf explants. Scientia Hort, 135: 194–201. https://doi.org/10.1016/j.scienta.2011.11.028.

Yang X, Zhang X. 2010. Regulation of somatic embryogenesis in higher plants. Critical Rev. Plant Sci. 29(1): 36–57. https://doi.org/10.1080/07352680903436291.

Zanello CA, Cardoso JC. 2019. PLBs induction and clonal plantlet regeneration from leaf segment of commercial hybrids of *Phalaenopsis*. J. Hort. Sci. Biotechnol. 00(00), 1–5. https://doi.org/10.1080/14620316.2019.1600384.

Zhao P, Begcy K, Dresselhaus T, Sun MX. 2017. Does early embryogenesis in eudicots and monocots involve the same mechanism and molecular players? Plant Physiol. 173(1): 130–142. https://doi.org/10.1104/pp.16.01406.