

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

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Abstract. Dwiati M, Susanto AH, Budisantoso I. 2022. Somatic embryogenesis of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine': Application of NAA and TDZ. *Nusantara Bioscience* 14: 160-165. Intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' has produced a hybrid seedling with several characters potentially developing into plant individuals with flowers of better performance. Therefore, identical clones of the selected hybrid should be developed into PLBs using an *in-vitro* culture technique employing somatic embryogenesis supported by the application of plant growth regulators. This study aims to unveil the effect of NAA and TDZ in stimulating the formation of identical clones 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 plant growth regulators and the levels of concentrations of each substance. It was found that the combination of NAA and TDZ significantly affected the growth of the identical clones. Furthermore, the combination of NAA 0.5 mgL⁻¹ and TDZ 1.5 mgL⁻¹ resulted in clones that potentially differentiate into PLBs. This finding indicates that NAA and TDZ should be applied appropriately to stimulate somatic embryogenesis in the intergeneric hybrid.

Keywords: Intergeneric hybrid, NAA, *Phalaenopsis* 2166, TDZ, *Vanda*' Saint Valentine'

INTRODUCTION

The members of the 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 to be 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 are even listed in the IUCN Red List (Kurniawan et al. 2021). On the other hand, some wild orchid species can be potentially used as parental lineages to produce hybrid varieties of desirable better performances, including flower color, shape, and resistance (Li et al. 2021).

Intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' has resulted in several hybrid seedlings characterized phenotypically and molecularly. Based on the leaf shape, edge, and color, the intergeneric hybrids likely resemble *Phalaenopsis* 2166 as the female parent. However, some variations of leaf shape and color 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 using the *ndhE* partial gene revealed that 11 of the 14 hybrids obtained had the same sequences of the *ndhE* partial gene as that of *Phalaenopsis* 2166. The sequences are now registered in

the 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 the NCBI database as MH646651. One of the three hybrids, i.e., F1.14, has a partially 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 and Susanto 2021).

Furthermore, to develop the promising hybrid, an *in vitro* culture technique should be employed, by which the hybrid clones are grown using ½ MS media enriched with NAA and TDZ. In this case, NAA is used for stimulating clone formation, while TDZ is intended to promote the propagation of the somatic embryos (Mayer et al. 2010; Gantait and Sinniah 2012). Some previous studies on the stimulation of somatic embryogenesis using TDZ alone or in combination with NAA have been reported, such as those in *Phalaenopsis amabilis* (L.) Blume (Mose et al. 2017), *Dendrobium aqueum* Lindl. (Parthibhan et al. 2018), commercial *Phalaenopsis* hybrids (Zanello and Cardoso 2019), and *Paphiopedilum niveum* (Rchb.f.) Stein (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 plant growth regulators (NAA and TDZ) and their respective concentrations levels. 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 mgL⁻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

A half-strength modified MS was prepared as the basal medium, which was then supplemented with 2 gL⁻¹ peptones, 150 mL⁻¹ coconut water, 150 mL⁻¹ alkaline water, 75 mgL⁻¹ vitamin C, 0.50 mgL⁻¹ PVP, 0.25 mgL⁻¹ Na pantothenate, 0.25 mgL⁻¹ pyridoxine HCl, 2 gL⁻¹ active charcoal, and 20 gL⁻¹ sucrose. NAA solution was applied to the medium corresponding to the respective treatment. The 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; 0.15 MPa for 20 minutes. All the media were cooled at approximately 45°C and shaken gently for homogeneity. Each medium was added with TDZ according to the respective treatment and poured onto a Petri dish.

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% (v/v) ethanol for 5 minutes, followed by HgCl₂ for 5 minutes, and rinsed

three times with sterile distilled water. Finally, 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 in the dark at a temperature of 22°C and an air humidity of 90%. The clone growth was observed daily. Since the 30th 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.

Parameters

The parameters that were examined comprised the date of clone formation, the number of embryogenic clones formed (%), the thickness of clones formed (mm), clone diameter (mm), and clone color and consistency. All parameters were examined weekly from the date of explant incubation until the clones were 21 days old. Meanwhile, the development of somatic embryos was still examined 108 days after explant incubation.

Data analysis

The quantitative data obtained were analyzed using ANOVA. When a 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

Clone formation

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

Table 1. Identical clones formed in the dark condition on the 14th day after explant incubation

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

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

It was found in this study that clone formation of 100 % was 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. Therefore, it seemed likely that NAA of 0.5 mgL⁻¹ was the optimum concentration for promoting clone formation. In addition, the somatic embryo thus produced was 90 % (Table 1). It was also shown from the table that a combination of NAA 0.5 mgL⁻¹ and TDZ 1.0 mgL⁻¹ on the 14th day was the most optimum treatment in producing clone thickness and clone cell diameter, i.e., 0.763 mm and 0.312 mm, respectively. As a result, light green and friable clones were obtained.

The selected intergeneric hybrid clone's development stage began when the cells had reached their maximum size. On the 16th day, the sufficiently friable clones started to enlarge, gradually reaching their maximum size (Figure 1A). Then, the clone cells would enlarge, followed by the formation of initial globular structures, which the division of clone cells indicated. The next stage was characterized by forming several new cells inside the previous big clone cell, as shown in Figure 1B, which was found on the 21st day.

Clone development

Table 2 and Figure 2A show that the combination of NAA 0.5 mgL⁻¹ and TDZ 1.0 mgL⁻¹ resulted in light green and friable clones forming dome-like protuberances of 50 % on the 21st day. Here the explants started to be subjected to light for an hour, and on the 25th day, the light condition was prolonged for six hours. Then, on the 28th day, the somatic embryo cells under treatment began to differentiate (Figure 2B).

Further development of globular structures was observed in all treatments, except in control (NAA 0 mgL⁻¹ and TDZ 0 mgL⁻¹). On the other hand, 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 protuberance (Figure 2A).

On the 49th day, the somatic embryos developed, forming structures characterized by protuberances in some parts of the cell side (Figure 2B). These would then develop into structures resembling scutella (Figure 3B).

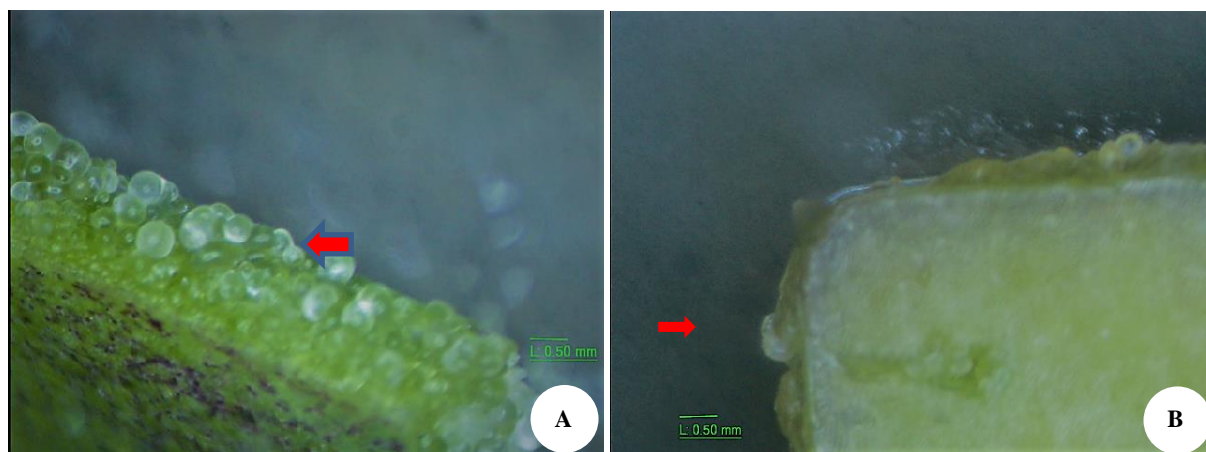


Figure 1. Development of identical clones of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' (A) diameter of clone cells with NAA 0.5 mgL⁻¹ and TDZ 1.0 mgL⁻¹ at 16th day; (B) clone obtained at 21st day

Table 2. Clones formed in the dark condition on the 21st day after explant incubation

Treatment		Clone color	Clone consistency	Further development	Clones undergoing further development (%)
NAA (mgL ⁻¹)	TDZ (mgL ⁻¹)				
0.0	0.0	whitish green	sticky	clone not develop	0 ^d
0.0	1.0	light green	compact	initial globular	30 ^{bc}
0.0	1.5	light green	compact	tree-like	20 ^c
0.0	2.0	light green	compact	initial globular	40 ^{ab}
0.5	0.0	light green	friable	dome-like	40 ^{ab}
0.5	1.0	light green	friable	dome-like	50 ^a
0.5	1.5	light green	friable	dome-like	30 ^{bc}
0.5	2.0	fresh green	friable	tree-like	30 ^{bc}
1.0	0.0	fresh green	friable	globular	35 ^b
1.0	1.0	dark green	friable	dome-like	40 ^{ab}
1.0	1.5	dark green	friable	last globular	50 ^a
1.0	2.0	dark green	friable	globular	35 ^b

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

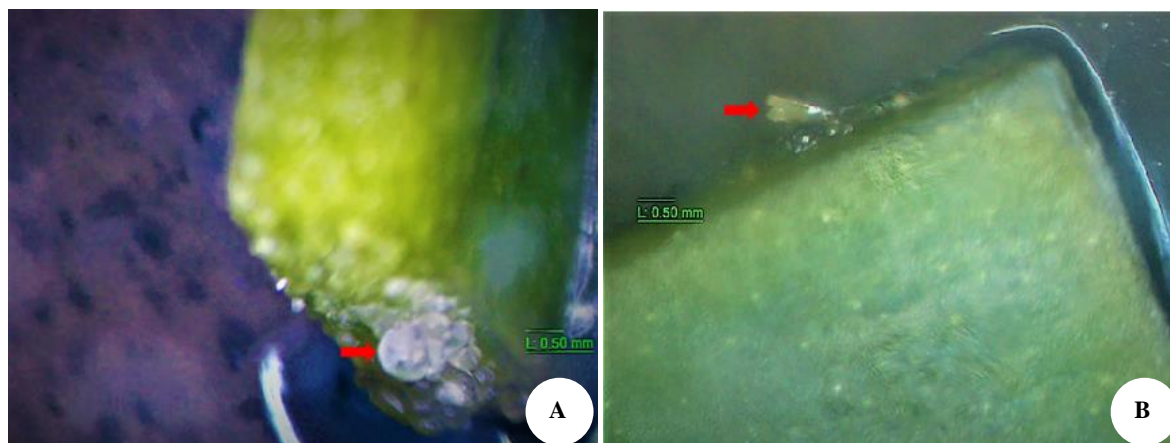


Figure 2. Further development of globular structures of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*'Saint Valentine' (A) dome-like protuberance with NAA 0.5 mgL⁻¹ and TDZ 1.0 mgL⁻¹ at 28th day; (B) somatic embryo with NAA 0.5 mgL⁻¹ and TDZ 1.5 mgL⁻¹ at 49th day

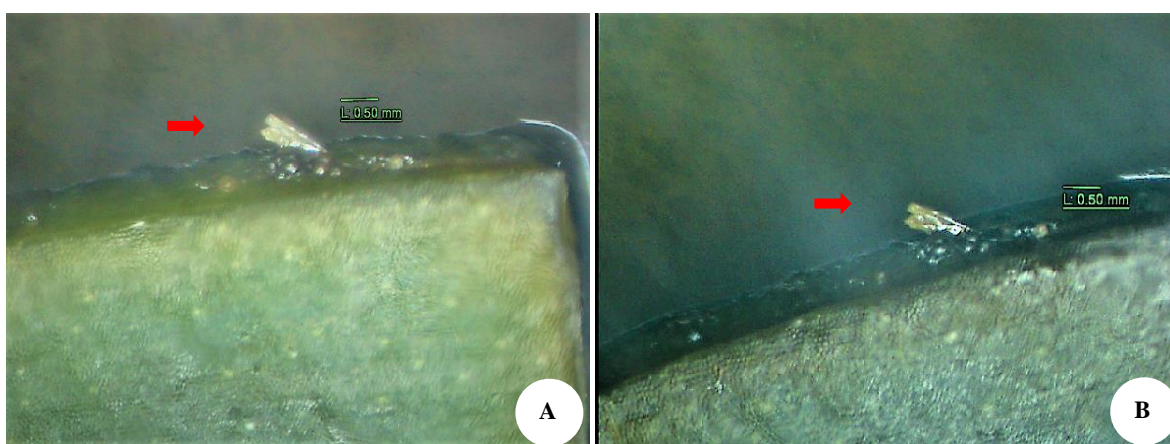


Figure 3. The next development of somatic embryos of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' (A) somatic embryo with NAA 0.5 mgL⁻¹ and TDZ 1.5 mgL⁻¹ under light condition for an hour on the 70th day; (B) somatic embryo with NAA 0.5 mgL⁻¹ and TDZ 1.5 mgL⁻¹ under 12 hour-light and 12 hour-dark conditions at 108th day

Discussion

Slightly different from our results in that 50% of clones were still formed in the medium without the supply of NAA and TDZ (Table 1), recalcitrant leaf explants in *Cymbidium eburneum* Lindl. were observed in the basal medium of MS. No regeneration occurred where the explants turned brown and died within 10 weeks (Sembi et al. 2020). A similar finding (no somatic embryo formation) was reported in leaf explant in *Spathoglottis plicata* Blume when cultured in MS medium without a plant growth regulator (Manokari et al. 2021). Another report on indirect somatic embryogenesis where calli of the intergeneric hybrids between *Aranda* Wan Chark Kuan' Blue' and *Vanda coerulea* Griff. ex Lindl. appeared from the incision scar relatively fast since the third day of explant incubation (Gantait and Sinniah 2012). Wounding is considered important in assisting both direct and indirect somatic embryogenesis because it stimulates cell division (Rojas-Herrera and Loyola-Vargas 2002)

Regeneration responses in the leaf explant culture of *C. eburneum* were observed in the applications of NAA and BA. The highest response (83.3%) was obtained using 2 mgL⁻¹ NAA and 0.5 mgL⁻¹ BA (Sembi et al. 2020). In our study, we use TDZ instead of BA to stimulate the further development of the clones into somatic embryos since TDZ has been generally used in orchid tissue culture to enhance somatic embryogenesis (Wu et al. 2012; Mahendran and Bai 2016). It was also reported that TDZ showed better efficacy over other purine types of cytokinins, such as BA, in inducing somatic embryogenesis in orchids (Bhattacharyya et al. 2018). The fastest PLB induction in the intergeneric hybrids between *Aranda* Wan Chark Kuan' Blue' and *V. coerulea* using leaf explants was observed in the treatment of TDZ 1.5 mgL⁻¹ (Gantait and 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), *P. amabilis* (Mose et al. 2017), and

P. niveum (Soonthornkalump et al. 2019). TDZ can replace cytokinin as a plant growth regulator and auxin in an in vitro culture media (Kou et al. 2016). As a plant growth regulator, TDZ should not exceed 3 mgL⁻¹. The high level of TDZ (3 to 5 mgL⁻¹) will inhibit cytokinin oxidase (Soonthornkalump et al. 2019). Variation in the application of growth regulators, especially auxin and cytokinin, in an in vitro culture media could affect somatic embryogenesis (Guo et al. 2011; Moradi et al. 2017).

Since the 30th day after incubation, the explants of the selected intergeneric hybrid of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine' were subjected to 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 the last globular structure on the 23rd day, and the embryos began to form coleoptelar (Pereira et al. 2019). Meanwhile, direct somatic embryogenesis from leaf explants in *Phalaenopsis* 'Little Steve' revealed that somatic embryos were formed on the 30th day after explant incubation in the dark (Kuo et al. 2005).

Somatic embryogenesis in *P. amabilis* was reported to begin in the 8th week when grown in NP media supplemented with TDZ. However, the most rapid somatic embryogenesis was obtained with TDZ 3.0 mgL⁻¹ on the 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 on the 21st day in the modified ½ MS medium. Meanwhile, *Vanda tricolor* Lindl. shoots produced from somatic embryogenesis showed the best development when subcultured in the New Dogashima (ND) medium without applying any plant growth regulator (Ashihah et al. 2022).

Regeneration of somatic embryogenesis in orchids began on the 15th to 30th day in the concentration of TDZ ranging between 0.001 and 5 mgL⁻¹ (Shen et al. 2018). In the last development stage of somatic embryogenesis, reduced auxin was needed, especially for stimulating PLB proliferation and differentiation (Yang and 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).

While no callus was formed in our study, pre-embryo is a further callus development in the indirect somatic embryogenesis, which two bipolar centers of meristems characterize. These structures will develop into the root and stem meristem, respectively (Seth et al. 2017; Shen et al. 2018). In addition, histological examination shows that callus resulting from somatic embryogenesis will develop sequentially into PLBs, which consist of some meristematic tissues undergoing further development to form roots, stems, 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 and

Cardoso 2019). Half-strength MS is the most common media used, in which N is in the form of nitrate (NH₄NO₃) in a sufficiently high concentration, i.e., 1.7 mgL⁻¹. In addition, KNO₃ of 1.9 mgL⁻¹ is also contained in ½MS media. Most plants absorb N in the form of nitrate. Both NH₄NO₃ and KNO₃ can be used to stimulate somatic embryogenesis (Zanello and Cardoso 2019). NP was reported as the media with N in the form of nitrate suitable for *P. amabilis*. This media contained NH₄NO₃ of 82 mgL⁻¹, KNO₃ of 424 mgL⁻¹, Ca(NO₃)₂·4H₂O of 443.04 mgL⁻¹, and Mg(NO₃)₂·6H₂O of 256.4 mgL⁻¹ (Mose et al. 2017). Nitrogen, in the form of either potassium nitrate or calcium nitrate, is very good at stimulating somatic embryogenesis, while that in the form of ammonium nitrate less stimulates 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* (Kunth) Lindl. without light would stimulate the regeneration of PLBs. Pre-embryos with no chlorophyll were formed in the dark condition, so somatic embryogenesis occurred in the absence of chlorophyll. After treatment with no growth regulator and incubation in the light condition, embryos would be greenly initiated to form PLBs (Zanello and 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 the proliferation of young PLBs cultured in the MVW media containing NAA 0.1 mgL⁻¹. Therefore, the increasing accumulation of endogenous auxin through exogenous auxin application in the early stages of somatic embryogenesis was needed. In the next stages of development, reduced levels 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 a 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 identical clones of the intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' that showed high potential of differentiating into PLBs. That indicates that applying NAA and TDZ could stimulate somatic embryogenesis in the selected hybrid.

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