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

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Manuscript received: 29 May 2022. Revision accepted: 11 August 2022.

**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.* 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, identical clones of the selected hybrid should be developed into PLBs by means of *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 had significant effect on the growth of the identical clones. The combination of NAA 0.5 $mgL^{-1}$ and TDZ 1.5 $mgL^{-1}$ resulted in the 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 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 and Susanto 2021).

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 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* (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 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 $mgL^{-1}$. 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. This was then supplemented with 2 $gL^{-1}$ 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 solution was applied to the medium 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. All the media were cooled at approximately 45ºC and shaked gently for homogeneity. Each medium was then added with TDZ according the respective treatment and was poured onto 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 $HgCl_2$ 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 clone 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.

### Parameters

The parameters that were examined comprised the date of clone formation, number of embryogenic clones formed (%), thickness of clones formed (mm), clone diameter (mm), and clone colour and consistency. All parameters were examined weekly since 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 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 clone 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 somatic embryo formation.

**Table 1.** Identical clones formed in the dark condition at 14$^{th}$ day after explant incubation

| Treatment | | Clone | Clone | Clone | Somatic | Clone | Clone diameter |
|---|---|---|---|---|---|---|---|
| NAA ($mgL^{-1}$) | TDZ ($mgL^{-1}$) | colour | consistency | formation (%) | embryos (%) | thickness (mm) | (mm) |
| 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 $^{d}$ | 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 non-significant difference after DMRT at α 0.05

It was found in this study that clone 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 clone formation. In addition, the somatic embryo thus produced was 90 percent (Table 1). It was also shown from the table that a combination of NAA 0.5 $mgL^{-1}$ and TDZ 1.0 $mgL^{-1}$ at 14$^{th}$ day was the most optimum treatment in producing clone thickness and clone cell diameter, i.e., 0.763 mm and 0.312 mm respectively. Light green and friable clones were obtained.

The development stage of the selected intergeneric hybrid clone began when the clone cells had reached their maximum size. At the 16$^{th}$ 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 was indicated by the division of clone cells. The next stage was characterized by the formation of several new cells inside the previous big clone cell as shown in Figure 1B. This was found at 21$^{st}$ day.

**Clone 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 clones forming dome-like protuberances of 50 percent at 21$^{st}$ day. Here the explants had started to be subjected to light for an hour, and at 25$^{th}$ day the light condition was prolonged for six hours. Then, at 28$^{th}$ day the somatic embryo cells under the treatment began to differentiate (Figure 2B).

Further development of globular structures 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 protuberance (Figure 2A).

At 49$^{th}$ day the somatic embryos got into the development stage forming structures which were characterized by the occurrence of protuberances in some parts of cell side (Figure 2B). These would then develop further 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 16$^{th}$ day; (B) clone obtained at 21$^{st}$ day

**Table 2.** Clones formed in the dark condition at 21$^{st}$ day after explant incubation

| Treatment | | Clone colour | 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 non-significant difference after DMRT at α 0.05

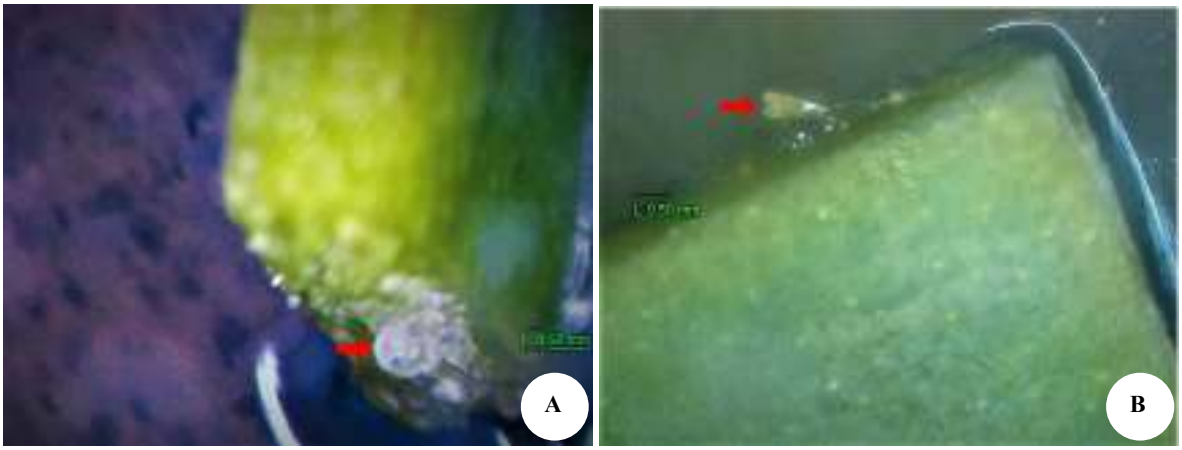


**Figure 2.** Further development of globular structures of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*'Saint Valentine' (A) dome-like protuberance with NAA 0.5 $mgL^{-1}$ and TDZ 1.0 $mgL^{-1}$ at $28^{th}$ day; (B) somatic embryo with NAA 0.5 $mgL^{-1}$ and TDZ 1.5 $mgL^{-1}$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

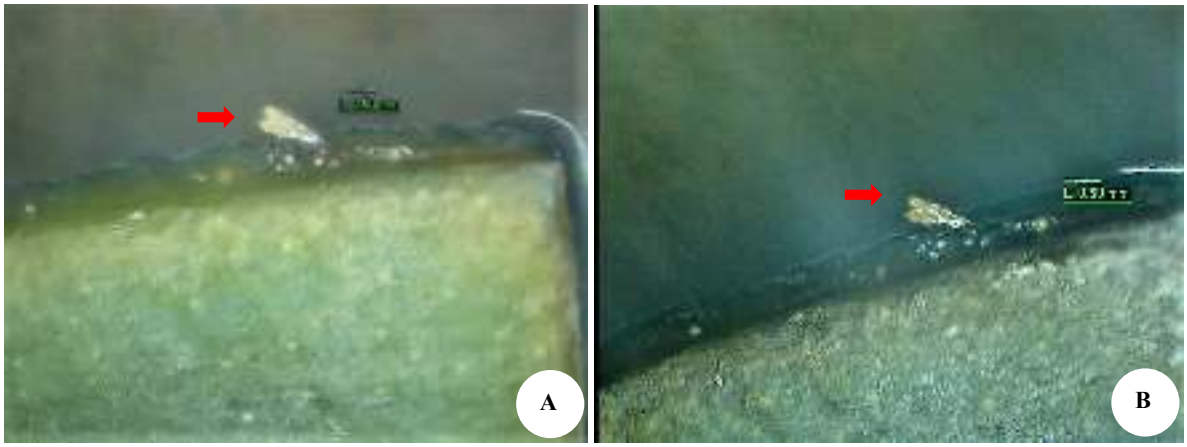


**Figure 3.** The next development of somatic embryos of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' (A) somatic embryo with NAA 0.5 $mgL^{-1}$ and TDZ 1.5 $mgL^{-1}$ under light condition for an hour at $70^{th}$ day; (B) somatic embryo with NAA 0.5 $mgL^{-1}$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

## Discussion

Slightly different from our results that 50% clones were still formed in the medium without 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). Similar finding (no somatic embryo formation) reported in leaf explant in *Spathoglottis plicata* Blume when cultured in MS medium in the absence of plant growth regulator (Manokari et al. 2021). Other 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 leaf explant culture of *C. eburneum* were observed in the applications of NAA and BA, where the highest response (83.3%) was obtained in the combination of 2 $mgL^{-1}$ NAA and 0.5 $mgL^{-1}$ BA (Sembi et al. 2020). In our study we use TDZ instead of BA for stimulating 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 be used in replace of cytokinin, so that it can be applied as plant growth regulator along with auxin in an in-vitro culture media

(Kou et al. 2016). As plant growth regulator, TDZ should be applied not exceed 3 $mgL^{-1}$. The high level of TDZ (3 to 5 $mgL^{-1}$) will inhibit cytokinin oxidase (Soonthornkalump et al. 2019). Basically, 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 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). Meanwhile, direct somatic embryogenesis from leaf explants in *Phalaenopsis* 'Little Steve' revealed that somatic embryos were formed at 30th day after explant incubation in the dark (Kuo et al. 2005).

Somatic embryogenesis in *P. 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* Lindl. 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).

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 and Zhang 2010). TDZ concentration and its interaction with light spectra were found highly determining direct somatic embryogenesis in *Phalaenopsis* orchids. The concentration of 3$mgL^{-1}$ 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 development of callus in the indirect somatic embryogenesis, which is characterized by two bipolar centers 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 meristematic 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 and Cardoso 2019). Half strength 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 and Cardoso 2019). NP was reported as the media with N in form of nitrate suitable for *P. 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* (Kunth) Lindl. 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 chlorophyll. After treatment with no growth regulator and incubation in the light condition, embryos would be green initiating 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 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 conclusions, 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. This indicates that somatic embryogenesis in the selected hybrid could be stimulated by proper application of both NAA and TDZ.

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