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The effectivity of thidiazuron and 1-naphthaleneacetic acid on somatic embryo induction in transgenic *Dendrobium phalaenopsis* Fitzg. carrying *35S::GR::AtRKD4*

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ABSTRACT *Dendrobium phalaenopsis* Fitzg. (also known as the Larat orchid) is an endemic orchid from Larat Island, Eastern Indonesia. Its beautiful flowers mean that many plants are taken for commercial purposes, leading to the rapid decline of populations in their natural habitats. The objectives of this study were to determine which organs of the transgenic Larat orchid carrying the *35S::GR::AtRKD4* construct, together with which concentrations of the plant growth regulators (PGRs) auxin and cytokinin, are suitable for the induction of somatic embryos (SEs). In this study, the *AtRKD4* gene in Larat orchids was confirmed using PCR with specific primers for the *AtRKD4* and *HPT* genes. Thidiazuron (TDZ) (1, 3 and 5 mg/L) in combination with 1-naphthaleneacetic acid (NAA) (0.5 and 1 mg/L) were used on new phalaenopsis (NP) medium to induce SEs from leaves, pseudobulbs and roots. The *AtRKD4* transgenes were detected as being stably integrated into the DNA genome of transformant plants using specific primers for *AtRKD4* and *HPT* genes, and positive results were obtained using *ACTIN* gene primers as internal controls for PCR. Pseudobulbs produced 19 to 20 SEs from 108 pseudobulb explants (89–100%), a higher number than produced in explants of the other organs studied. Among the PGR treatments, the best results were obtained in NP medium supplemented with a combination of 1 mg/L TDZ and 1 mg/L NAA, 100% of the explants of which produced SEs (2.11 ± 1.36). No significant difference was found between the morphology of the SEs produced from the non-transformant Larat orchid pseudobulb explants and the *35S::AtRKD4* carrier transformant.

KEYWORDS *Dendrobium phalaenopsis* Fitzg; *AtRKD4*; somatic embryos; NAA; TDZ

1. Introduction

The Larat orchid (*Dendrobium phalaenopsis* Fitzg.) has a very attractive flower with high frequency of flowering and resistance to drought; as a result, it is widely cultivated as an ornamental potted plant and for cut flowers used in traditional religious rituals. Since its beauty and uniqueness it has been widely exploited, leading to scarcity in its natural habitats (Mahadi 2017) and the threat of extinction in Indonesia (Ivakdalam and Pugesehan 2016). Conversion of forests to plantations and agricultural land is also a cause of decreasing orchid habitat (Zulkaidha et al. 2019). The Larat orchid is included in the Appendix II category of CITES 2019, which means that the taking of these orchids directly from the natural environment is prohibited because of excessive exploitation by the community of the presence of this plant in its natural habitat will reduce the number of this plant. Therefore, it is necessary to carry out mass propagation by *in vitro* culture for both commercial use and ex situ conservation.

Orchid conservation can reduce the threat of extinction, both through conventional and tissue culture approaches. Orchid propagation technology has been widely developed in the forms of tissue culture and genetic engineering. Tissue culture methods are considered effective for obtaining large plant yields relatively quickly. In addition, this method can improve the quality of flower development in orchids (Zulkaidha et al. 2019). Orchid propagation through tissue culture and genetic transformation has been reported by Setiari et al. (2018), in which the *AtRKD4* embryogenesis gene from *Arabidopsis thaliana* was inserted into the protocorm genome of *D. phalaenopsis* using *Agrobacterium tumefaciens* carrying T-DNA with the *35S::GR::AtRKD4* construct.

The *RKD4* gene can induce the formation of SEs. This gene encodes a protein that has the RWP-RK motif, the amino acid sequence consisting of arginine (R), tryptophan (W), proline (P), arginine (R) and lysine (K), in early stages of embryo initiation in *Arabidopsis* (Chardin et al. 2014). The *RKD4* gene is appropriate for SE induction because it is one of the genes that have a role in early embryo development. The *RKD4* gene can be activated after being expressed by the YDA MAP kinase signalling cas-

cade (Bayer et al. 2017). In this way, *RKD4* is attached to the signalling of *YDA*, *WOX* and *PIN1* genes, contributing to embryo development. According to Zulwanis et al. (2020), it has shown the expression of the *AtRKD4* transgene induced by dexamethasone (DEX) or TDZ, with the highest expression level at five days in the culture of Larat orchid carrying T-DNA with a *35S::GR::AtRKD4* construct. *AtRKD4* was able to induce SE formation in Larat orchid treated with PGR. In this further research we would like to determine the ability of organs of Larat orchid to form SEs using PGR combinations of the auxin 1-naphthaleneacetic acid (NAA) and the cytokinin thidiazuron (TDZ). In the *Phalaenopsis amabilis* orchid, Mose et al. (2020) reported that a combination of TDZ and NAA effectively induced direct somatic embryogenesis, with the best concentration was 3.0 mg/L TDZ and 1.0 mg/L NAA. There are several objectives of this research, which are consist of: (1) to detect the stability of integration of *AtRKD4* as a transgene in the genome of transgenic Larat orchid; (2) to cultivate Larat orchid using TDZ and NAA; and (3) to analyse the phenotypic development of SEs using an anatomical approach.

2. Materials and Methods

2.1. Plant materials

The plants used in this study were two-year- and five-month-old transgenic (T) Larat orchids carrying *35S::GR::AtRKD4* construct and non-transformant (NT) plant specimens obtained from previous research in which its had undergone three rounds of *in vitro* propagation from the original transformants. A total of 150 plantlets were grown *in vitro* under continuous light conditions at a temperature of 25 ºC ± 1 ºC.

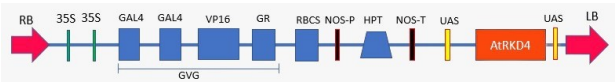

FIGURE 1 Construct of T-DNA carrying *35S::GR::AtRKD4* in plasmid pTA7002 (Mursyanti et al. 2015)

2.2. Induction of somatic embryos

NP medium (Islam et al. 1998) supplemented with 0.5 mg/L or 1 mg/L NAA (N) (N0 = 0 mg/L, N1 = 0.5 mg/L, N2 = 1 mg/L) and 1, 3 or 5 mg/L TDZ (T) (T0 = 0 mg/L, T1 = 1 mg/L, T2 = 3 mg/L, T3 = 5 mg/L) was used for the induction of SEs, providing 12 treatment combinations (Table 1). Each treatment was repeated three times to give 36 experimental units. Orchid plants that were known to carry the *AtRKD4* gene were used as explants for SE induction. The explants used were pseudobulbs cut into three parts and planted in treatment mediums. The cultures were continuously maintained at 25 ºC ± 1 ºC under continuous light.

Morphological development during somatic embryo formation was observed every week for eight weeks.

2.3. DNA isolation and detection of the insertion AtRKD4

The DNA isolation protocol was performed using CTAB (cetyl trimethylammonium bromide) solution following the method of Murray and Thompson (1980). Leaf materials in amounts of about 30–50 mg were used for DNA isolation. Detection of the *AtRKD4* transgene was carried out by direct PCR amplification with oligonucleotide specific primers as shown in Figure 1 and according to Mursyanti et al. (2015). In Figure 1, by using a pair composed of *AtRKD4* forward primer (‘5-GTTCATTTCATTTGGAGAGGACG-3) and *AtRKD4* reverse primer (5-CTTCCATATCTAGGAGAGAATCAAG-3), the expected amplified DNA fragment of *AtRKD4* was 382 bp. The fragment of *HPT* gene (545 bp) was amplified using HPTF1 (‘5-TCGGACGATTGCGTCGCATC-3) and HPTR1 (‘5-AGGCTATGGATGCGATCGCTG-3) primers. As internal positive control, a fragment from the *ACTIN* gene (114 bp) was amplified using *ACTIN* F1 (‘5-GTATTCCCTAGCATTGTTGGT-3) and *ACTIN* R1 (‘5-CAGAGTGAGAATACCTCGTTTG-3). The *HPT* gene was used is a gene for resistance to the hygromycin antibiotic. PCR reactions to amplify the expected fragments were carried out under conditions as described in the protocol of Bioline MyTaqTM HS Red Mix (UK), as follows: pre-denaturation at 95 ºC for 1 min; denaturation at 95 ºC for 15 s; annealing at 57.8 ºC for *AtRKD4* for 15 s, at 61 ºC for HPT for 15 s, and at 51 ºC for *ACTIN* for 15 s; elongation stage at 72 ºC for 10 s; hold for 12 ºC for 35 PCR cycles. The PCR results were analysed using 1% agarose gel for detection of *HPT* and *ACTIN* genes on TAE 1 X buffer. *AtRKD4* was detected using 1.4% agarose gel with 100 volts for 18 min, then observed and visualized using a UV transilluminator. The DNA marker is 100 bp DNA ladder (Geneaid, US).

2.4. Anatomical study

Anatomical study was conducted using the embedding method according to the procedure of Sutikno (2018). Pseudobulbs that had formed SEs were fixed using the paraffin method by being put into FAA solution (90 mL 70% alcohol, 5 mL formalin, 5 mL glacial acetic acid) in flask bottles for 24 h, then dehydrated using multilevel 70–100% alcohol solution followed by dealcoholisation with alcohol mixture 3:1, xylol:paraffin mixture 1:9. The sam-

TABLE 1 Addition of TDZ and NAA to NP media for induction of somatic embryos from Larat orchids

Treatments*	N0	N1	N2
T0	T0N0	T0N1	T0N2
T1	T1N0	T1N1	T1N2
T2	T2N0	T2N1	T2N2
T3	T3N0	T3N1	T3N2

*T0-3 = NP medium + 0–3 mg/L TDZ; N0-3 = NP medium + 0–3 mg/L NAA

ples were then infiltrated into pure paraffin at 57 ºC for 24 h, and the paraffin blocks were sliced with a rotary microtome. The samples were multilevel stained with 1% safranin in 70% alcohol. The anatomical samples were observed using a light microscope (Olympus, Japan) connected to an Optilab viewer 2.2 (Miconos, Indonesia).

2.4.1 Data analysis

All experiments with pseudobulb explants were repeated three times for twelve experiments. Univariate ANOVA was performed using a factorial treatment design (Compton 1994). The results were presented as mean ± standard error. Significant differences among the approaches were examined using Duncan's multiple range test (DMRT) at α=0.05 using SPSS v. 25.0 (IBM, United States).

3. Results and Discussion

3.1. Growth analysis of transformant and non-transformant Larat orchid (Dendrobium phalaenopsis Fitzg.)

In this study, growth observations were carried out on nine transformant (T) and non-transformant (NT) plants, respectively. The T and NT plantlets were grown on NP0 *in vitro* medium and growth was measured every two weeks for comparison.

The growth parameters are plant height, number of leaves, leaf length, leaf width, number of roots, root length, number of shoots, and shoot length. Based on these growth parameters, the T plants showed better growth rates than the NT plants, in that the leaves of T plants were longer than those of NT leaves. The results presented in Table 2 showed that the differences in the parameters were not significant. The result of this study are in line with research conducted by Semiarti et al. (2018) comparing the growth of *Phalaenopsis* "Sogo Vivien" orchid transformant plants carrying *35S::GR::AtRKD4* to that of non-transformant plants. Vegetative growth in transformant plants with the gene inserted is similar to that of NT plants, reflecting the expression level of the inserted gene. No different was found in the growth of *P. amabilis* orchid between NT plants and T plants carrying *35S::GR::AtRKD4* in ex vitro conditions (Perdana et al. 2017).

It was also found that there were no significant differences in morphological characteristics between NT Larat orchids and T Larat orchids that carry the *35S::GR::AtRKD4* gene. This is the expected result in orchids, i.e. that the number of embryos increases but the phenotype of the plant does not change. Waki et al. (2011) reported that this gene is specifically important in the beginning of embryogenesis. Therefore, it can be expected that in mass plant propagation, orchid plants will be produced that are uniformly the same as the parent.

3.2. Confirmation the stability of integration of the AtRKD4 gene in Larat orchid T-DNA transformants carrying 35S::GR::AtRKD4

The Larat orchid has morphological characteristics such as modified short stems and horizontal stems. Stem branching is sympodial, the main stem being upright and cylindric to fusiform and velamens being present on the root surfaces. No morphological differences were observed between the NT and T Larat orchids (Figure 2). Furthermore, after explant selection, the *AtRKD4* transgene was isolated from the genomic DNA of NT and T Larat orchids. The isolated genomic DNA showed clear DNA bands on 1 kb markers. The orchid genomic DNA was used as a template for the amplification process using specific primers for *AtRKD4*, *HPT* and *ACTIN* (Figure 3)

Detection of *AtRKD4*, *HPT* and *ACTIN* genes was carried out by PCR using specific primers, as shown in Figure 3. The fragments of the *ACTIN* 114 bp genes were amplified in the NT and T specimens. The fragments of *HPT* 545 bp and *AtRKD4* 382 bp genes were amplified in the T specimen carrying *35S::GR::AtRKD4*.

The genes detected were DNA fragments for *AtRKD4* (382 bp), *HPT* (545 bp) and *ACTIN* (114 bp). The results showed that the *AtRKD4* and *HPT* genes were found in all analysed T plants, but not in NT plants. This finding

TABLE 2 Morphological characteristics after eight weeks of T and NT Larat orchids grow from NP medium.

Parameters	Averages						Sig. (2- tailed T-test) P value
	Initial measurement		Growth difference		Growth percentage (%)		
	NT	T	NT	T	NT	T	
Plant height	15.76 ± 3.68	17.90 ± 3.00	2.49	2.35	15.80	13.13	0.22
Number of leaves	2.89 ± 0.74	3.44 ± 0.50	0.11	0.00	3.81	0.00	0.33
Leaf length	9.35 ± 4.95	9.30 ± 3.70	0.51	0.24	5.45	2.58	0.66
Leaf width	1.37 ± 0.44	1.37 ± 0.49	0.10	0.19	7.30	13.87	0.08
Number of roots	1.67 ± 0.67	2.11± 0.87	0.11	0.22	6.59	10.43	0.55
Root length	2.81 ± 1.41	3.54 ± 2.11	0.29	0.36	10.32	10.17	0.39
Number of shoots	0.00 ± 0	0.00 ± 0	0.56	0.89	56.00	89.00	0.36
Shoot length	0.00 ± 0	0.00 ± 0	5.76	7.73	576.00	773.00	0.38

is reinforced by the results showing that the *ACTIN* gene can be amplified in all plants, both T and NT. It suggested that the *AtRKD4* gene was stably integrated in this generation. The explants carrying *AtRKD4* genes have the ability to produce SEs after being induced by TDZ and NAA. TDZ and NAA are PGR compounds that can stimulate plant development and induce T-DNA with glucocorticoid response (GR). Zulwanis et al. (2020) obtained explants of *D. phalaenopsis* that after being induced by TDZ showing cDNA of *AtRKD4* on induction at one, three, five and seven days. The results of *HPT* gene amplification also showed that the T plants carried the hygromycin resistance gene, as shown by Setiari et al. (2018). *HPT* can be used as a selectable marker in T-DNA of *A. tumefaciens* and proves that the positive T plants are resistant to hygromycin. Likewise, the presence of *ACTIN* can be used as an indicator that the genomic DNA of plants, both NT and T, is in good condition. It has been proven that 114 bp of DNA fragments from the *ACTIN* gene as a housekeeping gene must always be found in all plants (Semiarti et al. 2011; Zulwanis et al. 2020; Puspitasari et al. 2020; Lee et al. 2020). In this study, T-DNA containing the glucocorticoid (GR) induction system was used. The GR induction system is a gene expression system widely used in plants in which the chimeric GVG transcription factor consists of the yeast GAL4 binding domain (BD), the Herpes simplex VP16 activation domain (AD), and the mouse glucocorticoid receptor. Induction is based on the presence of a GR domain to constitutively localize GVG protein expression to the cytoplasm in the absence of an inducer. The chimeric GVG protein remaining in the cytoplasm will be bound by a 90 kDa heat shock protein (*HSP90*) complex and cannot enter the nucleus (Picard 1993). After the addition of PGRs such as TDZ and NAA, the chimeric protein can be released from its interaction with *HSP90* and can be localized to the nucleus to exert its function as a transcription factor. The presence of TDZ and NAA is thought to be able to trigger the endogenous steroid hormone in Larat orchid, thus stimulating the expression of the *AtRKD4* transgene. Plants contain endogenous steroid hormones known as brassinosteroids. The signalling of brassinosteroids plays a role in stimulating cell division, cell elongation and cell enlargement (Tang et al. 2016; Nolan et al. 2017; Planas-Riverola et al. 2019; Li and He 2020). Based on this study, the success of the *AtRKD4* gene carrying *35S::GR::AtRKD4* was triggered by complex interaction signalling between TDZ, NAA and brassinosteroids. Thus, further research is needed to prove whether the signals from these three hormones are indeed necessary in the formation of SEs.

3.3. The effect of NAA and TDZ and its combinations on induction of somatic embryogenesis in the Larat orchid

The use of TDZ and NAA for SE induction over a period of eight weeks of culturing revealed that pseudobulb was the most effective explant for SE formation in all treatment media (Table 3). The highest percentage of SE formation

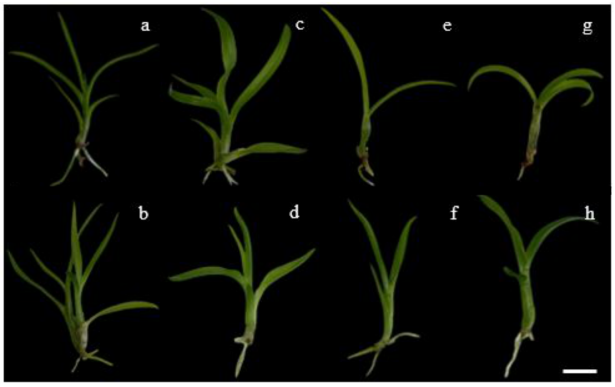

FIGURE 2 Morphology of Larat orchid NT and T plants: a and b: non-transformant; c–h: transformant (Scale bar = 1 cm)

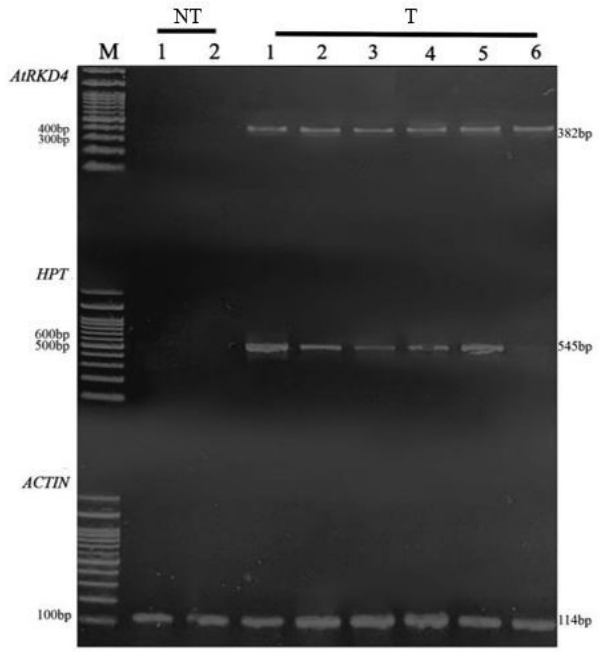

FIGURE 3 Integration of *AtRKD4*, *HPT* and *ACTIN* genes in NT and T Larat orchid (M: 100 bp DNA ladder marker; Lanes 1–2 (NT): non-transformant Larat orchid: Lanes 1–6 (T): Larat orchid transformant carrying *35S::GR::AtRKD4*)

(100%) was shown in pseudobulb explants induced on NP medium with added PGR in a combination of 1 mg/L TDZ + 1 mg/L NAA (2.11± 1.36). Single treatment with 1 mg/L TDZ produced similar number of SEs (2.22 ± 1.39) but not all pseudobulb explants could produce SE (only 89%), the addition of 1 mg/L NAA pseudobulb was able to produce one SE in root explants (0.09 ± 0.37a). This is in accordance with the results of previous studies reported by Mose et al. (2017), Febryanti et al. (2020), Zulwanis et al. (2020), and Puspitasari et al. (2020), which showed that pseudobulb explants were the most responsive in SE formation. Although DMRT testing showed that in the formation of SEs in pseudobulb explants from media with 1 mg/L TDZ have similar result with the media with a combination of 1 mg/L TDZ + 1 mg/L NAA, and a significant difference in relation to other treatment media, but the per-

centage of SE formation the addition of a combination of 1 mg/L TDZ + 1 mg/L NAA was higher (100%). The effect of various concentrations of TDZ, NAA and their combination on the amount of SE based on the DMRT test and the percentage of SE formation from various types of explants of Larat transformant orchids is visualized in Figures 4 and 5.

Somatic embryogenesis is enhanced by the addition of PGRs such as cytokinin and auxin alone or in combination (Ji et al. 2011). The role of PGRs such as TDZ in inducing SEs in orchid plants has been proven in several studies in which TDZ is used either singly or in combination to induce SE formation (Feng and Chen 2014; Balilashaki and Ghehsareh 2016; Mahendran and Bai 2016; Mose et al.

TABLE 3 The effect of various PGRs and their combinations on the formation of SEs from various types of explants of Larat orchid over eight weeks' cultivation

Treatments (mg/L)	Types of explants	Explant	SEs formation	SEs formation (%)	Number of SEs
T0N0	Pseudobulb	9	7	78	0,88 ± 0,60bc
(NP0)	Leaves	9	0	0	0,00 ± 0,00
Control	Root	9	0	0	0,00 ± 0,00b
T0N1	Pseudobulb	9	15	100*	1,75 ± 0,88ab
(0 TDZ + 0.5 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Root	9	0	0	0,00 ± 0,00b
T0N2	Pseudobulb	9	18	100*	2,00 ± 0,866ab
(0 TDZ + 1 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Root	9	1	100*	0,09 ± 0,37a
T1N0	Pseudobulb	9	20	89*	2.22 ± 1,39a
(1 TDZ + 0 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Root	9	0	0	0,00 ± 0,00b
T1N1	Pseudobulb	9	16	100*	1.77 ± 0.97ab
(1 TDZ + 0.5 NAA)	Leaves	9	0	0	0.00 ± 0.00
	Roots	9	0	0	0.00 ± 0.00b
T1N2	Pseudobulb	9	19	100*	2,11± 1,36a
(1 TDZ + 1 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T2N0	Pseudobulb	9	10	56	1,11 ± 1,16abc
(3 TDZ + 0 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T2N1	Pseudobulb	9	14	89	1,55 ± 1,01abc
(3 TDZ + 0.5 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T2N2	Pseudobulb	9	15	89	1,66 ± 1,00ab
(3 TDZ + 1 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T3N0	Pseudobulb	9	10	78	1,11 ± 0, 92abc
(5 TDZ + 0 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T3N1	Pseudobulb	9	11	56	1,33 ± 1,58abc
(5 TDZ + 0.5 NAA)	Leaves	9	0	0	0,00± 0,00
	Roots	9	0	0	0,00 ± 0,00b
T3N2	Pseudobulb	9	10	33	0,44 ± 0,72c
(5 TDZ + 1 NAA)	Leaves	9	0	0	0,00 ± 0,00
	Roots	9	0	0	0,00 ± 0,00b

* = Highest percentage of SE formation.

2020; Zulwanis et al. 2020; Puspitasari et al. 2020). PGRs such as TDZ are able to stimulate endogenous steroid hormones in plants that will activate *AtRKD4* gene expression (Zulwanis et al. 2020). In SEs from pseudobulb explants on NP medium supplemented with combinations of TDZ (1, 3, and 5 mg/L) and NAA (0.5 and 1 mg/L), TDZ plays an important role in stimulating production of endogenous cytokinin and as an inhibitor of cytokinin oxidase (as an enzyme) that can eliminate the activity of free adenine cytokinin. The role of TDZ can improve other cell sites in relation to both endogenous and exogenous cytokinin (Guo et al. 2011). TDZ affects plant physiological processes such as cellular development, energy, nutrients, transport and metabolic changes in plant membranes (Ouyang et al. 2016), and physiological and biochemical biological processes in cells can be induced or enhanced by the use of TDZ (de Melo Ferreira et al. 2006).

As shown in Table 3, single NAA treatment consisted of two dosages: 0.5 mg/L and 1 mg/L without TDZ. These treatments were able to induce SEs of Larat orchids in pseudobulb explants. A single concentration of NAA (0.5 and 1 mg/L) was able to produce SE formation in 100% of specimens (15 and 18 SEs respectively). This is in accordance with the research of Sherif et al. (2018), which found that NAA alone with a concentration of 0.5–1 mg/L could stimulate direct nodal induction of somatic embryogenesis (36.5%) in *Anoectochilus elatus* Lindl. explants. Root explants produced SEs in the single treatment of 1 mg/L NAA (0.09 ± 0.37a). The development of root explants was characterized by a globular structure at the base of the root.

The best treatment in this study based on the percentage of the number of explants that produced SE supported by the DMRT test results was pseudobulb explants treated with a combination of 1 mg/L TDZ and 1 mg/L NAA, indicated by 100 percent explants producing SE as much as 2.11 ± 1.36a. When TDZ and NAA are combined there is significant increase in the percentage frequency of somatic embryogenesis and higher numbers of embryos. In *Anoectochilus elatus*, it was reported that after eight weeks of cultivation, the maximum number of somatic embryos was 31.3 with 76.4% SE induction on a combination medium of 4.54 mg/L TDZ and 2.69 mg/L NAA (Sherif et al. 2018). Puspitasari et al. (2020) found that NP + TDZ 2 mg/L + DEX 12 µM medium was the best treatment to produce SEs with 100% production in pseudobulb explants. In a study by Febryanti et al. (2020) it was stated that the combination of PGRs increased the formation of SEs, including DEX 15 µM + TDZ 1 mg/L which induced the formation of the best somatic embryos in stem explants of transformant *Dendrobium lineale*.

Auxin is a powerful plant hormone required for the initiation of SE development. In this research, the addition of 1 mg/L TDZ to the culture medium in combination with auxin significantly increased the number of explants that formed SEs and the number of SEs. This is in accordance with Kim et al. (2019) that reported the induction frequency of SEs can be increased by increasing the level of NAA in the medium from 8 to 16 µM.

3.4. *Morphological and anatomical analysis of transformant Larat orchid (Dendrobium phalaenopsis Fitzg.) after induction medium treatments*

In vitro propagation of transformant Larat orchids from several types of explants indicates that SEs can be produced directly without callus formation (Puspitasari et al. 2020). In this study, the optimum treatment for SE induction was observed in the second week of SE development. Morphological observations are presented in Figure 4 and anatomical observations in Figure 5.

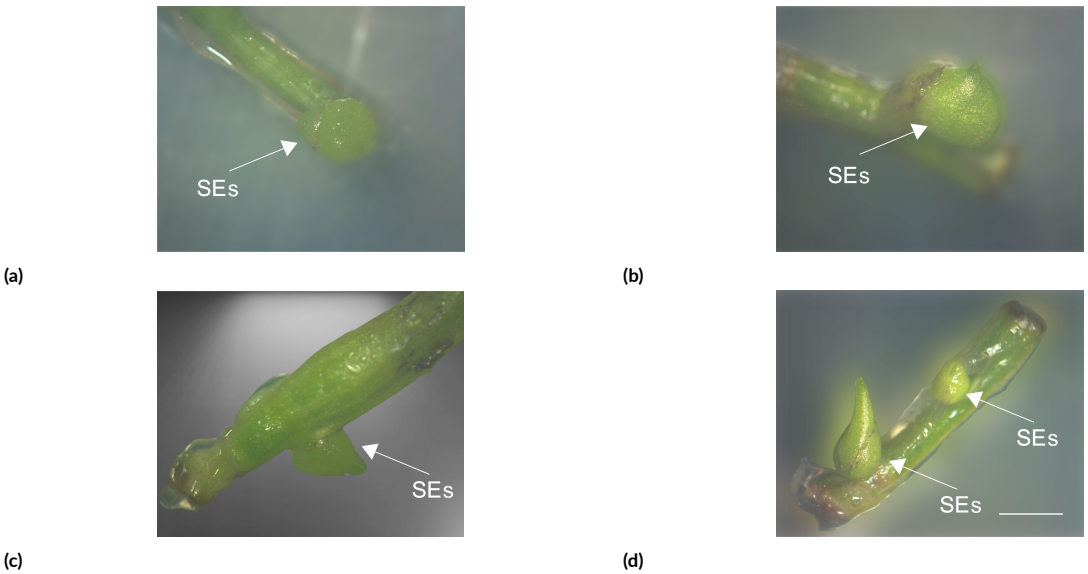

FIGURE 4 Morphology of SEs on pseudobulb explants of Larat orchid after being induced in a single treatment and in combinations of TDZ and NAA for two weeks. a) TDZ 0 mg/L + NAA 1 mg/L); b) TDZ 1 mg/L + NAA 1 mg/L; c) TDZ 1 mg/L + NAA 0.5 mg/L; and d) TDZ 1 mg/L + NAA 0 mg/L. Scale bar = 1 cm.

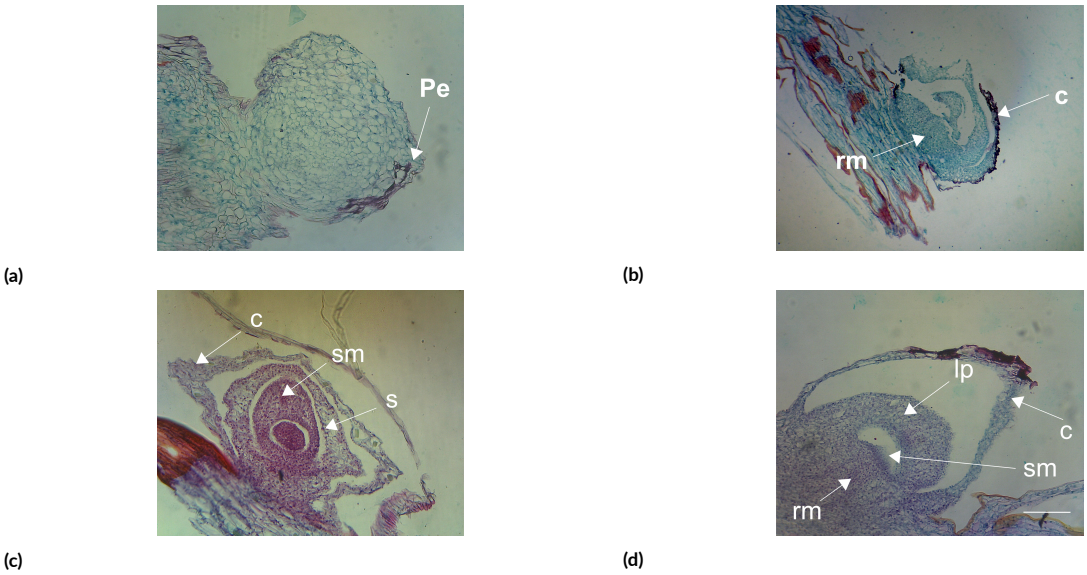

FIGURE 5 Anatomic structures of the best SEs from pseudobulb explants of Larat orchid after two weeks' induction in treatment combinations or single applications of TDZ and NAA. a) NAA 1 mg/L treatment; b) TDZ 1 mg/L + NAA 1 mg/L treatment; c) TDZ 1 mg/L + NAA 0.5 mg/L treatment; d). TDZ 1 mg/L treatment. Notes: Pe = proembryo; c = coleoptile; rm = root meristem; sm = shoot meristem; sc = scutellum; lp = leaf primordia. Scale bar = 100 µm.

In Figure 4, it can be seen that the morphology of Larat orchid SEs pseudobulb explants develops rapidly. In the second week the SEs develop, swell and are globular in form (Figure 4a); a shoot meristem then begins to develop on the upper surface of the SE (Figures 4b and 4c); then shoot primordia begin to appear that will develop into new shoots (Figure 4d). Setiari et al. (2018) have also reported the morphology of embryonic development from seeds of Larat orchid. The development of orchid seeds is characterized by swelling of the embryo and change in colour from yellow to green during the germination phase (Phase 1); the embryo then becomes swollen and begins to enlarge but is still wrapped in the testa (seed coat) (Phase 2); the larger embryo will then emerge from the testa and become rounded, forming the protocorm (Phase 3); the protocorm then forms a bipolar structure and is flat with apical bud meristems that develop at the upper surface (Phase 4); shoot primordia then begin to develop (Phase 5); and finally shoot primordia rise and grow into shoots (Phase 6).

The anatomical structure shown in Figure 5 represents SEs from pseudobulb explants in the optimum treatments. Longitudinal cross-section of the SEs in Figure 5a (NAA 1 mg/L) show slightly slow development, with the SE proembryo being globular in shape surrounded by protoderm and the basal forming of a suspensor. Figure 5b (TDZ 1 mg/L + NAA 1 mg/L) shows the development of the coleoptile (c) and root meristem (rm). Figure 5c (TDZ 1 mg/L + NAA 0.5 mg/L) shows the coleoptile (c) still wrapped around the shoot meristem (sm) and scutellum (S). Figure 5d supplemented with 1 mg/L TDZ shows the presence of coleoptiles covering the leaf primordia (lp), shoot meristems (sm), and root meristems (rm). The SEs are characterized by the presence of meristematic cells of different shapes and sizes. The outermost layer of embryonic cells is the protoderm layer. The protoderm is a precursor to epidermal meristems which are characterized by adjacent cell layers and a prominent nucleus (Meira et al. 2019). In the protoderm stage, the formation of apical or basal polarity is observed with the development of shoot apical meristems and root meristems in SEs (Oliveira et al. 2017). As shown in Figure 5, the somatic embryogenesis pattern of monocot plants such as Larat orchids are proembryo stages, globular embryos, scutellar embryos and coleoptilar embryos.

4. Conclusions

The *AtRKD4* gene was stably integrated in the genome of the Larat orchid transformant carrying T DNA with *35S::AtRKD4*. The best explants that could form SEs were pseudobulbs grown on NP medium with the addition of 1 mg/L TDZ and 1 mg/L NAA which 100% explants produced SE with an average amount (2.11 ± 1.36). There was no significant difference between the morphology of SE produced from the non-transformant Larat orchid pseudobulb explants and the *35S::AtRKD4* carrier transformant.

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Authors' contributions

ES designed and controlled the research and wrote the manuscript. MI conducted the isolation of genomic DNA, PCR, data compiling, morphology and anatomy analysis and wrote the manuscript. FP conducted growth analysis and compiled data.

Competing interests

The authors declare no competing interests.

References

Balilashaki K, Ghehsareh MG. 2016. Micropropagation of *Phalaenopsis amabilis* var. 'Manila' by leaves obtained from *in vitro* culturing the nodes of flower stalks. Not. Sci. Biol. 8(2):164–169. doi:10.15835/nsb.8.2.9782.

Bayer M, Slane D, Jürgens G. 2017. Early plant embryogenesis — Dark ages or dark matter? Curr. Opin. Plant Biol. 35:30–36. doi:10.1016/j.pbi.2016.10.004.

Chardin C, Girin T, Roudier F, Meyer C, Krapp A. 2014. The plant RWP-RK transcription factors: Key regulators of nitrogen responses and of gametophyte development. J. Exp. Bot. 65(19):5577–5587. doi:10.1093/jxb/eru261.

de Melo Ferreira W, Barbante Kerbauy G, Elizabeth Kraus J, Pescador R, Mamoru Suzuki R. 2006. Thidiazuron influences the endogenous levels of cytokinins and IAA during the flowering of isolated shoots of Dendrobium. J. Plant Physiol. 163(11):1126–1134. doi:10.1016/j.jplph.2005.07.012.

Febryanti NLPK, Nurliana S, Gutierrez-Marcos J, Semiarti E. 2020. The expression analysis of AtRKD4 transgene in *Dendrobium lineale* rolfe transgenic orchid carrying 35S::GR::AtRKD4 for micropropagation. In: AIP Conf. Proc., volume 2260. p. 060021. doi:10.1063/5.0015876.

Feng JH, Chen JT. 2014. A novel in vitro protocol for inducing direct somatic embryogenesis in Phalaenopsis aphrodite without taking explants. Sci. World J. 2014:1–7. doi:10.1155/2014/263642.

Guo B, Abbasi BH, Zeb LL A ad Xu, Wei YH. 2011. Thidiazuron: a multidimension plant growth regulator. Afr. J. Biotechnol. 10(45):8984–9000. doi:10.5897/AJB11.636.

Islam MO, Ichihashi S, Matsui S. 1998. Control of growth and development of protocorm like body derived from callus by carbon sources in *Phalaenopsis*. Plant Biotechnol. 15(4):183–187. doi:10.5511/plantbiotechnology.15.183.

Ivakdalam LM, Pugesehan DJ. 2016. Keragaman jenis tanaman anggrek (Orchidaceae) di Cagar Alam Angwarmase, Kabupaten Maluku Tenggara Barat. J. Agroforestri 11(3):163–168.

Ji A, Geng X, Zhang Y, Wu G. 2011. Advances in somatic embryogenesis research of horticultural plants. Am. J. Plant Sci. 02(06):727–732. doi:10.4236/ajps.2011.26087.

Kim DH, Kang KW, Sivanesan I. 2019. Influence of auxins on somatic embryogenesis in *Haworthia retusa* Duval. Biologia (Bratisl). 74(1):25–33. doi:10.2478/s11756-018-0151-1.

Lee MH, Lee J, Choi SH, Jie EY, Jeong JC, Kim CY, Kim SW. 2020. The effect of sodium butyrate on adventitious shoot formation varies among the plant species and the explant types. Int. J. Mol. Sci. 21(22):8451. doi:10.3390/ijms21228451.

Li Z, He Y. 2020. Roles of brassinosteroids in plant reproduction. Int. J. Mol. Sci. 21(3):1–16. doi:10.3390/ijms21030872.

Mahadi I. 2017. Multifikasi tunas anggrek larat (*Dendrobium phalaenopsis* Fitzg.) dengan pemberian hormon IAA dan BAP terhadap pertumbuhan secara in vitro. Eksakta 2(XVII):1–6.

Mahendran G, Bai VN. 2016. Direct somatic embryogenesis of *Malaxis densiflora* (A. Rich.) Kuntze. J. Genet. Eng. Biotechnol. 14(1):77–81. doi:10.1016/j.jgeb.2015.11.003.

Meira FF, Luis ZG, de Araújo Silva-Cardoso IM, Everson Scherwinski-Pereira J. 2019. Developmental pathway of somatic embryogenesis from leaf tissues of macaw palm (*Acrocomia aculeata*) revealed by histological events. Flora Morphol. Distrib. Funct. Ecol. Plants 250:59–67. doi:10.1016/j.flora.2018.11.011.

Mose W, Daryono BS, Indrianto A, Purwantoro A, Semiarti E. 2020. Direct somatic embryogenesis and regeneration of an Indonesian orchid *Phalaenopsis amabilis* (L.) Blume under a variety of plant growth regulators, light regime, and organic substances. Jordan J. Biol. Sci. 13(4):509–518. URL https://jjbs.hu.edu.jo/files/vol13/n4/Paper%20Number%2013.pdf.

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. doi:10.1016/j.hjb.2017.11.005.

Murray MG, Thompson WF. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8(19):4321–4326. doi:10.1093/nar/8.19.4321.

Mursyanti E, Purwantoro A, Moeljopawiro S, Semiarti E. 2015. Induction of somatic embryogenesis through overexpression of ATRKD4 genes in Phalaenopsis "Sogo Vivien". Indones. J. Biotechnol. 20(1):42–53. doi:10.22146/ijbiotech.15276.

Nolan T, Chen J, Yin Y. 2017. Cross-talk of Brassinosteroid signaling in controlling growth and stress responses. Biochem. J. 474(16):2641–2661. doi:10.1042/BCJ20160633.

Oliveira EJ, Koehler AD, Rocha DI, Vieira LM, Pinheiro MVM, de Matos EM, da Cruz ACF, da Silva TCR, Tanaka FAO, Nogueira FTS, Otoni WC. 2017.

Morpho-histological, histochemical, and molecular evidences related to cellular reprogramming during somatic embryogenesis of the model grass *Brachypodium distachyon*. Protoplasma 254(5):2017–2034. doi:10.1007/s00709-017-1089-9.

Ouyang Y, Chen Y, Lü J, Teixeira Da Silva JA, Zhang X, Ma G. 2016. Somatic embryogenesis and enhanced shoot organogenesis in *Metabriggsia ovalifolia* W. T. Wang. Sci. Rep. 6:24662. doi:10.1038/srep24662.

Perdana NGA, Mose W, Lawrie MD, Gutierrez-Marcos J, Semiarti E. 2017. Stable transformant of *Phalaenopsis amabilis* somatic embryo carrying 35S::GR::AtRKD4 develops into normal phenotype of transgenic plant. J. Tropical Biodiversity Biotechnology 06(02):jtbb59210. doi:10.22146/jtbb.59210.

Picard D. 1993. Steroid-binding domains for regulating the functions of heterologous proteins in Cis. Trends Cell Biol. 3(8):278–280. doi:10.1016/09628924(93)90057-8.

Planas-Riverola, Gupta A, Betegon-Putze I, Bosch N, Ibanes M, Cano-Delgado AI. 2019. Brassinosteroid signaling in plant development and adaptation to stress. Development 146(5):dev151894. doi:10.1242/dev.151894.

Puspitasari F, Sutra CL, Sundari D, Wirajagat GC, Setiari N, Gutierrez-Marcos J, Semiarti E. 2020. Stability of AtRKD4 gene integration in the genome of Dendrobium phalaenopsis fitzg. Transformants induces somatic embryogenesis. Propag. Ornam. Plants. 20(4):129–138.

Semiarti E, Indrianto A, Purwantoro A, Machida Y, Machi C. 2011. *Agrobacterium*-mediated transformation of Indonesian orchids for micropropagation. London, United Kingdom: IntechOpen. doi:https://doi.org/10.5772/24997.

Semiarti E, Mursyanti E, Suyoko A, Perdana FSW, Widyastuti CT, Subchan AN. 2018. Stability of T-DNA integration in *Phalaenopsis* "Sogo Vivien" transgenic orchid carrying 35S::Gal4::AtRKD4::GR. Biol. Med. Nat. Prod. Chem. 7(1):5–13. doi:10.14421/biomedich.2018.71.5-13.

Setiari N, Purwantoro A, Moeljopawiro S, Semiarti E. 2018. Micropropagation of *Dendrobium phalaenopsis* orchid through overexpression of embryo gene AtRKD4. Agrivita 40(2):284–294. doi:10.17503/agrivita.v40i2.1690.

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. doi:10.1007/s11240-017-1330-4.

Sutikno. 2018. Plant Microtechnic Handbook for Plant Structure and Development Laboratory. Yogyakarta, Indonesia: Biology Faculty, Universitas Gadjah Mada.

Tang J, Han Z, Chai J. 2016. Q&A: What are brassinosteroids and how do they act in plants. BMC Biol. 14(113):1–5. doi:10.1186/s12915-016-0340-8.

Waki T, Hiki T, Watanabe R, Hashimoto T, Nakajima K. 2011. The arabidopsis RWP-RK protein RKD4 triggers gene expression and pattern formation in early embryogenesis. Curr. Biol. 21(15):1277–1281. doi:10.1016/j.cub.2011.07.001.

Zulkaidha Z, Muslimin M, Hapid A, Toknok B. 2019. Upaya Konservasi Tanaman Hias Anggrek Melalui Perbanyakan Secara Vegetatif dan Kultur Jaringan. In Seminar Nasional Hasil Penelitian dan Pengabdian Kepada Masyarakat (SNP2M) p. 217–221.

Zulwanis, Setiari N, Gutierrez-Marcos J, Semiarti E. 2020. The expression of AtRKD4 transgene during induction of somatic embryogenesis in transgenic *Dendrobium phalaenopsis* orchid carrying 35S::GR::AtRKD4. AIP Conf. Proc. 2260:060015–1–060015–6. doi:10.1063/5.0015873.