




## SCIENTIFIC ARTICLE

## TDZ and 2,4-D on *in vitro* propagation of panda plant from leaf explants

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### Abstract

The panda plant (*Kalanchoe tomentosa* Baker) is a popular ornamental succulent. The optimal method for *in vitro* plantlet propagation was investigated. Effects of plant growth regulator on growth and development of leaf explants were determined using various concentrations of Thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 0.01, 0.1, 0.2 and 0.5 mg L<sup>-1</sup>. A concentration of 0.01 mg L<sup>-1</sup> 2,4-D gave the highest plantlet quality derived from direct organogenesis. Higher concentrations of 2,4-D promoted callus proliferation. The lowest concentration of 0.01 mg L<sup>-1</sup> TDZ induced shorter shoots, while higher concentrations resulted in greater callogenesis and inhibit root production. After *in vitro* culture, plantlets from the optimal treatment were acclimatized by exposure to growth in sand, sand with coconut husk (2:1), sand with potting soil (2:1) and sand with perlite (2:1). Highest survival percentage (100%) was found in plantlets grown in sandy soil, the most well-drained material of those selected materials. Results demonstrated an alternative production method for panda plantlets using plant tissue culture techniques.

**Keywords:** *Kalanchoe tomentosa*, *in vitro* propagation, plant growth regulator, plantlet acclimatization, succulent plant.

### Resumo

#### TDZ e 2,4-D na propagação *in vitro* de planta de panda a partir de explantes de folhas

A planta panda (*Kalanchoe tomentosa* Baker) é uma suculenta ornamental popular. O método para a propagação de plântulas *in vitro* foi investigado. Os efeitos do regulador de crescimento de plantas no crescimento e desenvolvimento de explantes de folhas foram determinados usando várias concentrações de Thidiazuron (TDZ) e ácido 2,4-diclorofenoxyacético (2,4-D) a 0; 0,01; 0,1; 0,2 e 0,5 mg L<sup>-1</sup>. A concentração de 0,01 mg L<sup>-1</sup> de 2,4-D proporcionou a maior qualidade de mudas derivada da organogênese direta. Concentrações mais elevadas de 2,4-D promoveram a proliferação de calos. A menor concentração de 0,01 mg L<sup>-1</sup> de TDZ induziu brotações mais curtas, enquanto concentrações maiores resultaram em maior calogênese e inibiram a produção de raízes. Após o cultivo *in vitro*, as plântulas do tratamento ótimo foram aclimatadas por exposição ao crescimento em areia, areia com casca de coco (2:1), areia com solo para envasamento (2:1) e areia com perlita (2:1). A maior porcentagem de sobrevivência (100%) foi encontrada em mudas cultivadas em solo arenoso, o material mais bem drenado dos materiais selecionados. Os resultados demonstraram um método alternativo de produção de mudas de panda usando técnicas de cultura de tecidos de plantas.

**Palavras-chave:** *Kalanchoe tomentosa*, propagação *in vitro*, regulador de crescimento vegetal, aclimação de plântulas, planta suculenta.

### Introduction

The panda plant, *Kalanchoe tomentosa* Baker, belongs to the family Crassulaceae that comprises 35 genera and some 1400 species. This perennial succulent has distinct hairy velvet leaves and is found naturally in rocky areas of central and southern central Madagascar. The attractive characteristic of the leaves has promoted *K. tomentosa* as an indoor ornamental plant. This succulent is usually used as

potting plant and container gardening (Smith et al., 2019). Alternatively, the panda plant has an important substance that can be applied for medicinal uses such as anticancer and antidiabetes (Aisyah et al., 2015; 2020). propagation of *K. tomentosa* is usually handled by leaf and stem cutting, however, the donor plants can be deteriorated. With an increasing demand on ornamental succulents (Stead, 2016), the plantlet production may be inadequate. An alternative method that can produce numerous plantlets in short time is

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archived by plant tissue culture. *In vitro* propagation has been reported in several *Kalanchoe* species (Kulus, 2015; Kale et al., 2018; Cui et al., 2019) but there are few reports for panda plant (Frello et al., 2002; Khan et al., 2006). For success in plant micropropagation *in vitro*, suitable techniques must be followed as many factors affect plant tissue culture including the effects of plant growth regulators (PGRs).

Thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) as synthetic PGRs are widely used for tissue culture in many plant species. Optimal concentration of PGRs enhances high crop yield. In *Kalanchoe* species, TDZ applied to induce the production of numerous shoots depending on concentration and explant cultivar (Sanikhani et al., 2006). TDZ also induced explants of a *Kalanchoe* species to regenerate callus (Kertrung and Junkasiraporn, 2018); however, the effect of TDZ concentration on *in vitro* culture of *K. tomentosa* Baker has not yet been examined. Another synthetic PGR, 2,4-D is also used for callus induction in many plant species (Machakova et al., 2008) and its effects have not been reported on *in vitro* culture of *K. tomentosa*. Understanding about the effects of those PGRs on explant responses may improve the plantlet production *in vitro* of the panda plant. One of the limitations on plant tissue culture is plantlet exposure *ex vitro* because high mortality may be occurred in this process. For the successfully propagation, the plantlet acclimatization *ex vitro* should be investigated (Vahdati et al., 2017).

Thus, here, the effects of different concentrations of TDZ and 2,4-D were examined on *in vitro* propagation of *K. tomentosa* leaf explants to optimize plantlet production. Also, the optimal planting materials for plantlet acclimatization were investigated. The improvement of *K. tomentosa* plantlets by plant tissue culture techniques was reported here.

## Material and Methods

### Plant materials and media preparation

Plant materials were purchased from a local market and grown in a greenhouse for one week. Four leaves were collected from each plant as the fourth to the seventh leaves from the shoot apex. The leaves were washed in tap water with 1% (v v<sup>-1</sup>) commercial surfactant (Sunlight®) and rinsed in running tap water for 30 min. Then, the cleaned leaves were surface-disinfected by a series of 70% (v v<sup>-1</sup>) ethanol for 1 min, 0.01% (w v<sup>-1</sup>) mercuric chloride solution with 1% (v v<sup>-1</sup>) Tween 20 for 5 min, and 10% commercial hypochlorite solution (Clorox®) for 10 min. disinfected leaves were washed aseptically three times in distilled water. Ventral sides of the leaves were placed on the surface of semi-solid MS (Murashige and Skoog, 1962) basal medium supplemented with 0.5 ml L<sup>-1</sup> plant preservative mixture (PPM™; Plant Cell Technology). The explants were then incubated at 25 ± 2 °C under 40 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity (provided by cool white LED tubes) and 12 h light photoperiod. Regenerated shoots were subcultured on

MS basal medium and aseptic plants were used as explant material in further experiments.

The widely used MS basal medium was applied containing 30 g L<sup>-1</sup> sucrose (MitrPhol®), 0.1 g L<sup>-1</sup> myo-inositol (Phytotechnology Laboratory®) and 7 g L<sup>-1</sup> agar (AA, Pearl Mermaid®). The pH of the medium was adjusted to 5.8 before autoclaving (121 °C, 15 psi and 20 min).

### Effects of plant growth regulator on growth and development of leaf explants

Leaf explants at about 5×5 mm<sup>2</sup> from aseptic culture were randomly applied in the experiment, and cultured on MS media supplemented with different concentrations (0, 0.01, 0.1, 0.2 and 0.5 mg L<sup>-1</sup>) of TDZ and 2,4-D. Each treatment was applied to one explant per bottle. All treatments were incubated at 25 ± 2 °C at 40 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity and 12-h light photoperiod. Growth and development parameters of the explants were observed after culture for 4, 8 and 12 weeks.

### Plantlet acclimatization *ex vitro*

Healthy plantlets (new shoots with active root), induced by MS with 0.01 mg L<sup>-1</sup> 2,4-D, about 2 cm high from the previous experiment were applied in this experiment (Figure 4a). Each plantlet individuals were separated carefully from each other by scalpel. The plantlets were rinsed in running tap water and then submerged in 0.5% (w v<sup>-1</sup>) fungicide solution for 15 min. Disinfected plantlets were transplanted into four different planting materials including sand, sand with coconut husk (2:1), sand with potting soil (2:1) and sand with perlite (2:1). A total of 25 plantlets were randomly assigned for each treatment. After transplantation, the plantlets were grown in a greenhouse (27 to 30 °C ambient temperature, 50% to 60% relative humidity and 50% (sunlight) transparency). The plantlets were watered by a spray pump for 1-h twice a day. Survival rate and plantlet quality were recorded weekly for 10 weeks.

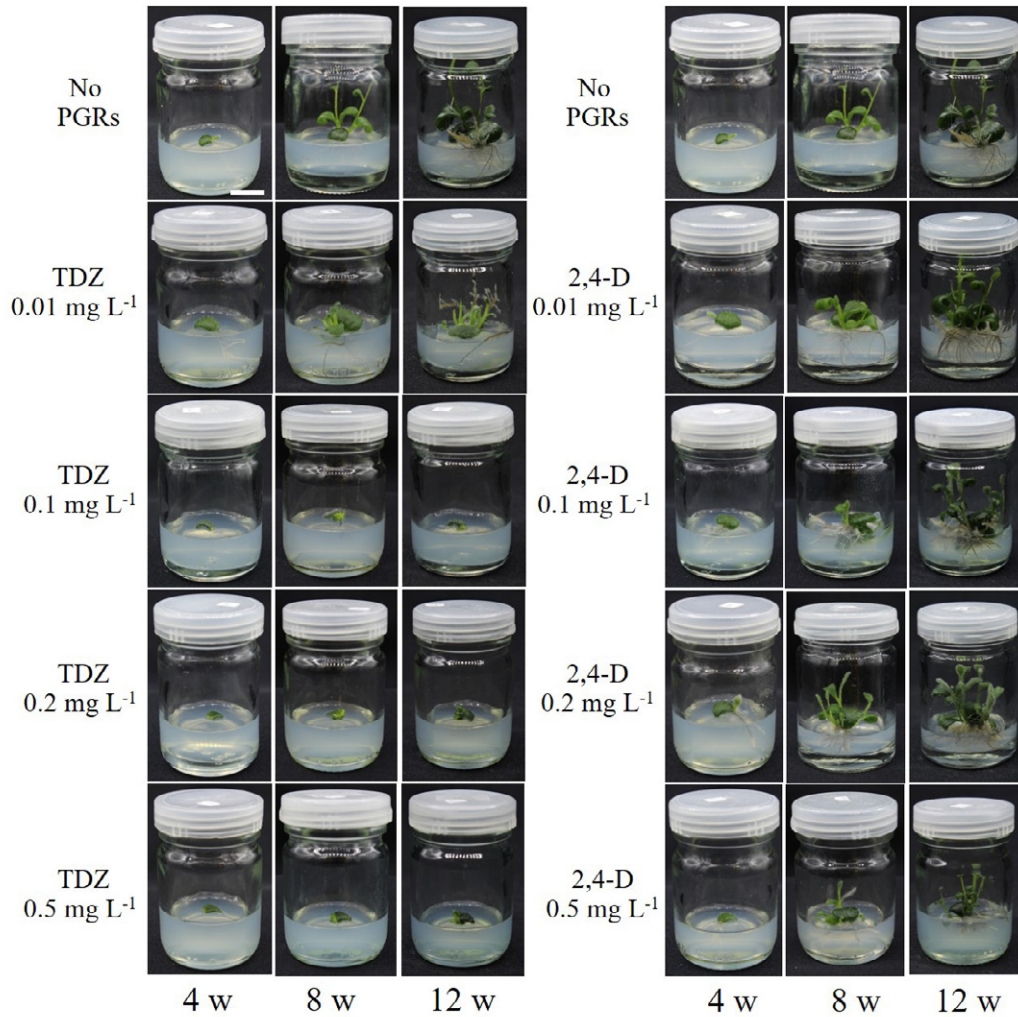
### Statistical analysis

All growth and development parameters of the *in vitro* experiment were analyzed by one-way ANOVA and post-hoc using Duncan's new multiple range test (DMRT) with SPSS version 22. To normalize the data distribution, the percentage data were arcsine transformed before analysis.

## Results

### Effects of plant growth regulator on growth and development of leaf explants

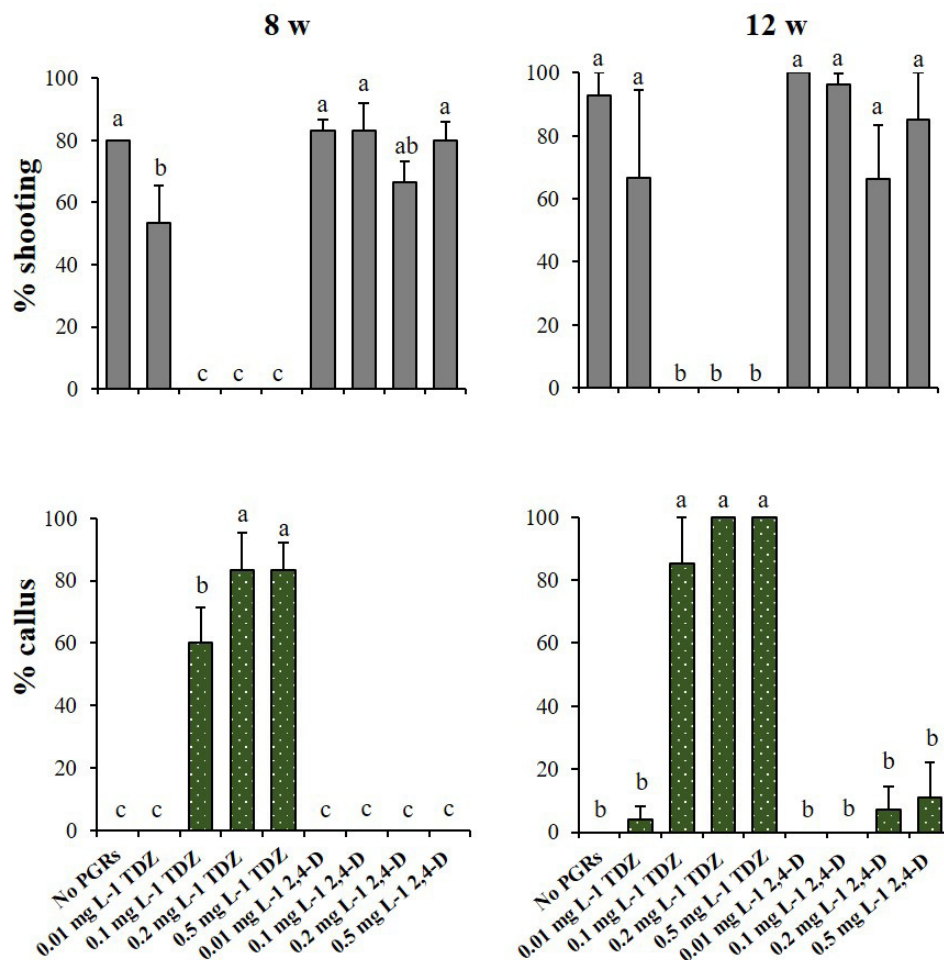
After culture for 4 weeks, direct organogenesis was observed in most explants except those cultured on MS medium supplemented with 0.2 and 0.5 mg L<sup>-1</sup> TDZ. Direct organogenesis was displayed as the occurrence of root(s) from the cut end of the explants (Figure 1).



**Figure 1.** Growth and development of *Kalanchoe tomentosa* explants cultured on different PGR treatments after 4, 8 and 12 weeks (bar = 1 cm).

After culture for 8 weeks, shoot regeneration ranging from 53.3 to 83.3% was found in 0.01 mg L<sup>-1</sup> TDZ, 0.01, 0.1, 0.2 and 0.5 mg L<sup>-1</sup> 2,4-D and the control treatments (Figures. 1 and 2).

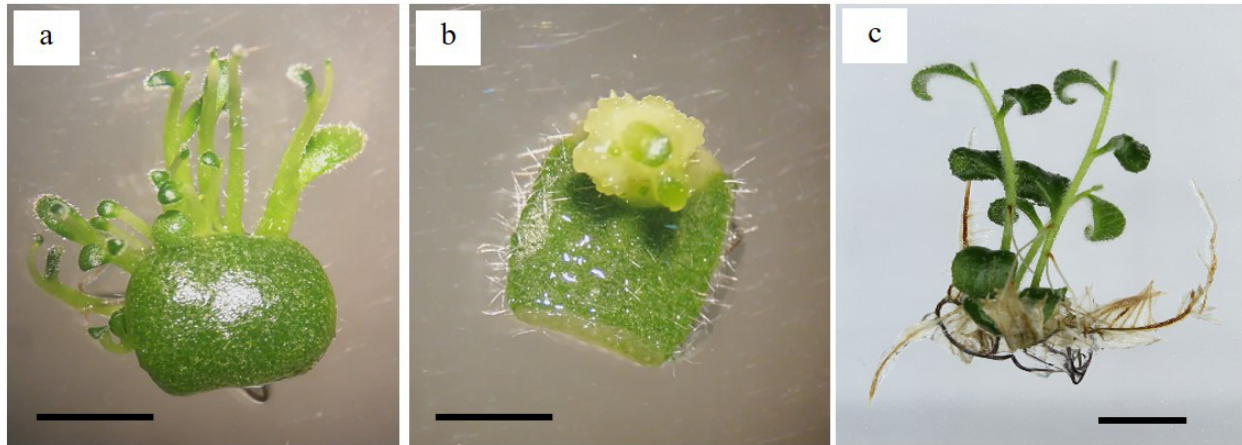
Highest shoot number (1.9 shoots) was observed in the 0.01 mg L<sup>-1</sup> 2,4-D treatment; however, there were no significant differences between 0.01 mg L<sup>-1</sup> TDZ, 0.1 and 0.2 mg L<sup>-1</sup> 2,4-D and the control treatments (data not shown).



**Figure 2.** Growth and development of *Kalanchoe tomentosa* explants; percentage of shoot regeneration (top) and percentage of callus formation (bottom), cultured on different PGR treatments after 8 weeks (left) and 12 weeks (right). Bars and error bars show mean  $\pm$  SE of three replications (ten explants per replication at week 8 and nine explants per replication at week 12). Different letters above the bar indicate significant differences by DMRT at  $p \leq 0.05$ .

Shoots emerged from the cut scars of the explants (Figure 3a). The remaining treatments (0.1, 0.2 and 0.5 mg L<sup>-1</sup> TDZ) had no shoot production and showed callus formation (Figures 1 and 2). Highest percentage (83.3%)

of callus formation was found in 0.2 and 0.5 mg L<sup>-1</sup> TDZ treatments followed by 0.1 and 0.5 mg L<sup>-1</sup> TDZ (Figure 2). All calluses were friable and emerged from cut scars of the explants and did not differentiate (Figure 3b).



**Figure 3.** Shoot regeneration (a; bar = 5 mm), callus formation (b; bar = 5 mm), and healthy plantlet (c; bar = 1 cm) of *Kalanchoe tomentosa*.

At week 12 of culture, shoot regeneration was observed on MS medium supplemented with 0.01 mg L<sup>-1</sup> TDZ, 0.01, 0.1, 0.2, 0.5 mg L<sup>-1</sup> 2,4-D and the control except for 0.1, 0.2 and 0.5 mg L<sup>-1</sup> TDZ (Figure 1). Shoot

regeneration ranged from 66.3 to 100% (Figure 2). Highest shoot number (4 shoots) was found in 0.01 mg L<sup>-1</sup> TDZ; however, no significant differences were found among shoot regeneration treatments (Table 1).

**Table 1.** Growth and development of *Kalanchoe tomentosa* explants cultured on different PGR treatments after 12 weeks.

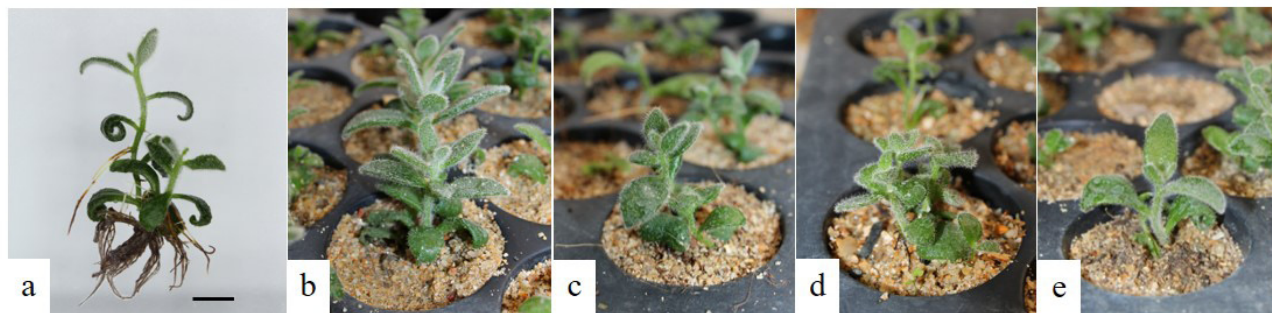
Treatment (mg L <sup>-1</sup> )	Shoot number	Plant height (cm)	No. of leaves per explants	% Rooting	No. of root per explants	Root length (cm)	
No PGR	2.8 ± 0.0 a	2.03 ± 0.28 a	7.5 ± 0.6 abc	100.0 ± 0.0 a	3.6 ± 0.5 b	2.36 ± 0.17 a	
TDZ	0.01	4.0 ± 1.9 a	0.77 ± 0.31 b	4.8 ± 2.4 c	66.7 ± 28.0 a	2.0 ± 0.9 b	2.12 ± 1.10 a
	0.1	0.0 ± 0.0 b	0.00 ± 0.00 c	0.0 ± 0.0 d	7.3 ± 3.6 b	0.1 ± 0.0 c	0.05 ± 0.02 b
	0.2	0.0 ± 0.0 b	0.00 ± 0.00 c	0.0 ± 0.0 d	0.0 ± 0.0 b	0.0 ± 0.0 c	0.00 ± 0.00 b
	0.5	0.0 ± 0.0 b	0.00 ± 0.00 c	0.0 ± 0.0 d	0.0 ± 0.0 b	0.0 ± 0.0 c	0.00 ± 0.00 b
2,4-D	0.01	3.6 ± 0.7 a	1.98 ± 0.15 a	11.6 ± 1.8 a	100.0 ± 0.0 a	6.6 ± 0.8 a	1.86 ± 0.08 a
	0.1	3.2 ± 0.6 a	2.05 ± 0.16 a	9.8 ± 1.1 ab	100.0 ± 0.0 a	3.9 ± 0.5 b	1.61 ± 0.31 a
	0.2	2.4 ± 0.9 ab	1.25 ± 0.35 b	6.2 ± 2.3 bc	66.7 ± 17.0 a	2.2 ± 0.8 b	1.14 ± 0.40 ab
	0.5	3.1 ± 0.7 a	1.31 ± 0.25 b	6.4 ± 1.4 bc	85.3 ± 14.6 a	2.9 ± 0.7 b	1.25 ± 0.35 ab

Data are mean ± SE of three replications (nine explants per replication). Different letters indicate significant differences analyzed by DMRT at  $p \leq 0.05$ .

Callus formation was observed in all treatments, except for 0.01 and 0.1 mg L<sup>-1</sup> 2,4-D and the control. The percentage of callus formation varied from 4.2 to 100% (Figure 2). The root regeneration without shooting was found on explants treated by MS medium with 0.1 mg L<sup>-1</sup> TDZ. The three treatments that induced the highest plant height, leaf number and rooting percentage were 0.01 and 0.1 mg L<sup>-1</sup> 2,4-D and the control but with no significant differences. MS medium supplemented with 0.01 mg L<sup>-1</sup> 2,4-D (Figure 3a and Table 1) induced the healthiest plantlets of *K. tomentosa* with a significant difference in root numbers per explant.

#### Plantlet acclimatization *ex vitro*

Ten weeks after the acclimatization of healthy plantlets of *K. tomentosa*, survival percentage varied from 50% to 100%. Highest survival percentage (100%) was found for plantlets grown in sand, while survival percentages of plantlets grown in sand with coconut husk, sand with potting soil and sand with perlite were 88, 76 and 56%, respectively. Plantlet quality in different planting materials is depicted in Figure 4b-e.



**Figure 4.** *Kalanchoe tomentosa* plantlets before transplantation (a) and after transplantation in different planting materials; sand (b), 2:1 sand with fine coconut husk (c), 2:1 sand with perlite (d), and 2:1 sand with potting soil (e) for 10 weeks (bar = 1 cm).

Throughout the acclimatization, survival percentages in each treatment were decreased except plantlets grown in sand. On 2:1 ratio of sand per perlite, the survival percentage

dramatically decreased in week 5 after acclimatization while other treatments the percentage slightly decreased to stable (Table 2).

**Table 2.** Weekly survival percentage of *Kalanchoe tomentosa* on different planting materials in the acclimatization for 10 weeks (n = 25).

Planting Materials	Week(s)										
	0	1	2	3	4	5	6	7	8	9	10
Sand	100	100	100	100	100	100	100	100	100	100	100
Sand:Coconut husk	100	88	88	88	88	88	88	88	88	88	88
Sand:Perlite	100	100	100	92	92	68	64	56	56	56	56
Sand:Potting soil	100	80	80	80	80	80	76	76	76	76	76

## Discussion

Low concentration (0.01 mg L<sup>-1</sup>) of TDZ induced new shoot regeneration, however, using TDZ resulted in shorter plantlets than MS without PGR treatment (Table 1). Higher concentrations of TDZ gave increased callus formation with faster callogenesis (Fig. 2). The same result was found in other ornamental succulents, where TDZ promoted callus formation (Liu et al., 2016; 2017). In *K. rhombopilosa* Mannoni & Boiteau, higher TDZ concentration increased the callogenesis rate and decreased the differentiation rate (Kertrung and Junkasiraporn, 2018). Callus induction might be due to that TDZ stimulated cell division in plant cell more than other PGRs. TDZ also increase the level of endogenous cytokinin by reducing catabolism, increasing synthesis, and conversing inactive cytokinin to active forms (Dinani et al. 2018). By contrast, TDZ induced shoots regeneration in *Kalanchoe blossfeldiana* Poelln. and *Sedum sarmentosum* Bunge, while callus formation was not recorded (Sanikhani et al., 2006; Kim and Sivanessan, 2016). In *K. tomentosa* Baker, MS medium supplemented with 0.25 mg L<sup>-1</sup> TDZ and 0.4 mg L<sup>-1</sup> indole-3-acetic acid (IAA) induced new shoot regeneration at only 30% (Frello et al., 2002). Responses of *Kalanchoe* species varied with TDZ and the optimal quantity should be examined in

each application. In our study, the present of root without shoot regeneration found on MS medium supplemented with 0.1 mg L<sup>-1</sup> TDZ might be due to the consequence of explants development. An evidence was supported by the organogenesis at week 4 that root was the first occurrence organ in most explants of this study. From our result also revealed that higher TDZ concentration reduced root development.

Low concentration (0.01 mg L<sup>-1</sup>) of 2,4-D promoted the highest quality plantlets, while higher concentrations increased the callogenesis rate (Figure 2). 2,4-D is usually used with cytokinins for callus induction. The induction of callogenesis was also confirmed in *K. blossfeldiana* Poelln. and *Digitalis lanata* Ehrh. (Kale et al., 2018; Bhusare et al., 2020). However, our results suggest that 2,4-D at low concentration may induce plantlet formation. 2,4-D is an auxin and plays a primary role in cell elongation and root initiation (Machakova et al., 2008). The response of explants in this study might be due to the auxin-cytokinin balance derived from exogenous auxin (2,4-D) and endogenous auxin cytokinin in plant cells.

Plantlet acclimatization *ex vitro* results indicated that planting material affected survival rate. Proper planting material should be a major concern for each application. Many reports of *Kalanchoe* species on plantlet

acclimatization *ex vitro* from *in vitro* culture have indicated over 90% survival rate (Khan et al., 2006; Sanikhani et al., 2006). Our results indicated that the best planting material for *K. tomentosa* was sandy soil. High plantlet quality observed in sand insists that well-drained planting material is appropriate for growing panda plant (Smith et al., 2019) while higher water absorbed materials reduce survival rate.

## Conclusions

Our result demonstrated an alternative production method for panda plantlets using plant tissue culture techniques. Experimental results identified 2,4-D as a candidate plant growth regulator (PGR) for leaf explant culture *in vitro* of panda plant. 2,4-D gave a highly quality plantlet with increased plant height, leaves and root numbers per explant than TDZ. The suggested medium was MS medium supplemented with 0.01 mg L<sup>-1</sup> 2,4-D. Our protocol produced 2-4 shoots per explant but might be compensated by lower disturbance on plant individuals when leaf explants were deployed. To produce calluses of panda plants, MS medium supplemented with 0.2 mg L<sup>-1</sup> TDZ is recommended. For *ex vitro* acclimatization, sand is suitable for promoting more survival rate.

## Author Contribution

**WP:** conceptualization, experiment development, data analysis and interpretation, manuscript writing and corrections, **ON:** data collection, preliminary analysis and interpretation, **AK:** review and editing.

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