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Refining a protocol for somatic embryogenesis and plant regeneration of *Phalaenopsis amabilis* cv. Jinan from mature tissues

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Abstract: *Phalaenopsis* is one of the most remarkable commercial genera in Orchidaceae family. Using immature explants for tissue culture of orchid results in undesired characteristics such as genetic variation and long-lasting juvenility. In this study, we optimized a protocol for somatic embryogenesis of *Phalaenopsis amabilis* cv. Jinan, by using mature thin cell layer (TCL) explants to minimize immature explant limitations. We examined somatic embryogenesis from two types of mature explants, viz. leaf lTCL and flower stalk nodes tTCL at various concentrations of thidiazuron (TDZ) (0, 1, 2, and 3 mg L^{-1}). The results demonstrated that lTCL explants cultured on medium supplemented with 3 mg L^{-1} TDZ produced the highest number of somatic embryos (SEs) (21.5 embryos per explant), the lowest yellowish SE (7%), the highest plantlet regeneration (20.5 plantlets per explant) and the highest survival rate (94%) in comparison with tTCL explants. This protocol can be used in commercial mass propagation of *Phalaenopsis* to overcome the limitations such as long-term juvenility and genetic variation of plantlets regenerated in vitro by using immature explants.

Key words: Acclimatization, mature explant, orchids, protocorm-like bodies (PLBs), thin cell layer (TCL), thidiazuron

1. Introduction

Phalaenopsis is commonly known as moth orchid. It is a genus in the family Orchidaceae. It has a high value in flower industry as both cut flower and pot plant (Zanello and Cardoso, 2019). Somatic embryogenesis is a developmental process that generates an embryo from a single somatic cell (Chen et al., 2019). In orchids, formation of protocorm-like bodies (PLBs) is equal with somatic embryogenesis in other plants (Teixeira da Silva, 2014). Leaf explants are the most regenerative tissue for induction of PLBs in *Phalaenopsis* (Zanello and Cardoso, 2019; Naderi Boldaji et al., 2021). Thin cell layer (TCL) explants composed of a few cells form somatic embryos in higher frequency compared to the thicker explants (Ekmekçigil et al., 2019). The TCL explants are excised either longitudinally (lTCL) or transversely (tTCL) from various plant tissues (Bhattacharyya et al., 2018). In different orchid species, TCL has been successfully employed via leaf explant in *Doritaenopsis* hybrids (Park et al., 2002), nodal segments in *Dendrobium sp.* (Parthibhan et al., 2018) and node tTCL in *Cymbidium sp.* (Vyas et al., 2010). Various plant growth regulators were found to have a positive effect on organogenesis or somatic embryogenesis from TCL explants, the most important of which are cytokinins e.g., benzyl adenine (BA) (Della Rovere et al, 2015) and thidiazuron (TDZ) (Botero Giraldo et al, 2015) and auxins e.g., indole butyric acid (IBA) (Mao et al, 2014) and naphthalene acetic acid (NAA) (Thingbaijam and Huidrom, 2014). TDZ has cytokine activity and promotes organ regenerations in vitro. Its positive effect has been reported in PLB production from tTCL explant culture of *Cymbidium* (Teixeira da Silva, 2014).

Generally, immature explants derived from leaves and nodal tissues of seedlings are used for mass propagation of orchids. However, this leads to undesirable features such as genetic variation and long-lasting juvenility (Antensari et al., 2014; Moradi et al., 2017). In the current study, we introduced for the first time an efficient protocol for somatic embryogenesis of *Phalaenopsis amabilis cv. Jinan* using TCL explants derived from mature tissues of leaves and flower stalk nodes.

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2. Materials and methods

The experiment was conducted in three major steps, *viz.* I) somatic embryo (SE) formation, II) plantlet regeneration, and III) acclimatization.

2.1. Plant material and culture conditions

2.1.1. Preparation of leaf lTLC explant

Mature adventitious shoots were excised from in vitro cultured inflorescence of *Phalaenopsis amabilis* cv. Jinan and cultured for 3 months on 1/2 MS basal medium containing 30 g L^{-1} sucrose and 7 g L^{-1} agar (Duchefa Biochemie, Haarlem, Netherlands). After that, young and well grown in vitro leaves (3-4 cm) close to the shoot tips (the newest leaves) were selected as explant source. Then, the leaves were cut (approximately 0.5 mm) longitudinally parallel to the veins, and submerged in 1/2 MS (Murashige and Skoog, 1962) liquid medium containing 0.5% polyvinylpyrrolidone (PVP-40, Sigma Chemical, St. Louis, Missouri, USA) for 10 min (Parthibhan et al., 2018), in order to reduce the exudation of phenolic compounds into the culture media. Then, the explants were cultured in petri dishes (100 mm × 13 mm) containing 20 mL of 1/2 MS medium supplemented with TDZ (0, 1, 2 and 3 mg L^{-1}), 30 g L^{-1} sucrose, 7 g L^{-1} agar (Duchefa Biochemie, Netherlands) and 1 g L^{-1} peptone. The petri dishes were sealed with parafilm (Santa Cruz BioTechnology, Dallas, Texas, USA) and incubated at 25± 2 °C and 45 µmol $m^{-2}s^{-1}$ light (Philips TL 33) for 60 days. Each petri dish included four lTLC explants, and five petri dishes were used per treatment.

2.1.2. Flower stalk node tTCL explant preparation

Under aseptic conditions, transverse thin cell layers (*ca.* 0.5 mm thick) were dissected from in vitro flower stalk nodes using a scalpel. After that, the explants were submerged in 1/2 MS liquid medium supplemented with 0.5% PVP-40 in order to reduce the secretion of phenolic compounds into the culture medium (Parthibhan et al., 2018). Then, the explants were cultured on 1/2 MS supplemented with different concentrations of TDZ (0, 1, 2, and 3 mg L^{-1}). The explants were then incubated at 25± 2 °C and 45 µmol $m^{-2}s^{-1}$ light intensity (Philips TL 33) for 60 days. Each petri dish included five tTLC explants and five petri dishes were used per treatment.

2.2 Evaluation of SE formation

The in vitro cultures of both leaves (lTLC) and flower stalk nodes (tTLC) explants were monitored every week. After 45 days, clear globular shaped protuberances (Figure 1) were formed only in the media containing 2 and 3 mg L^{-1} TDZ. The SEs were observed under a stereomicroscope (Zeiss, Germany) and the images were captured with a Sony digital camera (SX-530). After 60 days, SE forming explants and the number of SE per explants were recorded. After 90 days data on number of yellowish embryos formed and regenerated plantlets were collected. The percentage of responding explants to SE formation was calculated according to the following formula (Eq.1).

Responding explant to SE formation(%)=(Number of explants with SE)/(Total number of explants)×100 (1)

2.3 Plantlet regeneration and acclimatization

Two weeks after PLB formation, the protocorm-like bodies (PLBs) were transferred to 1/2 MS medium (plant growth regulators-free) supplemented with 1 g L^{-1} activated charcoal (Duchefa Biochemie), 1 g L^{-1} peptone, 7 g L^{-1} agar (Duchefa Biochemie) and 30 g L^{-1} sucrose. Every 30 days, the explants were subcultured to fresh media. Finally, the in vitro rooted plantlets (4–5 cm in height) were transplanted into the pots containing cocopeat:

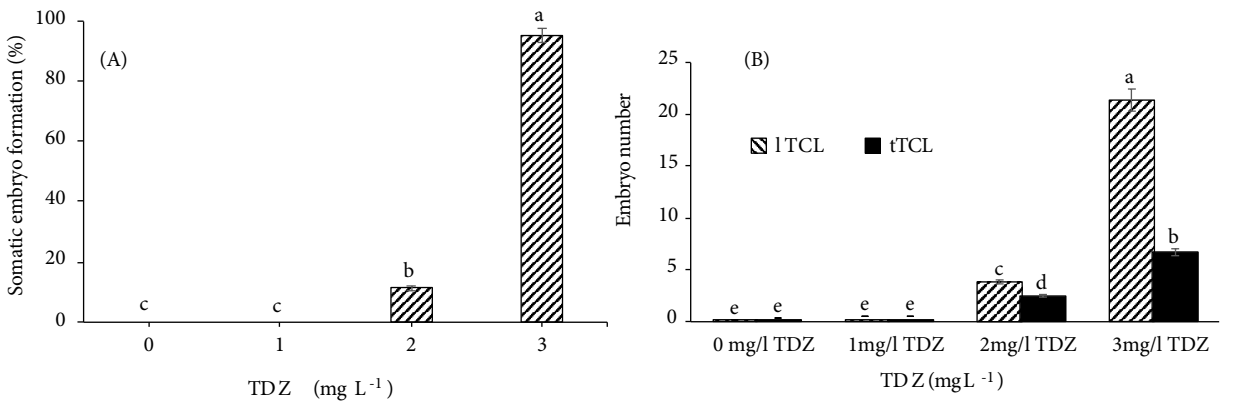

Figure 1. SE formation in *Phalaenopsis amabilis* cv. Jinan. A–B, at the edge of the lTCL explants; C–D, at the middle part of tTCL explants. (scale bar size; A–B = 0.5 mm; C–D = 2mm).

charcoal: perlite (3:1:1) and were kept in a greenhouse under high humidity (70%–80%), average temperature of 25/20 °C (day/night) and 500 µmol $m^{-2}s^{-1}$ light for 16h per day. Evaluation of plantlet regeneration was calculated according to the following formula (Eq.2).

Plantlet Regeneration (%)=(Number of regenerated plantles)/(Total number of SE)×100 (2)

Subsequently, the survival rate of plantlets was calculated according to the following formula (Eq. 3).

Survival Rate (%)=(Number of survived Plantlets)/(Total number of Plantlets)×100 (3)

2.4 Statistical analysis

All experiments were conducted as a completely randomized (CRD) factorial design with five replications and five explants per replicate. The data were analyzed by ANOVA and means were compared by Duncan's multiple range test at $P \leq 0.01$ and $P \leq 0.05$. The statistical analyses were performed using SAS software (Version 9.0). To predict optimal response (Kovalchuk et al., 2017), correlation coefficient (r), coefficient of determination (r^2), and regression between parameters were analyzed (Figure 2). Since the data were normal, Pearson test was used. Also, correlation among data was interpreted using the structures described by Akin et al. (2017a and 2020) and Kovalchuk et al. (2018).

3. Results

3.1. Somatic embryo (SE) formation

After six weeks, only at the media containing 2 and 3 mg L^{-1} TDZ, clear globular shaped protuberances appeared on the edge and middle part of the lTCL explants and only on the middle of the tTCL explants. In the control and at medium with 1 mg L^{-1} TDZ, SE did not form and the explants gradually became yellowish and subsequently died (Figure 3 and Table 1). The maximum responding explants to SE formation (95.7%) were measured in tTCL explants cultured on the medium with 3 mg L^{-1} TDZ. lTCl explants cultured on medium with 3 mg L^{-1} TDZ showed the highest number of SE (21.5 embryos per explant) and regenerated plantlets (20.5 plantlets per explant).

3.2. Plantlet regeneration and acclimatization

The somatic embryos were cultured on media with different concentrations of TDZ for regeneration of plantlets (Figure 4). The largest numbers of yellowish embryos (41.6%) were obtained through lTCL explants cultured on 1/2MS medium with 2 mg L^{-1} TDZ. These numbers declined to 7% at medium with 3 mg L^{-1} TDZ using lTCL explant. The highest plantlet regeneration (93%) occurred with lTCL explants cultured on medium containing 3 mgL^{-1} TDZ (Figure 5A). Furthermore, plantlet regeneration increased by 24% and 13.5% at medium with 3 mg L^{-1} TDZ compared to medium with 2 mg L^{-1} TDZ using lTCL and tTCL explants, respectively. At the end of this phase, the regenerated plantlets were transplanted to a greenhouse. The maximum survival rate (94%) was recorded in lTCL explants cultured on medium with 3 mg L^{-1} TDZ. The survival rate rose by 16% in plantlets derived from lTCL explants compared to tTCL explants (Figure 5B).

3.3 Relationship among properties

According to the Durbin–Watson test index (1.98), confidence interval 95% ($\alpha = 0.05$) and coefficient of determination ($r^2 = 0.98$), regression between parameters with dependent variable (Y6: survival rate) is reliable. Regression analyses (Table 2) showed that, between the

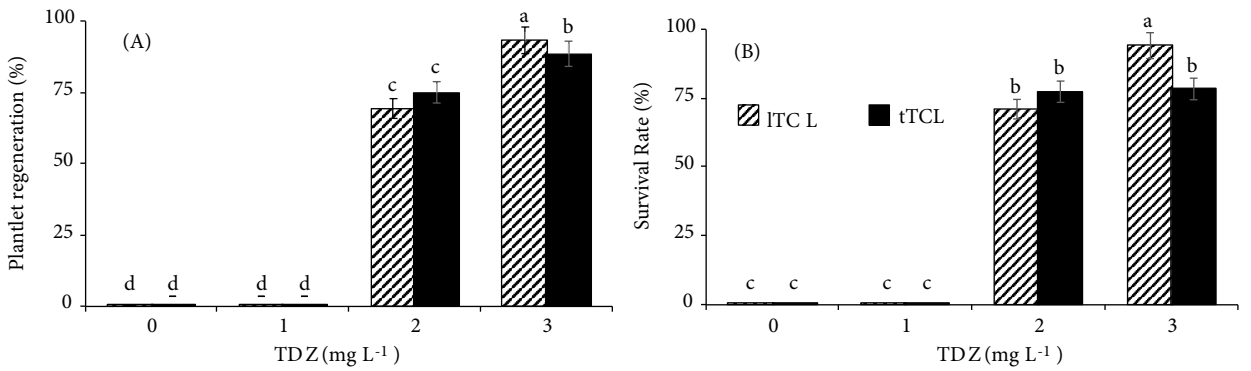

Figure 2. Predicted value and the regression between dependent variable (Y6: survival rate) by parameters (Y1: responding explant; Y2: somatic embryo number; Y3: yellowish somatic embryo number; Y4: number of plantlet; Y5: plantlet regeneration) occurred in lTCL and tTCL during acclimatization of *Phalaenopsis amabilis* cv. Jinan. (Durbin–Watson: 1.98; Adjusted R Square: 0.98).

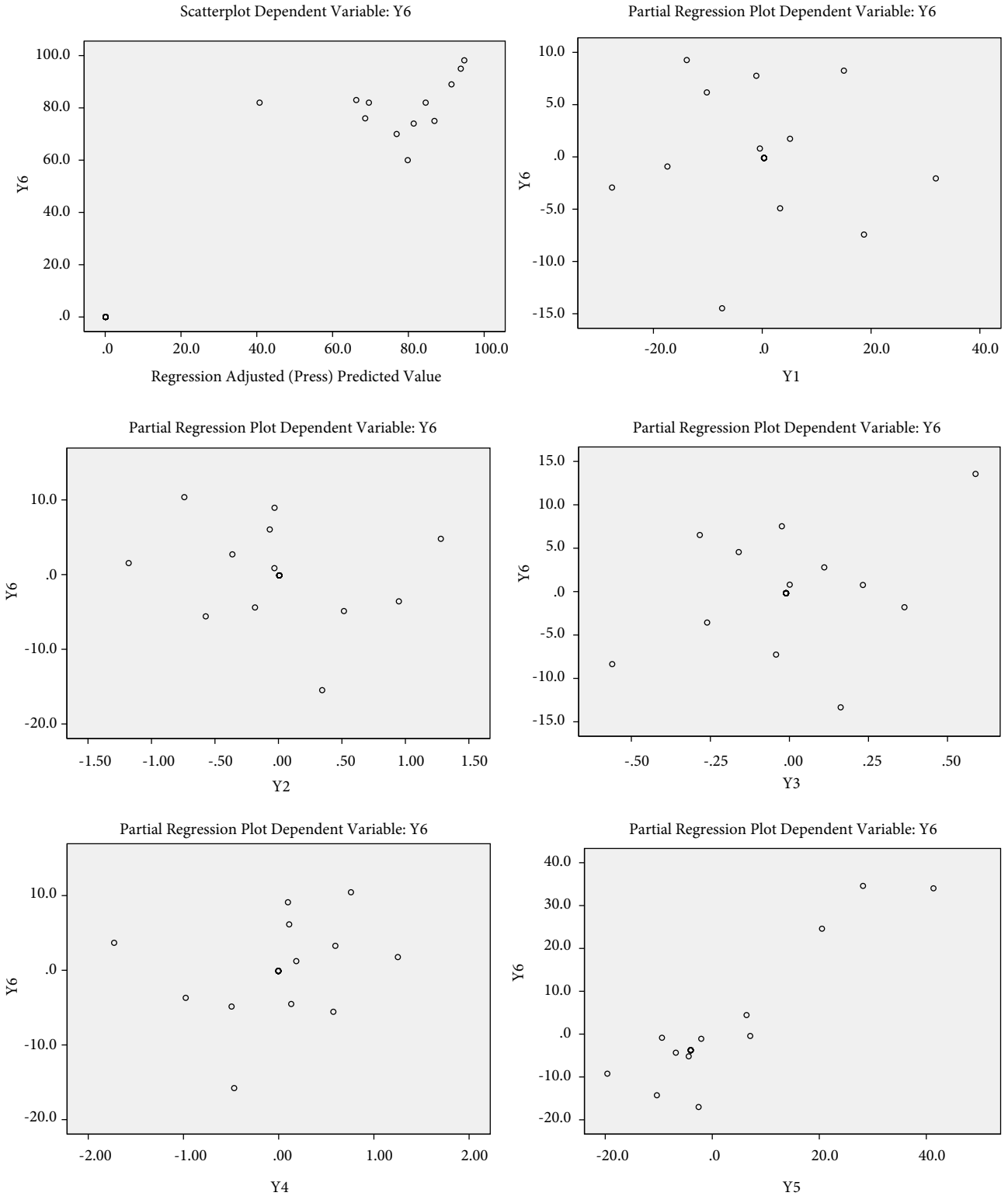

Figure 3. Influence of various concentrations of TDZ on SE formation (A) and SE number in lTCL and tTCL explants of *Phalaenopsis amabilis* cv. Jinan. (B). Bars with the same letters illustrate no significant difference at 1% probability using Duncan's multiple range test.

Table 1. Effect of various TDZ concentrations and two types of mature explant on SE formation and plantlet regeneration of *Phalaenopsis amabilis* cv. Jinan.

Tissue	TDZ	Responding explant (%)	No.SE	No. Yellowish SE	No. Plantlet
lTCL	0	0	0 d	0 c	0 d
	1	0	0 d	0 c	0 d
	2	10.17	3.84 c	1.6 a	2.74 bc
	3	94.83	21.37 a	1.5 a	20.28 a
tTCL	0	0	0 e	0 c	0 d
	1	0	0 e	0 c	0 d
	2	12.33	2.5 d	0.8 b	1.93 c
	3	95.7	6.7 b	0.84 b	4.23 b
	Explant (E)	NS	**	**	**
ANOVA	Hormone (H)	**	**	**	**
	E×H	NS	**	**	**

In each column, treatment followed by same letter(s) demonstrate no significant differences at 5% probability level (Duncan's multiple-range test), *p < 0.05, **p < 0.01. NS: Nonsignificant.

survival rate with plantlet regeneration (coefficient β = 0.92), plantlet number (β = 0.37) and somatic embryo number (β = 0.38) were significantly related. The linearity regression suggests that an increase in the number of embryos is correlated with the survival rate.

4. Discussion

Somatic embryo formation can be affected by physiological maturity and morphological structure of explant (Horstman et al., 2017). In this study, percentage of responding explants to SE formation was not statistically significant between lTCL and tTCL explants. The highest SE number was obtained through lTCL explants. It seems that fewer lignified cells, and the least latex and phenol components in the leaves assist them to be much more preferable explant for SE formation (Balilashaki et al., 2015 and Adero et al., 2019). With this in mind, the leaf-explant consists of larger cut surface and perhaps higher vascular bundles activity to uptake plant hormones and other medium components in comparison with flower stalk node explants. Apart from the explant type, higher SE numbers were achieved in the medium supplemented with 3 mgL^{-1} TDZ. Thidiazuron has cytokinin-like activity, which is involved in the accumulation of ions and enzymes along with the expression of SE formation genes in the targeted tissues (Balilashaki et al., 2020). The notable role of TDZ in SE formation was reported in other orchideous plants, especially in *Phalaenopsis* (Mose et al., 2017), *Doritaenopsis* (Park et al., 2002), *Oncidium* (Mayer et al., 2010), *Epidendrum* (Chen et al., 2002), *Vanda* (Lang and Hang, 2006), and *Paphiopedilum* (Wattanapen et al., 2018). The SEs derived from lTCL and tTCL explants were cultured on 1/2 MS medium supplemented with different concentrations of TDZ (0, 1, 2, and 3 mgL^{-1}). Plantlet regeneration increased in higher concentration of TDZ. As a consequence, higher TDZ concentration improved the capability of somatic embryos to form plantlets. Similarly, the positive effect of TDZ has been reported on plantlet regeneration of *Phalaenopsis* (Kosir et al., 2004). The highest yellowish SE was recorded in medium supplemented with 2 mgL^{-1} TDZ. But the number of yellowish SE reduced at the highest concentration of TDZ. Besides, an increase in the number of yellowish embryos has been reported due to the fact that TDZ elevates ethylene and phenolic compounds in explants (Park et al, 2006).

Last but not least, the highest survival rate (94%) was obtained from lTCL explants cultured on medium containing 3 mg L^{-1} TDZ. In *Echinacea*, TDZ-induced regenerants showed higher accumulation of serotonin and melatonin. These compounds may play a key role in free radicals scavenging and subsequently increasing the survival rate of the plantlets (Jones et al., 2017). Undoubtedly, the survival rate of our study was much higher than previous studies in *Phalaenopsis* (Balilashaki et al., 2015) and *Paphiopedilum* (Soonthornkalump et al., 2019). Statistical approaches (regression) and prediction of relationships between parameters are needed for protocol optimization (Akin et al., 2016; Akin et al., 2017b; Eyduran et al., 2019). In this study, coupled with the higher survival rate in lTCL explants, there is a strong correlation between embryo numbers and plantlet regeneration with survival rate.

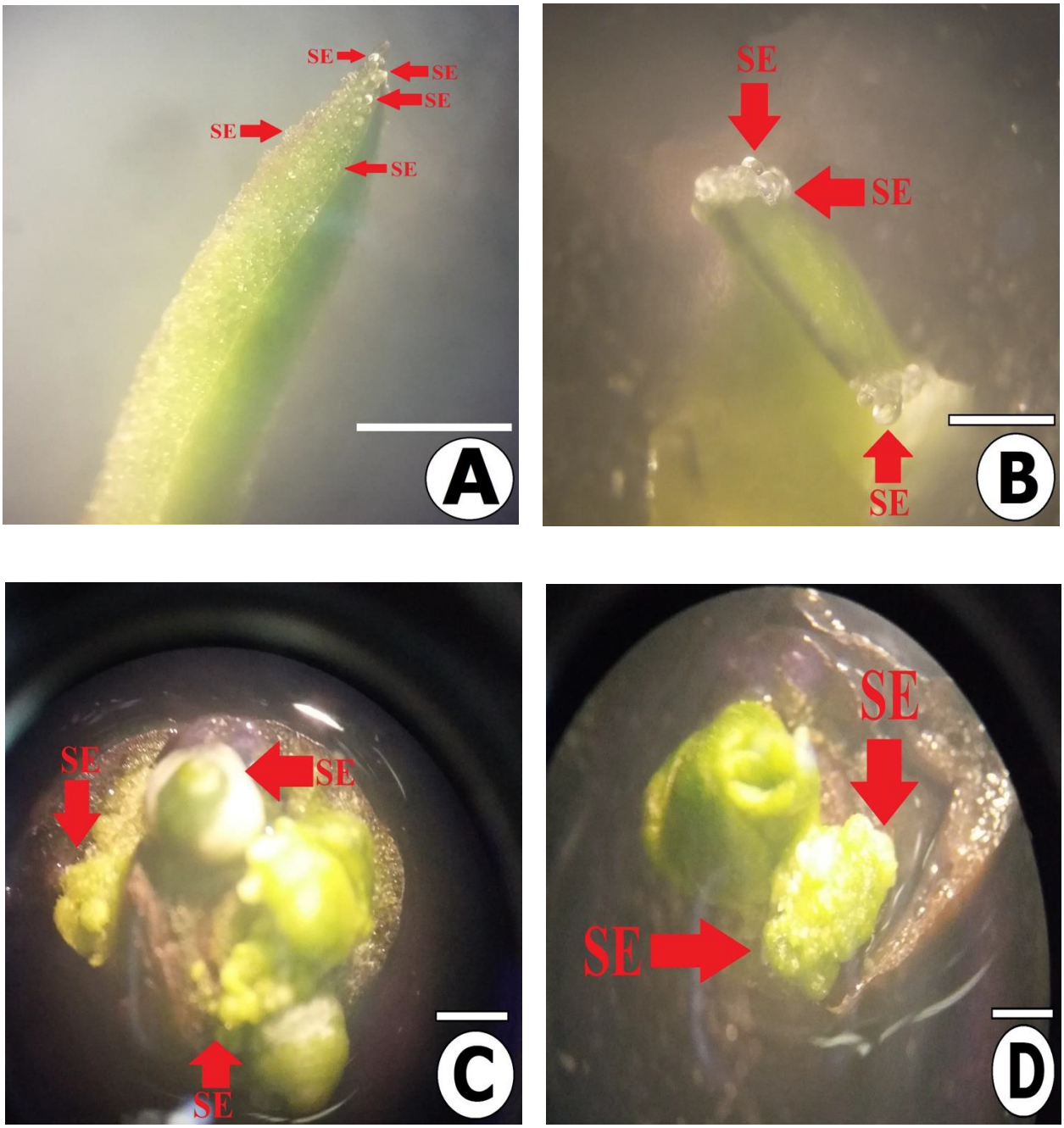

Figure 4. Plantlet regeneration and acclimatization in *Phalaenopsis amabilis* cv. Jinan. A. Cluster of SE (eight-week old SE); B. The plantlets regenerated from SE (four-week old plantlet); C. Acclimatization (acclimatized plantlets after six weeks) in a container containing cocopeat: charcoal: perlite (3:1:1); D. An acclimatized plantlet (after twelve weeks). (Bar scale size; A = 1 mm; B–D = 15 mm).

5. Conclusion

In *Phalaenopsis* tissue culture, mass propagation by using immature explants excised from seedling's juvenile tissues results in genetic diversity and long-lasting juvenility of plantlets. In this study, the explants were taken from mature leaves and flower stalk nodes to overcome the aforementioned obstacles. In summary, lTCL explants cultured on 1/2 MS medium supplemented with 3 mgL^{-1} TDZ, produced the highest number of SE, the lowest yellowish SE, the highest plantlet regeneration, and the highest survival rate. In conclusion, it could be an appropriate treatment to produce mature plantlets in multiplication of *Phalaenopsis*.

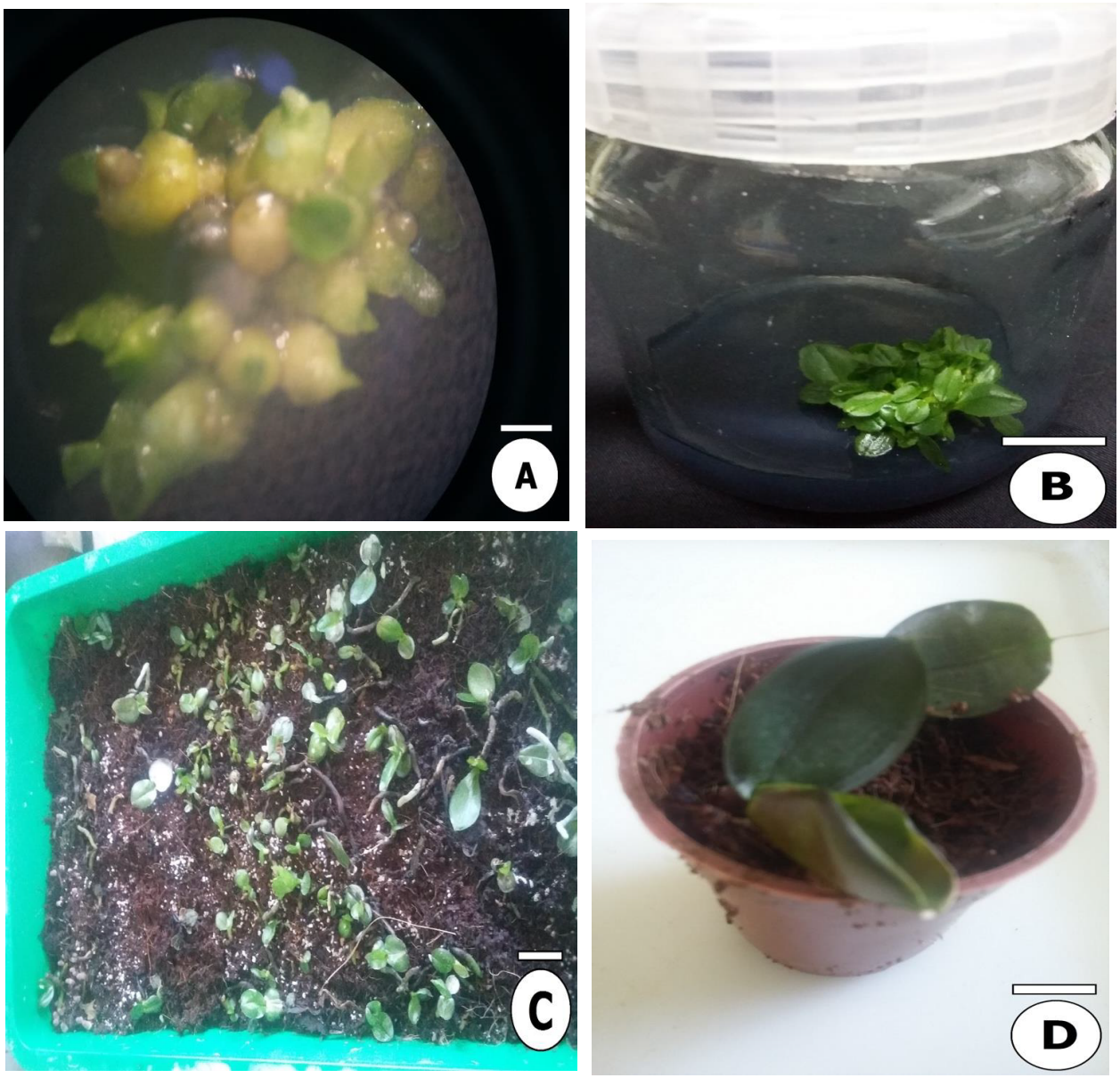

Figure 5. Influence of various TDZ concentrations on plantlet regeneration (A) and survival rate (B) in lTCL and tTCL explants of *Phalaenopsis amabilis* cv. Jinan. Bars with the same letters illustrate no significant difference at 5% probability using Duncan's multiple range test.

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Author contributions
RG performed the experiments and wrote the first draft. SDD and KV led and consult to the project. RG, SDD, KV, MM, and NA contributed to the commentary of the results. KV reviewed and proof read the final draft. All authors discussed the results and participated to the final manuscript.

Conflict of interest
No potential conflict of interest was reported by the authors.

Table 2. The coefficient and ANOVA results of regression between survival rate (dependent variable) with other parameters.

Model Beta	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	Std. Error	Beta			
(Constant)	0.095	1.604		0.059	0.036
Responding explant	–0.028	0.101	–0.027	–2.277	0.053
No.SE	–2.297	2.400	–0.385	–1.957	0.035
No. Yellowish SE	8.310	5.391	0.135	2.542	0.014
No. Plantlet	2.345	2.057	0.374	2.140	0.027
plantlet regeneration	0.913	0.089	0.924	10.209	.000
ANOVA	Sum of Squares	df	Mean Square	F	Sig.
Regression	39573.550	5	7914.710**	256.191	.000[b]
Residual	556.088	18	30.894		
Total	40129.638	23			

[a]Dependent Variable: survival rate; [b]Predictors: (Constant), Responding explant, No.SE, No. Yellowish SE, No. Plantlet, plantlet regeneration. *p < 0.05, **p < 0.01. NS: Nonsignificant.

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