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Efficient Method for Direct Embryogenesis in *Phalaenopsis* Orchid

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Abstract
Light spectrum is one of the environmental cues that influence plant growth and development. Light is a stimulating factor for induction of somatic embryos during tissue culture practices. To accelerate the direct embryogenesis, six different light spectra including: white (W), red (R), blue (B), green (G), red + blue (R+B) and red + far red (R+FR) together with dark condition (D), in combination with thidiazuron (TDZ) in four concentrations (0, 0.5, 1.5 and 3 mg L^{-1}) were used. Inter-simple sequence repeat was used for identification and genetic stability analysis of somatic regenerated plantlets. Intact protocorm explants showed higher potential for direct somatic embryogenesis (DSE) than the other explants. The rate of DSE was highly dependent on the concentration of TDZ and its interaction with light spectra. R and R + FR spectra with 3 mg L^{-1} TDZ on intact protocorms and R+FR with 3 mg L^{-1} TDZ were efficient treatments to induce DSE without somaclonal variation. G light spectrum has also significant effects on DSE of protocorm explants. The amplified products showed 26 scorable bands and regenerates were completely identical to the mother plant. In conclusion, this protocol provides way to regenerate plants through embryogenesis, and is a reliable protocol to obtain proper development and genetic stable *Phalaenopsis* embryos.

Keywords: Light spectrum, ISSR markers, protocorm like bodies, somatic embryo, wounding

Abbreviations: DSE, Direct somatic embryogenesis; PLB**,** protocorm like bodies; W**,** white; R, red; B, blue; G, green; R+B, red + blue; R+FR, red + far red; D, dark; TDZ, Thidiazuron; ISSR, Inter-simple sequence repeat.

Introduction

Phalaenopsis orchid is one of the ornamental plants with high economic value in international flower markets (Gow et al., 2009; Meilasari and Iriawati, 2016). Todays, orchids are propagated *in vitro* using different sample types such as seed (Stewart and Kane, 2006), shoot tip (Pant and Thapa, 2012), flower stalks (Park et al., 2002), protocorm-like bodies (PLBs) (Park et al., 2010; Zhao et al., 2008; Ng and Saleh, 2011). *In vitro* techniques are efficiently used to protect horticultural important and endangered orchid species (Bhattacharyya and Kumaria, 2014). In those methods, the generation of protocorm-like bodies (PLBs) that first indicated in the shoot-tip culture of Cymbidium orchid by Morel (1960), is vital for clonal micro-propagation of orchids (Arditti and Ernst, 1993). PLBs could be used as an experimental embryological system for physiological or molecular characterization and suitable material for regeneration, mass propagation, and

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commercial clonal production (Zimmerman, 1993; von Arnold et al., 2002).

Studies have been conducted on somatic embryogenesis (SE) in many orchid species such as *Phalaenopsis* (Gow et al., 2010; Meilasari and Iriawati, 2016), *Cymbidium* (Mahendran and Bai, 2012) and *Epipactis veratifilia* (Moradi et al., 2017). Direct formation of somatic embryos or embryogenic tissue from the explants without formation of an intervening callus phase, known as direct somatic embryogenesis (DSE), which is very practical but difficult or sometimes impossible to obtain it in most of the plant species (Finer, 1995). Therefore, exploring effective and accelerating factors on induction of DSE is very important to achieve efficient protocols. Type of explant (Guo et al., 2010) and plant growth regulator (PGR) (Grzyb et al., 2018), culture medium composition (Al-Khayri, 2010), genotypic properties of plants and lighting condition have been reported as factors influencing SE (Deo et al., 2010).

There are very little information about the effects of some of those effective factors such as light spectra (Gow et al., 2009; Chung et al., 2007) and wounding (Rojas and Loyola, 2002) on embryogenesis in *Phalaenopsis*. Promotion of SE by darkness has been reported in many plant species such as *Lilium ledebourii* and *Phalaenopsis* (Bakhshaie et al., 2010; Gow et al., 2010). The injury can influence morphogenesis response of tissue-cultured plantlets similar to plants in natural environment (Bhatia et al., 2005). Santarem et al. (1997) conducted the first study about the impact of wounding on initiation of soybean somatic embryos.

Plant development is influenced by light quality through photoreceptors such as phytochrome, cryptochrome and phototropin (Wang et al., 2013). Different light spectra can influence plant growth and development (Aliniaeifard et al., 2018; Bayat et al., 2018). Moghare and Naderi (2020) showed that the light condition affects morphological traits of ornamental plants.

Light spectrum are particularly important, and there are some reports on the influence of light quality on SE (Husaini and Abdin, 2007; Chen et al., 2014). It has been reported that SE can be influenced by light spectrum in *Phalaenopsis* (Gow et al., 2009), in *Carica papaya* (Ascencio-Cabral et al., 2008) and in *Agave tequilana* (Rodríguez-Sahagún et al., 2011).

Light quality can influence distinct morphological, anatomical, and physiological characteristics such as the formation of shoots, somatic embryos and etc. However, light quality and intensity are known to influence morphogenesis, differentiation and development of plant cells, tissues and organ (Li et al., 2013; Hosseini et al., 2019). Plant development is influenced by light quality through photoreceptors such as phytochrome, cryptochrome and phototropin (Wang et al., 2013). Light spectrum is particularly important, and there are some reports on the influence of light quality on SE (Husaini and Abdin, 2007; Chen et al., 2014). For examples, it has been reported that SE can be influenced by different light spectra in various plant speces including *Phalaenopsis* (Gow et al., 2009), *Carica papaya* (Ascencio-Cabral et al., 2008), *Dianthus caryophyllus* (Aalifar et al., 2019) and *Agave tequilana* (Rodríguez-Sahagún et al., 2011). Finding an appropriate and effective light recipe for accelerating the DSE protocol is an important goal of the researches based on the recent investigations (Aalifar et al., 2019).

PGRs play an important role in the dedifferentiation process of plants (Nazari et al., 2016). Cytokinins and auxins are the main PGRs that are mainly involved in the regulation of cell division and differentiation (Feher et al., 2003). TDZ has been generally used on orchid tissue culture to induce organogenesis and high frequency of SE (Wu et al., 2012). Currently, TDZ growth regulator are used

in orchid tissue culture to induce organogenesis and high frequency of somatic embryogenesis (Mahendran and Bai, 2016; Wu et al., 2012). In addition, several studies reported that TDZ can induce SE in *Phalaenopsis* orchid (Kuo et al., 2005; Gow et al., 2010; Khoddamzadeh et al., 2011; Meilasari and Iriawati, 2016).

It has been shown that genetic stability may be affected by various conditions of *in vitro* culture such as application of different PGRs, the number of subcultures and type of explant (Bairu et al., 2006; Raji et al., 2017). In contrast to the indirect embryogenesis resulting from the callus, the DSE has better genetic stability (Harini and Lakshmi, 1993). Today the propagation methods that provide genetic stability and uniformity, particularly in tissue culture, are required to produce clonal and true-to-type commercial plantlets. Genetic stability in embryogenesis has been evaluated by molecular marker analyses in regenerated plants (Khoddamzadeh et al., 2010; Samarfard et al., 2013; Bhattachryya et al., 2014).

Promotion of embryogenesis especially in direct form can increase the efficiency of plant regeneration and propagation, which can help future genetic improvement programs for *Phalaenopsis* and other relatives. Therefore, the aim of this study was to determine the effect of concentrations of TDZ, type of explant, different LEDs spectra and wounding on DSE induction during *in vitro* culture of *Phalaenopsis* in order to obtain genetic stable regenerated plantlets for improving the commercial mass clonal production of *Phalaenopsis*. This is the first report evaluating wide range of light spectra and wounding on induction of DSE in *Phalaenopsis*.

Materials and methods

Plant materials and culture conditions

For direct somatic embryogenesis (DSE), protocorms (in two forms of wounded and intact samples) and leaf segments of plantlet of *Phalaenopsis* 'Hong kong' in vegetative phase were used as explant and were cultured in half-strength modified MS (Murashige and Skoog, 1962) as basal medium. MS medium was supplemented with 100 mg L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.5 mg L^{-1} thiamine HCl, 2 mg L^{-1} glycine, 2 g L^{-1} peptone, 30 g L^{-1} sucrose and 4.8 g L^{-1} agar. The pH of the media was adjusted to 5.7 ± 0.1 prior to autoclaving for 20 min at 121 °C. To investigate the effect of TDZ on DSE, basal medium was supplemented with 0 and 3 mg L^{-1} TDZ for protocorms and 0, 0.5, 1.5 and 3 mg L^{-1} for leaf explants. Protocorms were used in two forms of cut and intact from of the end side.

The explants were maintained for two months for protocorm and two months for leaf explants under 16/8 h light/dark photoperiod and temperature of 25 ± 1 °C. The protocorm samples were also exposed to different light spectra including: W (400-700 nm), B (peak at 460 nm), R (peak at 660 nm), G (peak at 530 nm), a combination of R+B (50:50), and combination of R+ far red FR (70:30) using light-emitting diodes (LEDs) modules with all at the same light intensity of 80 µmol m^{-2} s^{-1} and also dark condition (D). Leaf explants were also exposed to R and R+FR light spectra and D condition. Wavelengths of the light treatments were measured by a Sekonic C7000 spectrometer (Sekonic Corp, Tokyo, Japan) in the range of 300–800 nm. After four weeks, induction of globular type embryos was evaluated by a binocular (Nikon, E200-Nikon Instruments lnc, Japan). For germination (growth) of somatic embryos, they were transferred into 1/2 MS hormone-free medium with the same environmental conditions as described above.

Histological observation

For histological observations, somatic embryos were initially fixed in formalin, ethanol, acetic acid (FAA) (95% v/v ethyl alcohol: glacial acetic acid: formaldehyde: water, 10:1:2:7), and then dehydrated in a tertiary-butyl alcohol, embedded in paraffin wax, sectioned to 10 µm thickness by microtome, then stained

with methylene blue. The observation and photography of samples were done under a digital microscope (Nikon, E200-Nikon Instruments lnc, Japan).

DNA extraction and assessment of genetic stability

Total DNA was extracted from 50 to 100 mg of leaves of PLBs dissected from the regenerated plantlets from PLBs and the mother plant using the CTAB method (Hasebe and Iwatsuki., 1990). Genetic stability of PLB-derived plants was evaluated by ISSR marker analysis as described by ÷ et al. (2007). A total of 12 primers were screened for effectiveness within ISSR reactions, using different DNA samples isolated from PLB-derived plantlets. PCR reaction was conducted in a volume of 25 µL (1 µL of DNA (50 ng DNA), 2 µL of primer, 12.5 µL of PCR master mix and 9.5 µL of nuclease-free water). The PCR program consisted of 35 cycles of 94 °C for 30 s, 51–56 °C (depending on the primers used) for 45 s, and 72 °C for 60 s with a final extension period of 72 °C for 10 min using a GeneAmp thermocycler (Applied Biosystems, California, USA) PCR machine. The PCR products were electrophoresed on 1% (w/v) agarose gel at 90 V for 80 min.

Statistical analysis

The experiment was conducted as a factorial experiment based on a completely randomized design with three factors including three concentrations of growth regulator, six light spectra for two types of explants with three replications of four explants. The data were subjected to analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at 0.05 probability level using the SAS 9.3 (SAS Institute Inc, Cary, NC, USA) software. ISSR markers were scored for the presence (1) or absence (0) of bands regarding the investigated samples (both regenerated plants from SE and mother plant). Electrophoretic DNA bands of low visual intensity that could not be readily differentiated as present or absent were not scored.

Results

Effects of PGRs and wounding on direct somatic embryogenesis

The embryos were formed directly following 4 weeks of culture on the protocorms and 8 weeks on the leaf explants (Fig. 1).

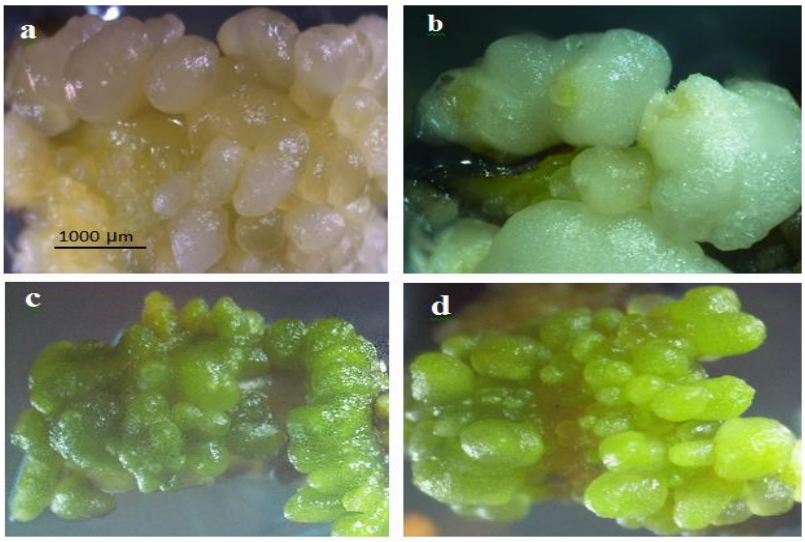

Fig. 1. Plant regeneration via somatic embryogenesis of *Phalaenopsis* under different light spectra and TDZ concentrations. a: Direct somatic embryogenesis formed on leaf in dark condition and 3 mg L^{-1} ; b: DSE matured on leaf explants 3 months after culture in media with 3 mg L^{-1} TDZ; c: Embryo formed under red light spectrum and 3 mg L^{-1} TDZ; d: Embryo formed under R + FR spectrum with 3 mg L^{-1} TDZ.

The results of leaf explants showed that TDZ and its different concentrations had significant effects on embryo formation and growth (**; $P < 0.01$). The highest DSE (19.9 protocorms) were obtained in media supplemented with 3 mg L^{-1} TDZ (Fig. 2). Intact protocorms had the maximum DSE 100% and 9.91 embryos per explant were observed (Fig. 2b). DSE on wounded protocorms was less than DSE in intact samples.

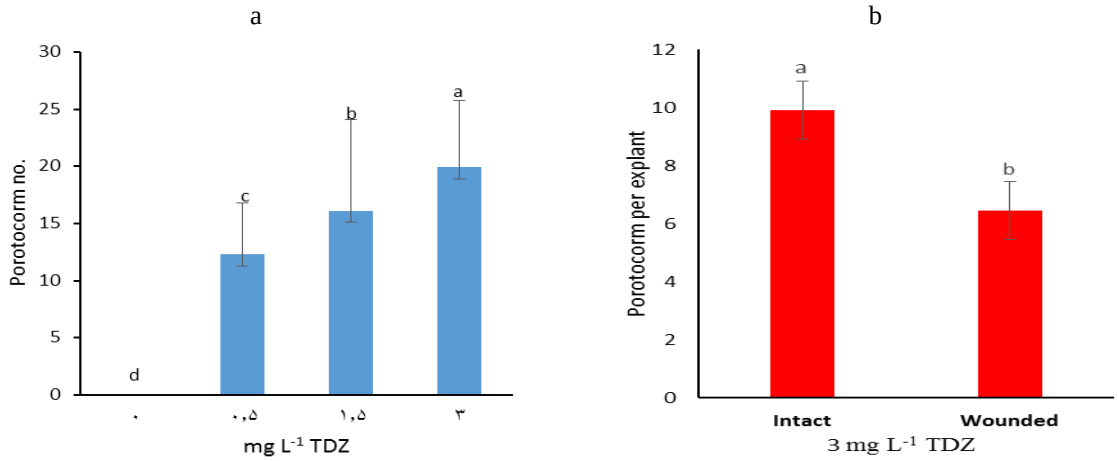

Fig. 2. Effect of TDZ concentrations on DSE of leaf and protocorm explants. (b) Effects of wounding on protocorm explants in *Phalaenopsis*. The data represent the average of three replications with standard error (±SE). Different letters indicate the statistically significant differences (Duncan multiple range test, p≤0.05).

Effects of light spectra on DSE

Exposing protocorm explants to different light spectra significantly influenced induction of somatic embryos (Fig. 3). Among the light spectra, highest DSE (100%) was observed under R and R+FR (Fig. 3b) spectra, while the lowest DSE was observed under R+B light. In general, higher embryogenesis was observed in samples that were exposed to lighting environment compared to the embryogenesis frequency in the darkness.

Although the efficiency of G light on DSE of protocorm explants was less than R and FR, it caused more DSE than the other light spectra (Fig. 3b).

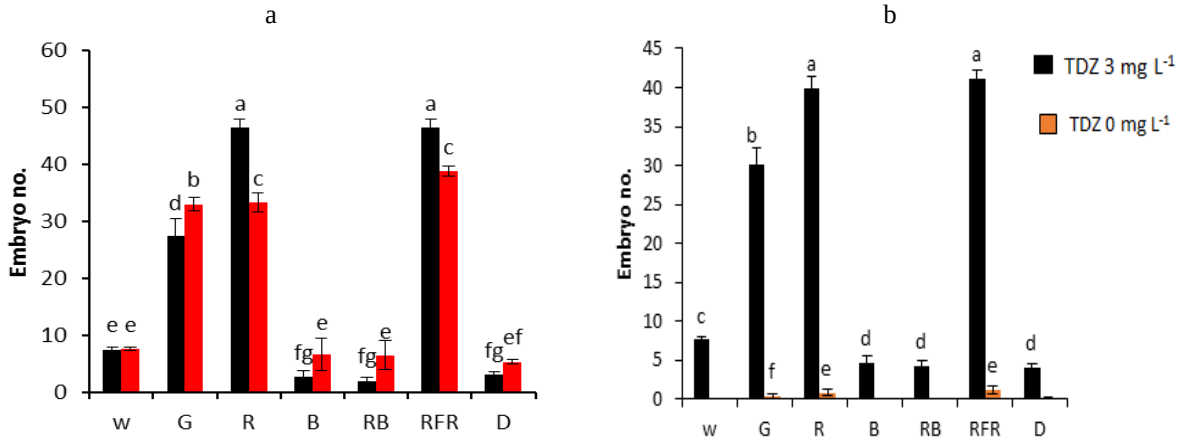

Fig. 3. Interaction between different light spectra and wounding (a) or TDZ (b) on embryogenesis of protocorms. The vertical bars shows the light treatment (w=white, G=green, R=red, RB=red + blue, RFR= red + far-red and D=dark). The data represent the average of three replications with standard error (±SE). Different letters indicate the statistically significant differences (Duncan multiple range test, p≤0.05).

Initiation of somatic embryos was observed directly on the leaf explants that were treated with TDZ, while no embryogenesis occurred in the control treatment (Fig. 4).

Best results were observed for the interaction of R+FR and 3 mg L^{-1} TDZ without significant difference with D and 1.5 mg L^{-1} TDZ. The results showed a decrease in the effect of darkness but increase in the effect of R-FR on DSE with highest TDZ concentration.

Comparison of the embryogenesis among the explants in 0 and 3 mg L^{-1} TDZ under different light spectra (R, RFR and D) showed significant effects of 3 mg L^{-1} TDZ on somatic embryogenesis on both protocorm and leaf explants. Intact protocorms that were cultured in medium containing TDZ under R or R-FR lights resulted in the highest number of protocorms.

Under D condition the highest number of protocorms was observed in leaf samples compared to the other explants (Fig. 5).

Wounded protocorms that were cultured under R or R + FR lights resulted in the highest number of embryo formation (Table 1).

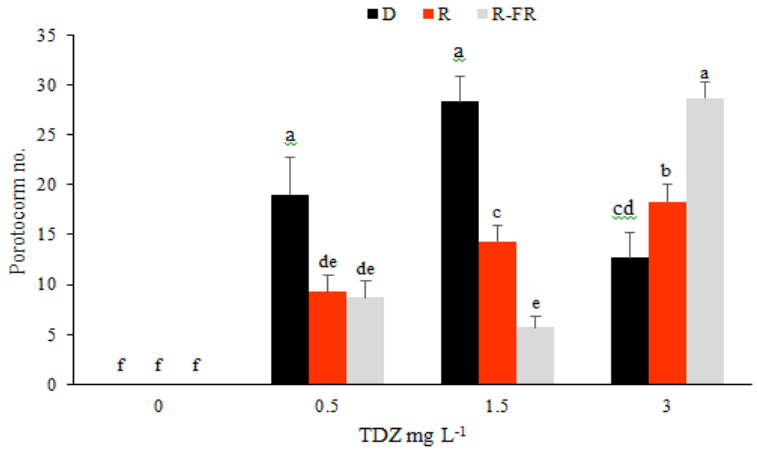

Fig. 4. Interaction between different light spectra and TDZ concentration on DSE on leaf explants in *Phalaenopsis*. Different concentrations of TDZ (0, 0.5, 1.5, 3 mg L^{-1}) were applied to leaf explants that exposed to red (R), red+far red (RFR) and darkness (D). The data represent the average of three replications with standard error (±SE). Different letters indicate the statistically significant differences (Duncan multiple range test, p≤0.05).

Table 1. Effect of light spectrum and wounding on direct somatic embryogenesis of *Phalaenopsis* 'Hong kong' (In. Pr.: Intact Protocorm, Wo. Pr.: wounded Protocorm)

Light spectrum	Explant	Mean number of embryos
W	Wo .Pr.	7.5^{ef}
	In. Pr.	7.6^{e}
G	Wo. Pr	27.3^{d}
	In. Pr.	33.0^{c}
R	Wo. Pr	46.5^{a}
	In. Pr.	33.3^{c}
B	Wo. Pr	2.6^{gh}
	In. Pr.	6.6^{et}
R + FR	Wo. Pr	46.5^{a}
	In. Pr.	35.8^{b}
R+ B	Wo. Pr	2.0^{gh}
	In. Pr.	6.5^{et}
Dark	Wo. Pr	3.0^{g}
	In. Pr	5.3^{f}

Values represent mean embryo number counted from 3 replicates. Means in a column with different letters (superscripts) are significantly different according to Duncan's ($P \leq 0.05$)

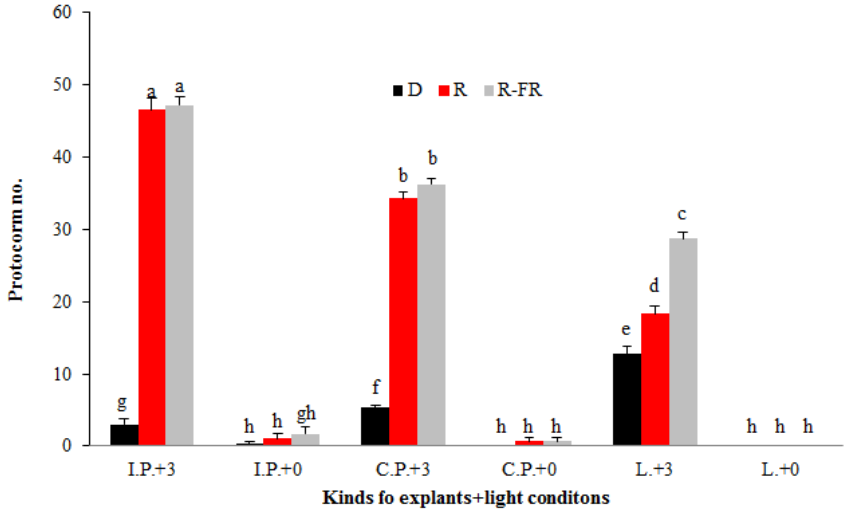

Fig. 5. interaction between different light conditions (R=red, R-FR= red + far red and D=dark) and TDZ concentrations (0 and 3 mg L^{-1}) on DSE of different explants (I.P.= intact protocorms, C.P.= cutted protocorms and L.= leaf section). Columns show direct somatic embryos (The number of embryos per treatment). The data represent the average of three replications with standard error (±SE). Different letters indicate the statistically significant differences (Duncan multiple range test, p≤0.05).

Histological observation

Histological observations showed that the somatic embryos formed directly from the surfaces of protocorm explants without an intervening callus phase (Fig. 6a). Indeed, different developmental stages of embryos were obtained on the protocorm explant (Fig. 6).

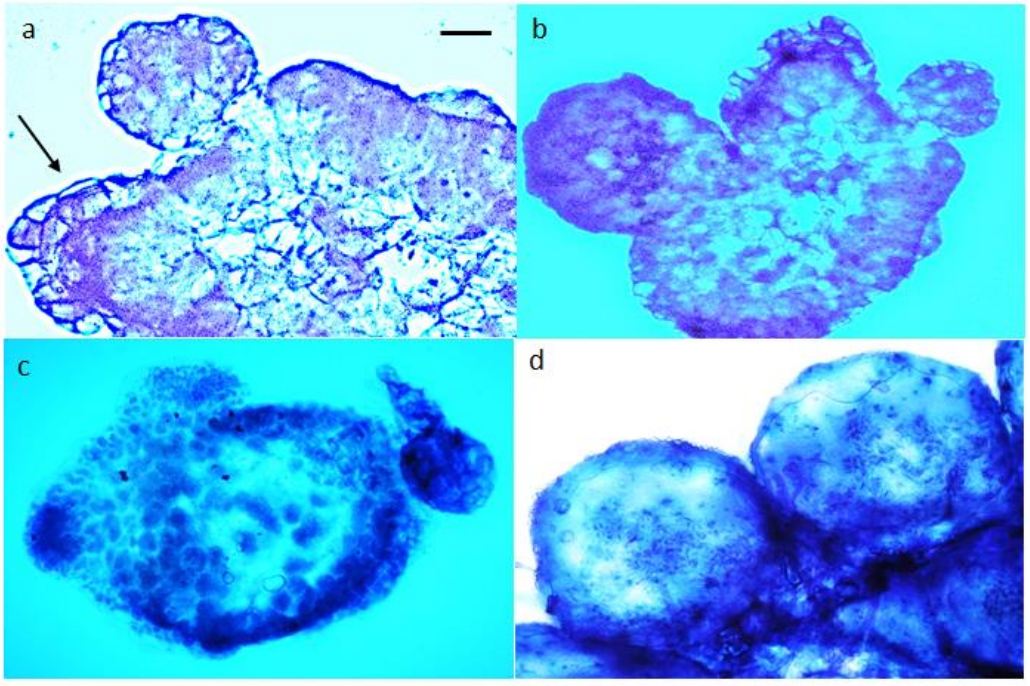

Fig. 6. Histological observation of direct somatic embryogenesis in *Phalaenopsis.* (a) Arrow shows Pre-embryonic cell formation (Scale bar=100 µm). (b) Direct embryogenesis and formation of globular embryos (Scale bar=100 µm). (c) Formation of globular embryos (Scale bar=100 µm), (d) Development of Globular embryo (Scale bar=1000 µm).

Genetic stability of somatic embryo-regenerated plants

A total of 10 ISSR primers were used to screen somaclonal variations. Out of 10 ISSR markers, only 5 primers (Table 2) gave distinct and scorable bands. The amplicons ranged in size from approximately 220 bp (primer DCP3) to 1220 bp (primer DCP2). The results of this study demonstrated that all the produced bands were monomorphic among the regenerants being analyzed with respect to the mother plant (Fig. 7). The amplification using all the 5 ISSR primers resulted in producing 26 distinct bands. The number of scorable and distinct bands generated by ISSR primers was varied from 3 (primer DCP4) to 7 (primer DCP3) with an average of 5.2 bands per marker. A representative of ISSR profile is showed in Figure 7.

Table 2. PCR amplicons obtained from ISSR primers in *in vitro*-raised *Phalaenopsis*

Primer ID	Primer sequence (5'–3')	Total bands amplified	No. of monomorphic bands	Band size (bp)
DCP1	(TC)8AG	6	6	400-1000
DCP2	(GA)8G	4	4	500-1220
DCP3	(GT)8AC	7	7	220-1100
DCP4	(GA)8CT	3	3	620-1000
DCP5	(GT)8C	6	6	250-950
Total bands		26	26	

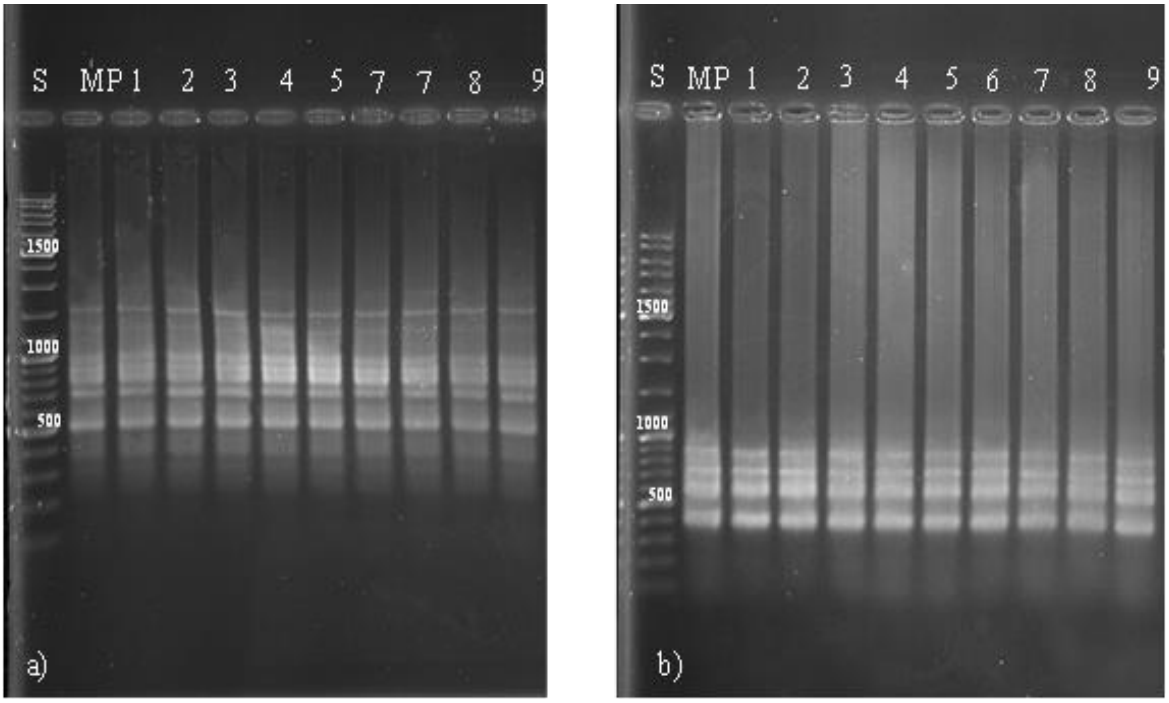

Fig. 7. Genetic stability assessment of *Phalaenopsis* with ISSR marker; profiles were obtained with primers DCP1 (a) and DCP2 (b). S size marker (100-2000 bp), MP mother plant, 1-9 regenerated plantlets obtained by somatic embryogenesis.

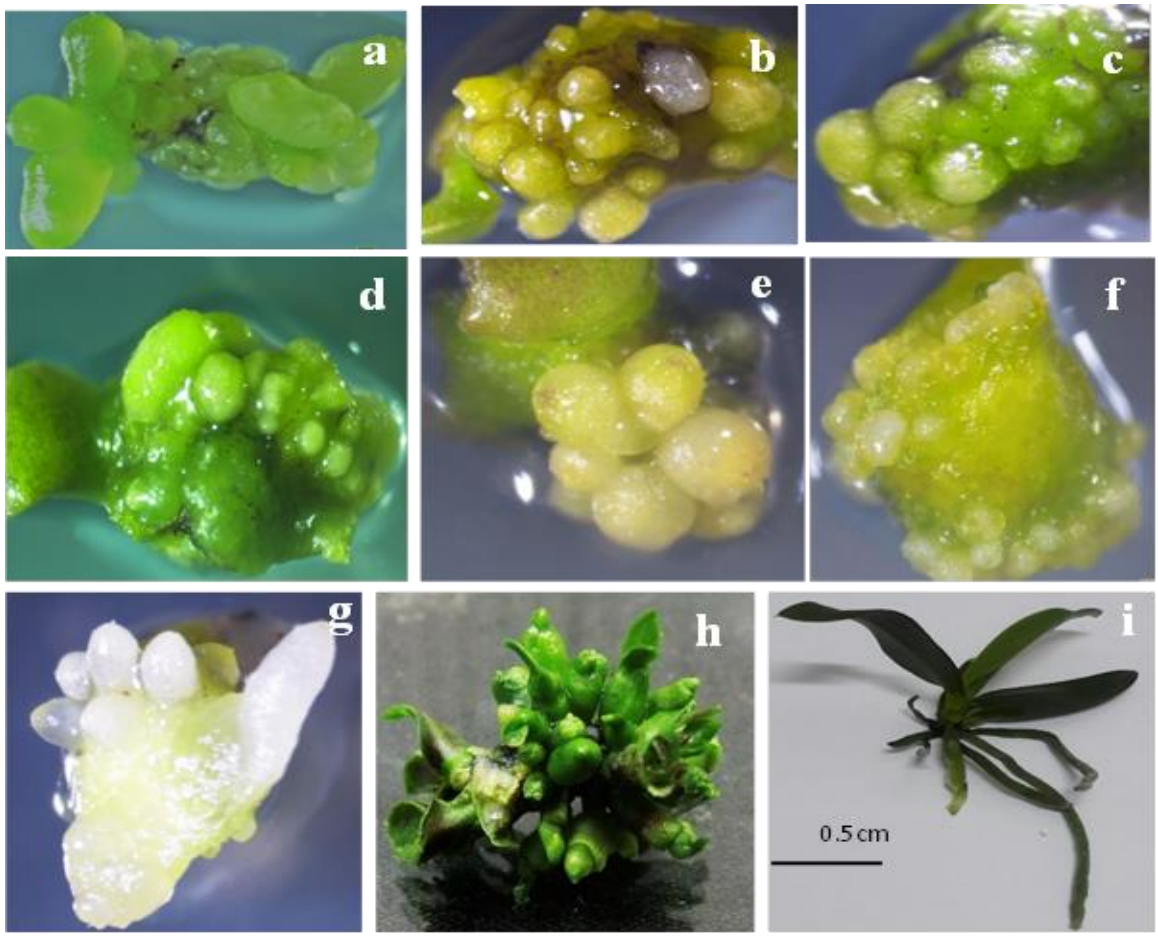

Fig. 8. Somatic embryogenesis (SE) on protocorms. (a) Red + Far-red, (b) Red, (c) Green, (d) White, (e) blue, (f) blue + red (1000 µm) light spectra, (g) dark (Scale bar=1000 µm), (h) SE germination, (i) Plant growth *in vitro* (Scale bar=0.5 cm).

Discussion

SE have many advantages, especially for propagation of plants with specific reproductive phases such as orchids (Mayer et al., 2010). SE approach can be used in genetic engineering as an effective system for plant regeneration as well (Naing et al., 2013). There are some studies that have used SE method to propagate *Phalaenopsis* (Chen and Chang, 2006; Feng and Chen, 2014). In our study, we have presented an efficient method for DSE in *Phalaenopsis* by investigating the effects of possible effective factors that influence the embryogenesis. Our result confirmed the importance of light spectrum, type of explant and presence of TDZ in culture medium for DSE in *Phalaenopsis*.

In some studies on *Phalaenopsis*, leaf samples have used for embryogenesis but in some other studies, the protocorms were used as the explants for DSE (Chen and Chang, 2004; Mose et al., 2017).

Wounding can play an important role in cell division and help to increase embryogenesis (Ludevid et al., 1990). There are some studies that investigated the role of wounding on plant embryogenesis. For instance, wounding increased somatic embryogenesis in leaf explants of *Coffea arabica* (Rojas-Herrera and Loyola-Vargas, 2002). Our results showed also the highest DSE in wounded protocorms (Table1).

In the present study, protocorm and leaf explants showed the highest DSE when incubated in media supplemented with 3 mg L^{-1} TDZ. Importance of PGRs and light as the effective factors that influence

embryogenesis have been reported (Mengxi et al., 2011). Many researchers have reported SE in different species of orchids such as *Phalaenopsis* (Gow et al., 2009) and *Xenikophyton smeeanum* (Mulgund et al., 2011) through TDZ application in the medium. The positive effect of TDZ as a growth regulator has been confirmed on somatic embryogenesis in *Phalaenopsis*. It has been reported that TDZ is an effective PGR on direct somatic embryogenesis in *Phalaenopsis* (Kuo et al., 2005; Gow et al., 2009; Guo et al., 2010; Meilasari and Iriawati, 2016). Mulgund et al. (2011) reported SE in different species of orchids such as *Xenikophyton smeeanum* through TDZ application in the medium. It has been reported that TDZ is an effective PGR on direct somatic embryogenesis in *Phalaenopsis* (Guo et al., 2010; Meilasari and Iriawati, 2016). Similar results have been also reported in other orchid species such as *Oncidium* (Chung et al., 2007), *Cymbidium* (Huan and Tanaka, 2004) and *Dendrobium* (Chen and Chang, 2001).

Promotion of SE by darkness (D) has been reported in many plant species such as *Lilium ledebourii*, *Phalaenopsis* and *Epipactis veratrifolia* (Bakhshaie et al., 2010; Gow et al., 2010; Moradi et al., 2017). In current study, dark conditions caused the formation of somatic embryos on leaf explants (Fig. 5). In a study on carnation it was shown that light spectra affects SE and it can play an inductive role in morphogenesis (Aalifar et al., 2019). Chung et al. (2010) observed embryogenic callus in *Oncidium* and determined that protocorm-like bodies (PLBs), which formed under 16 h photoperiod of tubular fluorescent lamps (FL) at 50 μmol m^{-2} s^{-1}. Chung et al. (2010) reported that R light can promote accumulation of carbohydrate as a source for embryo induction in *Oncidium* 'Gower Ramsey'. Furthermore, accumulation of soluble sugar, starch and free amino acid under R spectrum can positively affect induction of SE (Mengxi et al., 2011). Mengxi et al. (2011) showed that exposure to R light cause higher starch accumulation than D condition. Some authors have suggested that photoreceptors take role on the effects of different light spectra on development of plant tissues (Burritt and Leung, 2003).

There are few studies that investigated the role of light spectra on SE in *Phalaenopsis* (Park et al., 2010). Chung et al. (2010) found that combination of R+B+FR was the best light spectra for PLB formation. Promotion of SE by FR and R spectra in *Phalaenopsis* and *China Rose* (Torné et al., 2001; Park et al., 2010) has been also reported. We observed highest DSE on protocorm explants that were exposed to R and R+FR light spectra (Table 1). Chan. (2013) demonstrated that R light enhances SE in most of the species, while the effects of B light on SE is sometimes inhibitory.

Previous studies showed the importance of photoreceptors on morphogenetic responses under different light spectra (Kraepiel and Miginiac, 1997; D'Onofrio et al., 1998; Torné et al., 2001). Michler and Lineberger (1987) observed that R and G lights as well as the D cause the highest SE response on carrot compared to the W and B lights. In our study, G spectrum showed significant and interesting effect on DSE, compared to other light spectra (Fig. 3), which has not been reported earlier. Some of recent studies demonstrated the role of G spectrum in plant growth and development. Response to G light is typically a low-light response (Wang and Folta, 2013). A series of reports in the last decade have shown that not only G light specific responses exist, but also they are oftentimes mediated by well-described light sensing systems (Banerjee et al., 2007).

Phytochrome can be converted to the FR absorbing, biologically active form by G light. G light establishes a phytochrome equilibrium favoring the active Pfr form Hartmann (1967), and G light is sufficient to activate photoreceptors (Shinomura et al., 1996).

This observation is not surprising since phyA is extremely sensitive and abundant in the D-grown seedlings. However, the effect of G light and mechanism of its action on embryogenesis and its interaction with other light spectra such as FR, are the matters of further research.

ISSR markers were previously applied for the evaluation of genetic fidelity in different genus of orchids such as *Phalaenopsis* (Khoddamzadeh et al., 2011; Samarfard et al., 2013) and *Dendrobium* (Bhattacharyya et al., 2014). Our study assessed genetic fidelity of derived *Phalaenopsis* from PBLs using ISSR markers under D condition. Previous reports revealed that dissimilarity occurred between the mother plant and proliferated PLBs after 3 to 6 months in *in vitro* but no changes occurred between the mother plant and first-generation PBLs, which is in accordance with previous reports (Khoddamzadeh et al., 2011; Samarfard et al., 2013; Aalifar et al., 2019).

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Conflict of interest

No potential conflict of interest is reported on this study.

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