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Effects of plant growth regulators and activated charcoal on somaclonal variations of protocorm-like bodies (PLBs) of *Dendrobium* Sabin Blue orchid

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ABSTRACT

Protocorm-like bodies (PLBs) of *Dendrobium* orchids are emerging as a potential source of valuable secondary metabolites. This study examined the effect of four additives namely 1-naphthaleneacetic acid (NAA), kinetin, thidiazuron (TDZ), and activated charcoal (AC) used in culture medium on genetic variability in PLBs of *Dendrobium* Sabin Blue. Nine (9) ISSR primers and eleven (11) DAMD primers were used to assess the genetic variability of PLBs that were subcultured over a period of two years. We confirmed that the use of kinetin in culture medium for two years resulted in the highest rate of somaclonal variation in PLBs. On the other hand, TDZ and activated charcoal registered the lowest genetic variability in PLBs. The findings of this study suggest the importance of selecting additives used in the culture medium to maintain stable genetic lines of PLBs. We recommend that the assessment of somaclonal variations should be performed for long term maintenance of tissue cultures.

1. Introduction

Plant tissue culture is a prominent area in plant biotechnology due to its potential as a source of valuable secondary metabolites based on elite genetic resources. Nevertheless, scaling up of a reliable tissue culture system may be impaired owing to the prevalence of somaclonal variations (Larkin and Scowcroft, 1981). The pervasive occurrence of obscure defects caused by somaclonal variations can pose a serious limitation in plant tissue cultures (Salvi et al., 2001). Consequently, it is imperative to determine genetic fidelity of *in vitro* plant tissues for commercial application of a desired genotype for production of secondary metabolites. Genotypic and environmental interactions have been studied to determine cultivars with high stability to obtain plant secondary metabolites (Lal et al., 2018). *In vitro* raised plants are usually phenotypically normal despite occurrence of genomic changes rendering visual observation insufficient to detect somaclonal variations (Devi et al., 2014). Detection of somaclonal variations by morphological and physiological traits can be very tedious due to extensive observation on different developmental stages on the plants yet insufficient to detect somoclonal variation (Palombi and Damiano, 2002).

Single primer amplification reaction (SPAR) methods have been noted as an efficient tool to detect somaclonal variations in plantlets (Devi et al., 2014). Random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and direct amplification of minisatellite DNA region (DAMD) are the usual markers used for this purpose. These markers are utilised extensively for their ease to use and results reliability (Mishra et al., 2008; Fatima et al., 2015; Mahto et al., 2018; Sai Sudha et al., 2019). The use of such markers also facilitated screening of pest-tolerant variants and determination of genetic diversity of medicinal plants (Thilaga et al., 2017; Bharti et al., 2018). Using a single DNA marker for assessment of somaclonal variation is not sufficient as genetic variability may be undetected as the point mutations occur beyond the priming sites (Lakshmanan et al., 2007). More than one DNA amplification technique is recommended for better assessment of genetic variation so that different regions of the genome could be examined (Palombi and Damiano, 2002).

The PLBs of *Dendrobium* Sabin Blue used in this study were taken from the stock maintained in Plant Biotechnology Laboratory,

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University Sains Malaysia, Penang. The same mass of PLBs (0.5 g) were subcultured onto fresh medium every two months. In this study, ISSR and DAMD markers were used to assess somaclonal variations in protocorm-like bodies (PLBs) of *Dendrobium* Sabin Blue which have been subcultured every two months for two years in different plant growth regulators and activated charcoal (AC). ISSR and DAMD markers are part of PCR-based DNA fingerprinting methods that have been successfully used in determination of genetic variability in plants. Contrary to morphological observation which can time consuming, expensive, laborious and are subjected to plant developmental stages and environmental factors (Israeli et al., 1991; Smith and Hamill, 1993), these methods are relatively easy, cost effective, not affected by plant developmental stages and environmental factors and do not require DNA sequence prior knowledge (Verma and Rana, 2013). These two genetic markers have been widely used in assessing plant genetic fidelity such as regenerated *Nepenthes khasiana* (Devi et al., 2014) and *Withania somnifera* (Fatima et al., 2015), as well as micropropagated *Henckelia incana* (Prameela et al., 2015).

In our earlier study, half-strength Murashige and Skoog (1962) medium supplemented with (a) 4 mg/L 1-naphthaleneacetic acid (NAA), (b) 1.5 mg/L kinetin, (c) 4 mg/L thidiazuron (TDZ) and (d) 0.5 g/L activated charcoal (AC), respectively increased the proliferation of PLBs. This study was intended to assess the somaclonal variation effect caused by these four additives in the culture medium on the PLBs of *Dendrobium* Sabin Blue which had been subcultured every two month for two years. Determination of genetic fidelity of PLBs after two years of subculture is essential as alteration in genetic properties of PLBs could affect the yield of valuable secondary metabolites in our upcoming investigations.

2. Materials and methods

2.1. DNA extraction

Extraction of DNA from PLBs of *Dendrobium* Sabin Blue was performed by using Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada). PLBs were weighed for approximately 0.1 g and ground in liquid nitrogen with mortar and pestle. Lysis Buffer L (500 µL) and RNAse A (1 µL) was added to homogenised plant tissue in the mortar and subsequently transferred into a sterile 1.5 mL microcentrifuge tube. The tube was then incubated in 65 °C water bath for 10 min, after which 100 µL Binding Buffer I was added into the lysate. This was followed by incubating the mixture on ice for 5 min before transferring the lysate into a filter column for 2-min centrifugation at 14,000 rpm. The flow through of centrifugation was transferred to another 1.5 mL sterile microcentrifuge tube. Equal volume of 70% ethanol was vortexed with the flow through in the same microcentrifuge tube. Next, 650 µL of lysate was pipette to the spin column and centrifuged again for 1 min at 10,000 rpm. However this time, the flow through was discarded and the precipitate was added with 500 µL Solution WN. The spin column was then centrifuged for 1 min at 10,000 rpm and the flow through was discarded. This was followed by adding 500 µL of Wash Solution A into the spin column and centrifuged for 1 min at 10,000 rpm after which the flow through was discarded again. This washing step was repeated once again with centrifugation 14,000 rpm for 2 min. After the washing step, the column was detached from the collection tube and placed in a microcentrifuge tube. Membrane of the spin column was eluted with 100 µL of Elution Buffer B and incubated at room temperature for 1 min. Finally, the microcentrifuge tube with spin column was centrifuged at 10,000 rpm for 1 min and the extracted DNA was kept at −20 °C storage.

2.2. PCR amplification using ISSR marker

ISSR primers used for PCR amplification was purchased from First Base Laboratory (Malaysia) and molecular reagents used were purchased from Lucigen Corporation (USA). Nine (9) out of 15 ISSR primers screened for analysis showed clear and reproducible bands. DNA amplification was carried out according to Antony (2017) who performed PCR amplication of DNA extracted from the same plant used in this study. Total PCR amplification volume was 20 µL, containing 2.5 µL of genomic DNA (25–30 ng), 0.4 µL of deoxyribonucloetide triphosphate (dNTP) mix (0.2 mM), 2 µL of 1×PCR buffer, 1.2 µL magnesium chloride (1.5 mM), 0.5 µL Taq DNA polymerase, 1 µL primer (0.5 µM) and 12.4 µL autoclaved deionized water. The PCR amplification steps were set to begin with initial denaturation at 94 °C (4 min); followed by 40 cycles of denaturation at 92 °C (30 s), annealing at Tm-5°C (1 min), extension at 72 °C (2 min) and then a final extension at 72 °C (7 min).

2.3. PCR amplification using DAMD marker

From 12 DAMD primers screened for analysis, only 11 primers were selected for assessing polymorphism in this study because they produced well-separated and reproducible. The PCR amplifications were performed in volume of 20 µL and the molecular reagents composition amounts were identical as in DNA amplification using ISSR marker. PCR amplification steps was programmed with initial denaturation at 94 °C (2 min), followed by another 40 cycles of denaturation at 92 °C (1 min), annealing at T_m-5°C (2 min), extension at 72 °C (2 min), and concluded with a final extension at 72 °C (5 min).

2.4. Gel electrophoresis

PCR amplification products were separated by electrophoresis. Agarose (1%) was prepared in 40 mL 1 × Tris-Borate-EDTA (TBE) buffer with microwave heating and left to cool to room temperature before being stained with Redsafe nucleic acid staining solution (iNtRON Biotechnology, South Korea). This dissolved gel mixture was casted in Mini Gel Caster (Bio Rad Laboratories, USA) with 8-well comb and allowed to solidify at room temperature for 30 min. Following that, the solidified gel was submerged into the base of Wide Mini Sub-Cell® GT Agarose Gel Electrophoresis System (Bio Rad Laboratories, Inc., USA) which was filled with 1 × TBE buffer. Thermo Scientific GeneRuler 1 kb (Thermo Scientific, Lithuania) was loaded into the well to function as the molecular marker. PCR products (5 µL) were mixed with 3 µL 6× DNA Loading Dye (Thermo Scientific, Lithuania) and loaded into individual wells. Thereafter, the electrophoresis system was connected to the PowerPac™ Basic Power Supply (Bio Rad Laboratorie Inc., USA) and electrophoresed at 90 V for 90 min. Following that, the gel was visualized under UVIdoc HD5 Gel Imaging System (UVITec Limited, UK).

2.5. Determination of polymorphism

Clear and reproducible bands were manually determined as 0 for absence and 1 for presence of bands. The similarity index (SI) was evaluated between the PLBs cultured in medium devoid of plant growth regulators and PLBs subcultured in different types of plant growth regulators. SI was calculated based on Asnita and Norzulaani (2006) as the following:

$$SI = 2Nxy \;/\; (Nx + Ny)$$

SI = Similarity Index
N*xy* = Number of monomorphic bands between control PLBs and PLBs subcultured with different plant growth regulators
N*x* = Total number of bands in the control PLBs
‘ N*y* = Total number of bands in the PLBs subcultured in different plant growth regulators

The percentage of polymorphism in PLBs cultured in different plant growth regulators was determined according to Blair et al. (1999) using the following formula:

% polymorphism = (Total number of polymorphic bands/Total number of bands) X 100

3. Results

3.1. Genetic variability of PLBs based on similarity index (SI)

Band profiles of nine ISSR primers used to compare the DNA variation between PLBs subcultured in half MS medium supplemented with 4 mg/L NAA, 1.5 mg/L kinetin, 4 mg/L TDZ and 0.5 g/L activated charcoal with control PLBs subcultured in hormone-free medium are shown in Fig. 1 (only the results of three primers [UBC840, UBC864, I25] are shown in Table 1). The size of PCR amplified products was between 150 to 700 base pairs. SI for PLBs cultured in activated charcoal was consistently zero for all the three primers. The most sensitive ISSR primer to detect polymorphism was the UBC840 primer which scored zero in SI for PLBs cultured in all the four types of medium supplements, namely 4 mg/L NAA, 1.5 mg/L kinetin and 4 mg/L TDZ. UBC864 primer was comparatively less sensitive in detection of polymorphism was it scored high SI for PLBs cultured in NAA (0.67), kinetin (0.77) and TDZ (0.67). Using I25 primer, SI of PLBs in cultured in NAA, kinetin and TDZ was 0.50, 0.46 and 0.20, respectively. Thus, the order of primer sensitivity in the detection of polymorphism based on SI for PLBs maintained in NAA, kinetin and TDZ was UBC864 < I25 < UBC840.

Eleven (11) DAMD primers that could produce well-separated bands were used to examine genetic variability between PLBs subcultured in half-strength MS medium fortified with different supplements and control PLBs (Fig. 2). Band profiles for DNA of PLBs using three DAMD primers are shown in Table 2. PCR amplified products were between 100 and 700 base pairs. The URP9F primer consistently score zero SI for PLBs cultured in all four treatment media. The 6_2H (−) primer scored moderately high SI in PLBs treated in NAA (0.71), kinetin (0.75), TDZ (0.67) and activated charcoal (0.67). The primer that scored full or nearly full SI was the INS primer for PLBs in all treatment media except TDZ treatment. In general, the competence of these primers for detection of somaclonal variations are in the order of INS <6_2H (−) < URP9F.

3.2. Genetic variability of PLBs based on percentage of polymorphism

Different plant growth regulators and activated charcoal have caused different extend of somaclonal variations in subcultured PLBs. Based on ISSR marker analysis, kinetin produced highest percentage of polymorphism at 16.39% compared to other treatments (Table 3). There was not much difference in polymorphism caused by NAA, TDZ and activated charcoal, which was between 13.21 to 13.56%. DAMD band profile analysis showed similar results, in which kinetin caused the highest polymorphism at 47.89%. This was followed by NAA with 39.76% polymorphism, and TDZ and activated charcoal at 30.88 and 31.94%, respectively. DAMD marker analysis also indicated that NAA could cause very high rate of somaclonal variation besides kinetin. The ISSR and DAMD markers used to assess somaclonal variations in subcultured PLBs showed difference in sensitivity in detection of polymorphism. DAMD primers showed greater percentage of polymorphism and higher average number of polymorphic bands/primer as compared to ISSR primers. Somaclonal variations between PLBs subcultured in 4 mg/L NAA and PLBs subcultured without plant growth regulators was detected at 39.76% polymorphism by DAMD marker whereas ISSR marker detected only 13.33% polymorphism (Table 3). Average number of polymorphic bands/DAMD primer was 3, in contrast to 0.89 for ISSR marker. Polymorphism of PLBs subcultured in 1.5 mg/L kinetin was nearly 50% using DAMD marker but less than 20% with ISSR marker.

The average number of polymorphic bands/DAMD primer was three times more than that of ISSR marker in PLBs subcultured in NAA-enriched medium. Using DAMD primers, PLBs subcultured in 4 mg/L TDZ demonstrated 30.88% polymorphism and 1.91 polymorphic bands per primer compared to 13.56% polymorphism and 0.89 polymorphic bands/ISSR primers. DAMD band profile of PLBs cultured in 0.5 mg/L activated charcoal indicated 31.94% polymorphism with 2.09 polymorphic bands per primer. With ISSR band profile, only 13.21% polymorphism and 0.78 polymorphic bands per primer were obtained.

4. Discussion

4.1. Effect of plant growth regulators and activated charcoal

Mechanism of tissue-culture induced somaclonal variations has not been well understood, albeit some factors that are usually linked to the origin and frequency of somaclonal variations such as plant genotype, type of explant, culture medium composition, frequency of subcultures, donor plant age as well as tissue culture protocol (Veilleux and Johnson, 1998; Lakshmanan et al., 2007).

In this study, plant growth regulators and activated charcoal were separately used in maintenance and subculturing of *in vitro* PLBs of *Dendrobium* Sabin Blue for two years. The somaclonal variations of these subcultured PLBs were evident in the analysis of ISSR and DAMD marker analysis. The finding in this study is consistent with and Gimenez et al. (2001) and Bairu et al. (2006) who reported that long term *in vitro* culture and use of plant growth regulators could lead to somaclonal variations. However, effect of plant growth regulators on somaclonal variations has been rather inconsistent. Reuveni et al. (1993) reported that medium composition including plant growth regulators had no effect on genetic variations in micropropagated plants. Use of plant growth regulators such as high concentration of cytokinins did not affect genetic fidelity of tissue cultured banana (Venkatachalam et al., 2007). There is a possibility that use of additives in culture medium such as plant growth regulators and activated charcoal in this study produced interaction effect with long term subculture that lead to high rate of somaclonal variations in the PLBs.

Interestingly, the use of NAA, TDZ, kinetin and activated charcoal in culture medium for more than two years subcultures did not result in the same rate of somaclonal variations. The use of kinetin registered the highest percentage of polymorphism by both ISSR and DAMD band profile analysis compared to the other two PGRs and activated charcoal. Kinetin has been reported to cause extensive DNA hypomethylation in carrot root cultures (Arnhold-Schmitt, 1993). Transcriptome analysis of oil palm flower and fruit revealed that histone methylation which occurs during tissue culture can be disrupted and the effects can be stabilised and transmitted to regenerated plants (Shearman et al., 2013). It was also possible that the endogenous cytokinin has accumulated with each PLBs subculture in kinetin-enriched medium in this study. Accumulation of endogenous cytokinin in plant tissue resulted from its exogenous supply in culture medium had been reported (Zaffari et al., 2000). Endogenous cytokinin in *Musa* shoots increased substantially upon transferring from low benzylaminopurine concentration medium to high benzylaminopurine concentration. Improved proliferation rate caused by plant growth regulators can lead to somaclonal variation as was observed in bananas (Israeli et al., 1991; Damasco et al., 1998).

While speeding up the cell cycle for proliferation of PLBs, plant growth regulators in culture medium of this study could cause disruption of cell cycle. Since cell cycle involves duplication of genetic materials, application of plant growth regulators for proliferation of PLBs in this study increased the risk of genetic alteration during DNA transcription (Singh et al., 2012). This was supported by observation of mitotic abnormalities that occurred at every stage of cell division in common beans (*Phaseolus vulgaris*) (Lima et al., 2016). Abnormal somatic cells accounted for 19% of dividing cells with metaphase stage demonstrated the highest percentage of anomalies compared to other mitotic stages. Despite being cytokinins, the use of TDZ in medium for subculturing of PLBs did not result in high rate of somaclonal variation as in the case of kinetin. This is very likely due to differential effect of TDZ and kinetin in promoting proliferation of PLBs. TDZ was used in shoot cultures of *Dendrobium* Second Love hybrid and somaclonal

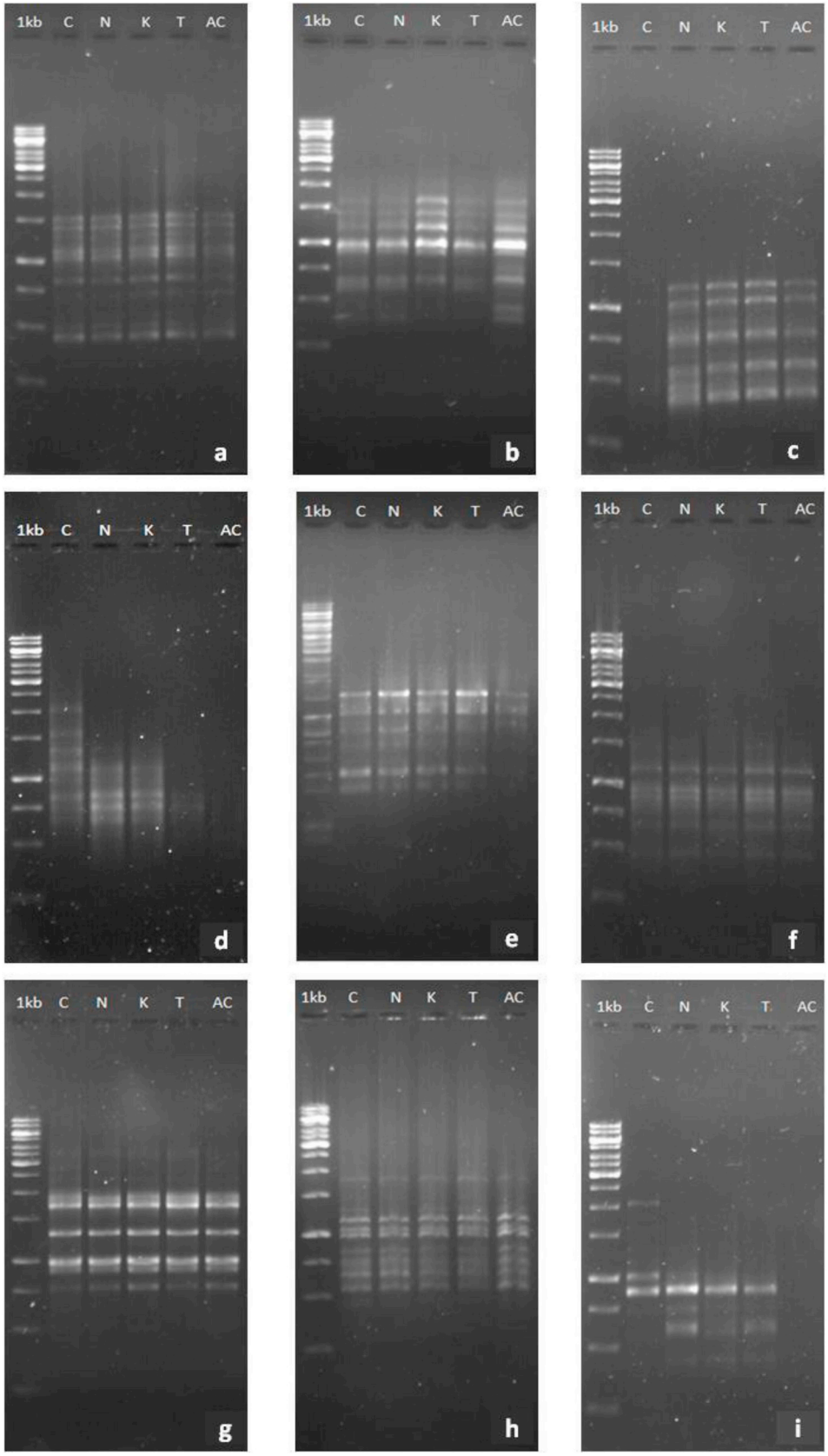

Fig. 1. ISSR marker band profiles for control PLBs (C) and PLBs subcultured in 4 mg/L NAA (N), 1.5 mg/L kinetin (K), 4 mg/L TDZ (T) and 0.5 g/L activated charcoal (AC) using different primers: UBC855 (a), UBC827 (b), UBC840 (c), UBC864 (d), I74 (e), I34 (f), I4 (g), I2 (h) and I25 (i).

Table 1
ISSR profile analysis of PLBs subcultured in different treatments.

Treatment	Primer	Length of amplified DNA fragments (bp)	Total bands in control PLBs	Total bands in treated PLBs	Total mono-morphic bands	Total poly-morphic bands	Similarity Index
NAA	UBC840	150–450	0	6	0	6	0.00
	UBC864	250–700	8	4	4	0	0.67
	I25	200–700	7	5	3	2	0.50
Kinetin	UBC840	150–450	0	6	0	6	0.00
	UBC864	250–400	8	5	5	0	0.77
	I25	200–400	7	6	3	3	0.46
TDZ	UBC840	150–450	0	6	0	6	0.00
	UBC864	250–700	8	4	4	0	0.67
	I25	200–700	7	3	1	2	0.20
AC	UBC840	150–450	0	6	0	6	0.00
	UBC864	250–700	8	0	0	0	0.00
	I25	200–700	7	0	0	0	0.00

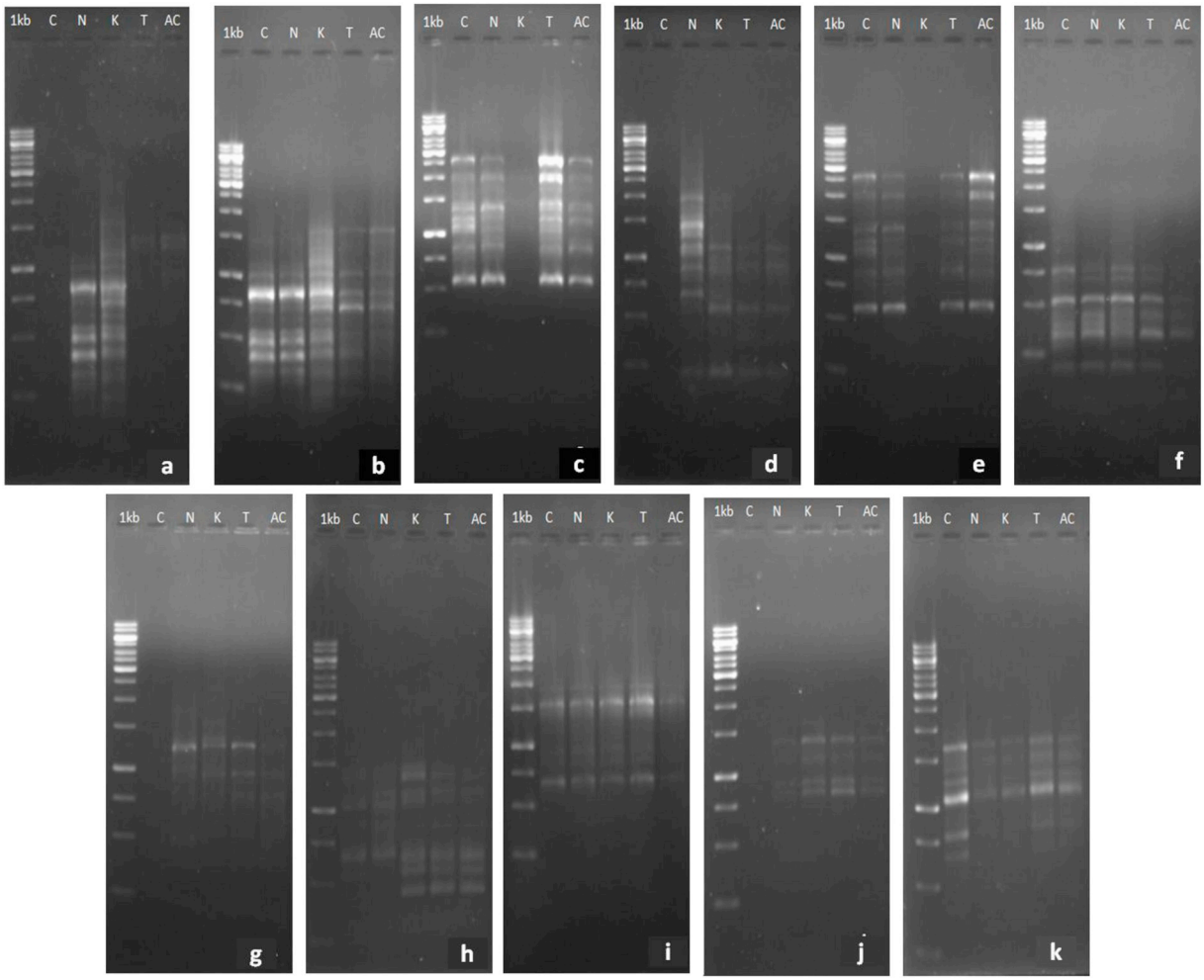

Fig. 2. DAMD marker band profiles for control PLBs (C), PLBs subcultured in: 4 mg/L NAA (N), 1.5 mg/L kinetin (K), 4 mg/L TDZ (T) and 0.5 g/L activated charcoal (AC) using different primers: URP9F (a), INS (b), URP4R (c), 33_6 (d), URP2R (e), M13 (f), 6_2H (+) (g), 6_2H (−) (h), YNZ22 (i), URP13R (j) and URP25F (k).

variation did not appear even after six subculture cycles, covering the span of 540 days (Ferreira et al., 2006). However regenerated Indian soybean showed morphological and agronomic variations from mother plant when it was cultured in TDZ-supplemented medium (Radhakrishnan and Ranjitha-Kumari, 2008).

Apparently the effect of plant growth regulators on somaclonal variations in plant tissue cultures could be species-specific. The effect of NAA on somaclonal variations in PLBs of this study was relatively low. This is consistent with the effect of NAA on leaf colour variants of *Caladium* (Ahmed et al., 2004). NAA caused the least colour variation in the leaf of *Caladium* compared to other plant growth regulators such as indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. Lower concentration of NAA in combination with lower concentration of benzylaminopurine induced 39.9% somaclonal variations in variegated *Saintpaulia* as compared to 4.9% in hormone-free medium (Matsuda et al., 2014). Activated charcoal which has been widely used in plant tissue cultures is distinct from elementary carbon for the elimination of its impurities and carbon surface oxidation (Budavari, 1996). Its application in plant tissue culture for various purposes is attributed to its unique adsorption capacity provided by an

Table 2
DAMD profile analysis of PLBs subcultured in different treatments.

Treatment	Primer	Length of amplified DNA fragments (bp)	Total bands in control PLBs	Total bands in treated PLBs	Total mono-morphic bands	Total poly-morphic bands	Similarity Index
NAA	URP9F	100–400	0	10	0	10	0.00
	6_2H (−)	200–500	8	6	5	1	0.71
	INS	100–500	10	11	10	1	0.95
Kinetin	URP9F	100–500	0	13	0	13	0.00
	6_2H (−)	200–500	8	8	6	2	0.75
	INS	100–500	10	14	12	2	1.00
TDZ	URP9F	400–500	0	3	0	3	0.00
	6_2H (−)	200–500	8	7	5	2	0.67
	INS	100–500	10	8	6	2	0.67
AC	URP9F	300–500	0	5	0	5	0.00
	6_2H (−)	200–500	8	7	5	2	0.67
	INS	100–500	10	10	9	1	0.90

Table 3
Comparative analysis of ISSR and DAMD markers for PLBs subcultured in different treatments.

Treatment	Band profile	ISSR (9 primers)	DAMD (11 primers)
NAA	Total bands	60	83
	Total polymorphic bands	8	33
	% polymorphism	13.33	39.76
	Average number of bands/primer	6.67	7.55
	Average number of polymorphic bands/primer	0.89	3
Kinetin	Total bands	61	71
	Total polymorphic bands	10	34
	% polymorphism	16.39	47.89
	Average number of bands/primer	6.78	6.45
	Average number of polymorphic bands/primer	1.11	3.09
TDZ	Total bands	59	68
	Total polymorphic bands	8	21
	% polymorphism	13.56	30.88
	Average number of bands/primer	6.56	6.18
	Average number of polymorphic bands/primer	0.89	1.91
AC	Total bands	53	72
	Total polymorphic bands	7	23
	% polymorphism	13.21	31.94
	Average number of bands/primer	5.89	6.55
	Average number of polymorphic bands/primer	0.78	2.09

extremely fine network of pores (Baker et al., 1992).

In orchid tissue cultures, activated charcoal has been used in concentration range between 0.01 – 2.0 g/L for various purposes (Thomas, 2008). Activated charcoal was used to promote orchid seedling growth and seed germination (Shiau et al., 2009; Thompson et al., 2006). It also promotes polarity in protocorms and supports rooting (Ket et al., 2004; Thompson et al., 2007). In this study, amplified DNA of PLBs subcultured in activated charcoal exhibited relatively low percentage of polymorphism. The tremendous surface area and volume of activated charcoal could change much of the physical and chemical properties of the culture medium and subsequently impact on the growth and development of the orchid tissue supported by the culture medium. Activated charcoal could alter medium pH and darken culture medium (Owen et al., 1991; Dumas and Monteuuis, 1995). The most critical role of activated charcoal being used in this study is probably adsorbing inhibitory substances in culture medium such as aromatics and phenolic compounds (Teixeria et al., 1994) to reduce part of stress conditions usually experienced by in vitro cultures. Activated charcoal could gradually release certain adsorbed substances such as nutrients besides releasing naturally occurring substances in the activated charcoal (Johansson et al., 1990) so that PLBs did not encounter stress due to nutrient depletion. By reducing in vitro stress on subcultured PLBs, the risk of alteration in their DNA was minimised, hence the low percentage of polymorphic bands of the amplified DNA primers. To date, this is the first report on the effect of activated charcoal on somaclonal variations on *in vitro* plant tissue cultures.

4.2. *Effect of subculturing*

In this study, the subculturing effect of PLBs of *Dendrobium* Sabin Blue for two years coupled with effect of different plant growth additives (NAA, TDZ, kinetin and AC) produced different trends of somaclonal variation. Based on ISSR markers analysis (Table 4), somaclonal variation due to subculturing and AC was the least with 13.21% polymorphism. Percentage of polymorphism increased as subculturing was carried out with NAA (13.33%), TDZ (13.56%) and kinetin (16.39%). DAMD marker analysis (Table 5) indicated that the lowest polymorphism was observed as a result of subculturing and the use of TDZ in culture medium (30.88%). The increased polymorphism effect was noted in the interaction between subculturing and AC (31.94%), NAA (39.76%) and kinetin (47.89%), respectively.

Number of subculture cycles has been known as one of the causes of somaclonal variations in plant tissue cultures. Repeated subcultures promote further multiplication of variants readily generated in the prior cycles. This is evident from reported findings that linked subculture effect to somaclonal variations in which percentage of somaclonal variations increased over lengthy subculture cycles. In micropropagation of Cavendish banana, percentage of somaclonal variations was 10% at the 5th subculture and increased to 41% at the 10th (Bairu et al., 2006). Genetic variations of regenerated *Nepenthes khasiana* increased from 5.65% after the first subculture to 10.87% in the third generation (Devi et al., 2014).

Despite the evidence of genetic homogeneity of *in vitro* plants ranging from 1 ½ to 10 years, reports of Goto et al. (1998) and Varshney et al. (2001) may not be reliable as only one DNA marker was used in

Table 4
Trend of subculturing effect coupled with plant growth additives on the percentage of polymorphism in PLBs based on ISSR markers.

	AC	NAA	TDZ	Kinetin
% polymorphism	13.21	13.33	13.56	16.39

Table 5
Trend of subculturing effect coupled with plant growth additives on the percentage of polymorphism in PLBs based on DAMD markers.

	TDZ	AC	NAA	Kinetin
% polymorphism	30.88	31.94	39.76	47.89

their work. As demonstrated in somaclonal variations analysis of micropropagated *Pinus pinea,* occurrence of somaclonal variations could be overlooked even with three DNA markers. However, polymorphism indicating somaclonal variations has been demonstrated using a single DNA marker in the case of tumeric regeneration, in which 16.5% polymorphism was detected by using 14 RAPD primers (Salvi et al., 2001). Nevertheless, using multiple markers for investigation of somaclonal variations is always preferred since different genomic regions could be targeted (Palombi and Damiano, 2002; Gribaudo et al., 2009).

There are other reports of long term subcutures that did not result in somaclonal variation using more than one DNA marker. Genetic stability of cassava (*Manihot esculanta*) stored *in vitro* for 10 years by subculturing every 12–18 months was confirmed with restriction fragment length polymorphism (RFLP) and RAPD markers (Angel et al., 1996). Genetic fidelity of shoot cultures of *Vanilla planifolia* subcultured for 10 years was also substantiated with RAPD and ISSR markers (Sreedhar et al., 2007). True-to-type *in vitro* clones of *Dendrocalamus asper* which has been subcultured every 25 days for two years was authenticated with genetic assessment using four types of marker, namely amplified fragment length polymorphism (AFLP), RAPD, ISSR and SSR (Singh et al., 2013). The stability of these *in vitro* cultures against genetic variations despite long subculture duration can be attributed to the respective genotype which could be inherently resistant to alteration in their genetic materials. The type of explant used also play an important part for establishing true-to-type long term *in vitro* cultures. Shoot explant used in the studies mentioned above has been found to be more resistant to genetic variation compared to unorganised callus (Varshney et al., 2001). As semi-organised structures, PLBs used in this study were expected to show polymorphism in DNA marker analysis but long term use of plant growth hormones has probably aggravated somaclonal variations.

4.3. Application of DNA markers for detection of somaclonal variations

Using more than one type of marker is critical to detect polymorphism that could arise in *in vitro* culture because each type of marker targets specific region of the plant genome. As more markers are used, plant genome could be more thoroughly screened for polymorphism. This study established that both ISSR and DAMD markers are suitable to assess genetic variation in PLBs of *Dendrobium* Sabin Blue. These markers efficiently and reliably produced discrete and polymorphic DNA band profiles extracted from PLBs cultures maintained in medium supplemented with different plant growth regulators and activated charcoal. However, DAMD marker is more competent than ISSR marker as it detected higher percentage of polymorphism than that detected by ISSR marker. Similar finding was reported by Devi et al. (2014) in which DAMD marker has been found to be more sensitive in evaluating genetic homogeneity of regenerated *Nepenthes khasiana* compared to ISSR and RAPD markers. Similarly, DAMD marker also detected higher percentage of polymorphism compared to ISSR marker in assessment of genetic diversity of Indian indigenous tumeric, *Cucurma longa* (Verma et al., 2015). The disparity in polymorphism percentage between DAMD and ISSR markers are due to the fact that these markers amplify different genomic regions, therefore permitting better chances for detecting genetic variations across the genome of *Dendrobium* Sabin Blue.

DAMD marker targets mini-satellite primers of the core sequences and the parts of genome containing variable number of tandem repeats (VNTR) (Heath et al., 1993). These tandem repeats of 10–60 base pairs DNA core sequence occur widely in both plant and animal species. On the other hand, ISSR marker amplifies DNA segments flanked by microsatellites, which are genomic regions consist of short DNA sequences also known as simple sequence repeats (SSR) (Ng and Tan, 2015). The differences between DAMD and ISSR markers are that DAMD marker targets minisatellites regions of the genome which can be 10 to 60 base pairs of DNA sequence whereas ISSR marker actually amplify inter-repeat regions of simple sequence repeats (SSR). SSR also known as microsatellites are usually between two to five DNA base pairs. Therefore, the relatively higher percentage of polymorphism detected by DAMD primers compared to that of ISSR primers in all the four PLBs cultures examined implies that mini-satellites genomic regions of *Dendrobium* Sabin Blue exhibited more genetic variability compared to inter-SSR regions of the same genome. The finding of this study ascertained kinetin to be more potent in inducing somaclonal variations in PLBs compared to NAA, TDZ and activated charcoal in culture medium. Therefore, the use of kinetin in long term maintenance of *in vitro* cultures should be reconsidered if the effect of somaclonal variation is not desired.

Assessment of genetic fidelity in *Dendrobium* Sabin Blue had shown high significance as PLBs can be exploited as heterosis genetic resources to obtain valuable secondary metabolites such as polysaccharides, alkaloids, aromatics and sesquiterpenes (Xu et al., 2010, 2013; Tan et al., 2014). There were multiple factors such as culture medium composition including plant growth regulators that act in coherent with *in vitro* culture micro-environment on the PLBs. The consequential extend of somaclonal variations depends on how *in vitro* plant tissues respond accordingly to these multi-faceted stress factors in their unnatural *in vitro* environment based on their genotypic properties.

5. Conclusion

The present study demonstrated that the use of different plant growth additives in culture medium to maintain the PLBs of *Dendrobium* Sabin Blue after repeated subculturing resulted in different extend of somaclonal variation. The use of different DNA markers, namely the ISSR and DAMD has established that (a) 4 mg/L TDZ and (b) 0.5 g/L AC posed less threat to genetic homogeneity of *in vitro* PLBs during long term subculture. On the other hand, using 1.5 mg/L kinetin in culture medium clearly compromised the genetic fidelity of *in vitro* PLBs. The findings of this study imply the necessity to examine the extend of somaclonal variation in *in vitro* cultures caused by culture medium additives during long term maintenance and subculture as the expected yield of the targeted secondary metabolites could be jeopardized. Other DNA markers such as Random Amplified Polymorphic DNA (RAPD) and Start Codon Targeted (ScoT) markers can be used in conjunction with ISSR and DAMD markers in this study to examine alterations in DNA sequence that occur in different regions of the DNA. In addition, somaclonal variations in PLBs of *Dendrobium* Sabin Blue may give rise to new hybrids that should be investigated for their potential to biosynthesize valuable compounds of interest.

Declaration of competing interest

The authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101426.

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