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Preliminary study on the effects of fusaric acid treated protocorm-like bodies of *Dendrobium* hybrid against *Fusarium proliferatum* and *Fusarium oxysporum*

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ABSTRACT

Aims: *Dendrobium*s are majorly affected by *Fusarium proliferatum* and *F. oxysporum*. The aim of this research was to utilise the mycotoxin, fusaric acid (FA) on *Dendrobium* hybrid to produce cultivars that are resistant towards these fungi.
Methodology and results: FA of concentrations 0.05, 0.10, 0.15 and 0.20 mM were transferred to sterilised half-strength Murashige and Skoog (MS) medium and inoculated with four weeks old thin cell layer (TCL) of protocorm-like bodies (PLBs) for eight weeks. It was deduced that PLBs treated with 0.10 mM of FA resulted in highest survival and shoot regeneration rate but the survival and regeneration rate began to decline as the concentrations of FA were increased. Histology and scanning electron microscopy (SEM) observation showed prominent cell damage and stomatal closure in PLBs treated with FA. Direct amplification of minisatellite DNA (DAMD) markers showed polymorphism in the FA treated PLBs compared to the control PLBs. In the leaf bridge bioassay, plantlets treated with 0.05 mM of FA showed most resistance towards both fungal species.
Conclusion, significance and impact of study: Therefore, this research is a preliminary screening study where the optimum concentration of FA was selected based on the reaction of treated TCL of PLBs towards these mutagens.

Keywords: Orchid, fusaric acid, tissue culture, resistant, stomatal closure

INTRODUCTION

Orchids have a wide use in the horticultural field, for medicinal, food and art purposes (Singh and Duggal, 2009). This increased demand for orchids is sustained by artificial propagation. Several tissue culture techniques have been developed for orchids but in recent years, thin cell layer (TCL) is used as another option of explant for micropropagation of orchids. TCL is a thin layer of cells which is totipotent and able to produce more number of explants compared to the whole protocorm-like bodies (PLBs) (Teixeira da Silva, 2013).

However, somaclonal variation is sometimes unavoidable when working with plants *in vitro.* In addition to the basic genetic implications of this phenomenon, the variation has proven advantageous in breeding programs of various crop plants (Fay, 1992). According to Kumawat, Kumar and Choudhary (2017), in somaclonal variation, some new alleles or mutation which was not available in germplasm may be isolated and these variations occur in rather high frequency.

These advantages of somaclonal variation can be put to use to overcome the problem of diseases and pests in orchids. *Dendrobium* orchids are prone to disease caused by *F. proliferatum* and *F. oxysporum* which cannot be eradicated because these fungi are resistant to most fungicide (Swett and Uchida, 2015). Hence, a valuable approach for improving the productivity of *Dendrobium* hybrid, *D5* (*Dendrobium* Waipahu Beauty × *Dendrobium* Burana White Big Flower) in this research is to select regenerated clones which are resistant or tolerant to fungal diseases through fusaric acid (FA) treatment. FA is a mycotoxin produced by most *Fusarium* spp. which can be used as a selecting agent for *in vitro* selection of resistant orchids (Mahlanza *et al.*, 2013). FA is a suitable method used in *in vitro* selection because has low to moderate toxicity to human, plant and environment and can cause alteration in the morphology and growth in the infected plants *per se* (Wang *et al.*, 2014).

Therefore, the objective of this research was to determine *in vitro* selection of disease resistant *Dendrobium* hybrid orchid PLBs using different concentrations of FA on the TCL of PLBs by investigating its morphology and direct amplification of minisatellite

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region DNA (DAMD) analyses. In order to test the treated plantlets for resistance or tolerance towards *F. proliferatum* and *F. oxysporum*, a leaf-bridge bioassay was conducted.

MATERIALS AND METHODS

In vitro cultures of *Dendrobium* hybrid were used for selection study. PLB were maintained in half-strength semi-solid MS medium (Murashige and Skoog, 1962). The TCL of healthy, four weeks old PLBs were used as explants. Longitudinal TCL (lTCL) of PLBs were prepared using Protocol 3 as described by (Teixeira da Silva, 2013).

Fusaric acid (FA) treatment of *Dendrobium* hybrid PLBs

Filter sterilised FA [Cayman Chemical Company, USA] was added to autoclaved half-strength MS medium. The selection medium was prepared in different concentrations comprising of 0.05, 0.10, 0.15 and 0.20 mM of FA. FA was added in pre-sterilised medium to prevent the formation of toxic compounds by the degradation of FA (Dehgahi, 2015).

The medium was poured in pre-sterilised Petri dishes and ten TCL of PLBs were inoculated in each Petri dish and six replicates were conducted for each FA concentration. Then, the TCL of PLBs were incubated for four weeks at 25 ± 2 °C under a 16 h photoperiod using cool white fluorescent lamps. The control treatment of the TCL of PLBs was inoculated in half-strength of MS medium without the addition of FA. The control was placed in Petri dishes and incubated under the same conditions.

Selection of FA-tolerant PLBs

This treatment was experimented for eight weeks or two selection cycles before the results were observed. PLBs surviving post FA treatment after the first four weeks which were green in appearance were selected and transferred to half-strength MS medium supplemented with the same concentration of FA for another four-week treatment cycle to produce PLBs which are resistant towards FA. At the end of selection process, PLB's survivability was scored based on colour of PLBs. Survived PLBs remained green, while dead PLBs turned dark brown or white with no green patches. The survival percentage of PLBs after selection process was calculated as follow:

$$\text{Survival (\%)} = \frac{\text{(Number of survived PLBs)}}{\text{total PLBs cultured}} \times 100\ \%$$

Survived PLBs were multiplied on half-strength MS medium without FA.

Statistical analysis

These results were statistically analysed using one-way ANOVA and differentiated with Tukey's range test at $P \leq 0.05$ with the IBM SPSS Statistics 22 program.

Histological analysis of PLBs

Control and FA treated PLBs were selected for histology analysis. The histological procedure applied in this treatment was modified from Vyas *et al.* (2010). Imaging, photography and observations of the slides were carried out with the aid of a light microscope [Olympus BX50, Olympus Optical Co. Ltd., Japan] fitted with a JVC K-F55B colour video camera [JVC Victor Company of Japan, Limited, Japan].

Scanning electron microscopy analysis of PLBs

The stomata behaviour between FA treated PLBs and the control were determined using scanning electron microscope (SEM) observation via the freeze drying method. The samples prepared were then viewed with the aid of a Leo Supra 50VP Field Emission scanning electron microscope [Carl Zeiss SMT, Germany]. Thirty (30) stomata were randomly chosen from the PLBs and mean width of stomata were recorded [CellSens Program]. Data was analysed using one-way ANOVA and differentiated with Tukey's test with the probability value set at 5 %.

DAMD-DNA analysis of PLBs

DNA extraction

For genomic DNA amplification and analysis, FA treated PLBs and the control were collected. DNA was extracted from these PLBs using Promega Wizard® Genomic DNA Purification Kit, based on the manufacturer's instructions.

Amplification reaction with DAMD primers

The treatments were examined using 19 primers based on the primer list reported by Bhattacharyya, Kumaria and Tandon (2015) and Devi *et al.* (2014). The primers were synthesised by Integrated DNA Technologies Company and is listed in Table 1.

PCR amplification was modified from a study by Zhou *et al.* (1997) using [BioRad T100] thermal cycler. The reaction mixture of 20 µL was prepared and contained 50 ng genomic DNA, 0.2 mM of dNTP mix [Next Gene Scientific], 2.5 mM $MgCl_2$, 1× PCR buffer, 1.0 U *Taq polymerase* [My TACG Bioscience Enterprise] and 0.3 mM of primers. PCR amplification was performed by initial denaturation at 94 °C for 2 min and 40 cycles of denaturation for 1 min at 92 °C, annealing for 2 min at 5 °C below each primer's melting temperature (TM), extension for 2 min at 72 °C and a final extension at 72 °C for 10 min.

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Table 1: List of DAMD primers

Primers	Sequence (5'-3')	G+C Content (%)	TM (°C)
M13	GAGGGTGGCGGCTCT	73.3	57.9
URP4R	AGGACTCGATAACAGGCTCC	55.0	56.1
6.2H (+)	AGGAGGAGGGGAAGG	66.7	52.4
URP6R	GGCAAGCTGGTGGGAGGTAC	65.0	60.6
URP17R	AATGTGGGCAAGCTGGTGGT	55.0	60.1
URP2F	GTGTGCGATCAGTTGCTGGG	60.0	59.1
HBV3	GGTGAAGCACAGGTG	60.0	50.0
YN73	CCCGTGGGGCCGCCG	93.3	67.2
URP9F	ATGTGTGCGATCAGTTGCTG	50.0	56.0
YNZ22	CTCTGGGTGTCGTGC	66.7	52.8
URP38F	AAGAGGCATTCTACCACCAC	50.0	54.5
HBV5	GGTGTAGAGAGGGGT	60.0	49.0
URP1F	ATCCAAGGTCCGAGACAACC	55.0	56.8
URP2R	CCCAGCAACTGATCGCACAC	60.0	59.1
URP13R	TACATCGCAAGTGACACAGG	50.0	54.9
URP32F	TACACGTCTCGATCTACAGG	50.0	53.0
URP25F	GGACAAGAAGAGGATGTGGA	50.0	53.9
URP30F	GGACAAGAAGAGGATGTGGA	50.0	53.9
6.2H (-)	CCCTCCTCCTCCTTC	66.7	50.4

Gel electrophoresis

DAMD bands were visualized in 1.5 % (w/v) agarose gel. The gel was casted in the 15 × 7 cm Mini Gel Caster [Bio-Rad Laboratories, Inc., USA]. 1.5 % agarose gel was prepared by dissolving 0.60 g agarose in 40 mL 1×Tris-Borate-EDTA (TBE) buffer using a microwave oven set at medium heating for four minutes. For electrophoresis, 3 µL of 1 kb marker [Thermo Fisher Scientific] and a mixture of 3 µL of the amplified DNA and 3 µL of 6×DNA loading dye (total 6 µL) were loaded into the wells.

A PowerPac™ Basic Power Supply [Bio-Rad Laboratories, Inc., USA] was attached to the electrophoresis system. The electrophoresis was allowed to run at 90 V for 90 minutes until the loading dye passed two-thirds of the gel's length. Then, the gel was viewed immediately under 302 nm UV transilluminator [Molecular Imager® Gel Doc™ XR+ System with Image Lab™ and Quantity One 1-D Analysis Softwares, Bio-Rad Laboratories, Inc., USA] for visualisation of the bands.

Determination of polymorphisms analysis

The determination of the DNA fragment patterns of each treated sample was performed by determining the similarity indices (SI) of the groups as compared to the untreated control. The visible bands were manually scored as 1 or 0 for the presence or absence of similar band. Similarity index was calculated according to Nei and Li (1979).

Leaf bridge bioassay

Three (3) months after the survived FA treated PLBs were transferred to MS medium, leaflets of size 1 to 1.5 cm were harvested. MS medium was filled into one of the two compartments in a two compartment Petri dish. Then, two (2) leaflets were placed far apart from each other on the medium. The petioles were embedded in the medium whereas; the leaves were raised above the partition and faced the other compartment of the Petri dish. The Petri dish was sealed with Parafilm with caution taken not to touch the leaflets with the lid of the Petri dish to avoid contamination. The incubated leaflets remained healthy for two weeks.

Spore inoculum of 10,000 spores/mL was prepared for *F. proliferatum* [VCG 1380] (Dehgahi *et al.*, 2016). The tip of the leaflets was wounded with a sterilised needle. Then, two (2) leaflets were inoculated by rubbing the conidial suspension on the tip of leaflet surfaces using a cotton bud. Control leaflets were not inoculated. Three replicates were conducted. Subsequently, the Petri dishes were sealed with Parafilm and kept in an incubator at 25 ± 2 °C with the photoperiod of 16 hours light and eight hours dark. The same procedure was applied for *F. oxysporum*. After four weeks, infected regions of leaflets were indicated by discolouration of the diseased tissues, beginning at the inoculation point was recorded. The leaflets were divided into five sections along the midrib. Each leaf section was numbered in increasing order from the leaf apex to the base and disease progress was scored using a 0 to 5 scale according to the absence or presence of disease symptom on each section (Mohd Pu'ad, 1999). Leaflets were deemed as resistant, intermediate and susceptible with scores of 0 to 2, 2 to 3 and more than 3, respectively.

RESULTS AND DISCUSSION

At the end of the second selection cycle, all the untreated controls survived. There was a slight increase in survival rate from 0.05 mM to 0.10 mM, but as the concentration of FA increased, it continued to decline until only 8.3 % of PLBs survived when treated with 0.20 mM. The highest survival rate was observed in TCL of PLBs treated with 0.10 mM where 53.3 % of the PLBs survived (Figure 1). PLBs treated with 0.05 to 0.20 mM FA also produced shoots. Therefore, this study indicated that higher concentrations of FA increased mortality of PLBs and reduced their growth compared to untreated FA control or lower concentrations treated PLBs. According to Singh and Upadhyay (2014) most studies claimed that FA only shows toxic effects at concentration greater than 0.10 mM. For example, Dehgahi *et al.* (2016) stated that survival rate for PLBs of *Dendrobium* sonia-28 at 0.05 mM FA was 20 % but only 1 % of PLBs survived at 0.20 mM FA.

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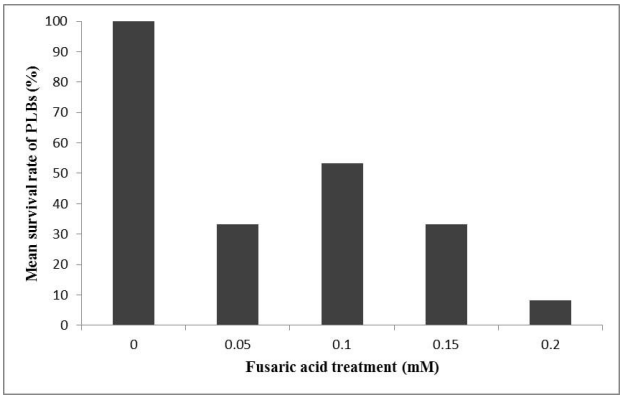
Figure 1: Effect of various concentrations of FA on the survival rate of *Dendrobium* hybrid PLBs at eighth week.



Moreover, histological observation and SEM analyses confirmed cell wall damage, stomatal closure and disruption of cell organelles in PLBs tested with higher FA concentrations. Histology analysis of control PLB cells was octahedral in shape and the cell walls were intact. There was also presence of meristematic cells, which indicates that the cells are actively dividing. In PLBs treated with 0.05 mM FA, even though there were some plasmolysed cells, most of the cells appear intact. There was also presence of meristematic cells with intact cell wall. In PLBs treated with 0.10 and 0.15 mM, some of the cells were ruptured. Cells in the meristematic region were plasmolysed and appeared densely stained because of the accumulation of cellular materials from the broken down cell membranes. The most severe damage was observed in PLBs treated with 0.20 mM FA where most part of the cell had ruptured and plasmolysed. The densely stained area showed almost no intact cells and accumulation of cellular material from broken down cell membranes were obvious (Figure 2a-e). According to Tai and Xu (2006), the ruptured plasma membrane causes decrease in membrane potential and the effect was more apparent with increasing concentrations and duration of exposure to FA. The changes in the membrane potential inhibit the uptake of nutrients and water by plants treated with FA.

The scanning electron microscopy analysis showed that the control PLBs had dense and compact cells. PLBs treated with 0.05 and 0.10 mM FA had compact and had larger cell size which indicates dividing cells. The damage on cell surface was only apparent when the PLBs were treated with 0.15 and 0.20 mM FA. The cells had clearly shrunken at these concentrations (Figure 3a-e). Besides, statistical analysis of mean stomata aperture of 30 stomata of PLBs proved that there was a definite decrease in the width of stomata aperture of FA treated PLBs except in PLBs treated with 0.05 mM FA (Table 2). Wu *et a*l. (2008) stated that one of the effects of FA on plant cells is decrease in cell division. According to Diniz and Oliveira (2009), a longitudinal cut of the apex or meristematic region of corn roots showed a progressive decrease of cellular division at 1.0 mM fusaric acid concentration. At 2.0 and 5.0 mM fusaric acid, the apex region was completely destroyed.

Table 2: Effect of various concentrations of FA on mean stomatal aperture (µm) of *Dendrobium* hybrid PLBs

Concentration of FA (mM)	Mean stomata aperture (µm)
Control	12.31 ± 4.97^{bc}
0.05	13.98 ± 5.25^{c}
0.10	7.73 ± 4.08^{a}
0.15	9.57 ± 3.73^{ab}
0.20	8.95 ± 5.37^{ab}

Letters a-c represents significant differences using Tukey's multiple range test at 5 % significance level.

DAMD analysis revealed mutant PLBs after FA inoculation compare to untreated PLBs. There were appreciable variations in plantlet structure and morphology relative to increasing FA concentration. Based on the results obtained by using 19 DAMD primers (Table 1), only 10 primers generated reproducible and clear bands from the FA treated and control PLBs (Table 3). This results in generating a total of 308 bands in the entire DAMD analysis. All the treated PLBs produced polymorphic bands. Total number of polymorphic bands is 123 bands (40 %). Similarity index (SI) for control and FA treated plants ranged from 0 to 1.0. The treatment which was completely monomorphic to the control was PLBs treated with 0.05 mM FA and obtained with primer URP13R. However, the PLBs treated with 0.05 mM FA also showed total polymorphism compared to the control with primer URP2F. This is due to each primer generated a unique set of amplification products and caused higher polymorphisms compared to others. The highest similarity index for treated PLBs were 1.00 (URP13R), 0.91 (URP13R), 0.83 (URP6R and URP13R) and 0.94 (HBV3) for PLBs treated with 0.05, 0.10, 0.15 and 0.20 mM FA respectively. Meanwhile, the lowest similarity index for treated PLBs was 0 (URP2F), 0.35 (URP2F), 0.22 (HBV3) and 0.20 (URP32F) for PLBs treated with 0.05, 0.10, 0.15 and 0.20 mM FA respectively. Nasir and Riazuddin (2008) stated that *Fusarium*-resistance in gladiolus plants cells which regenerated from cells *in vitro* was supported by polymorphism observed in resistant plants. Alteration of DNA could be induced by the reaction of reactive oxygen species with various cellular targets (Hossain *et al.*, 2007).

Disease symptoms in FA treated leaflets were high in the leaflets when inoculated with both *Fusarium* spp. There were no resistant FA treated leaflets towards these fungi produced. Only leaflets treated with 0.05 mM FA showed an intermediate tolerance towards both these fungi (Tables 4 and 5). Defence against FA by plant tissue is more vigorous in *Fusarium* resistant cultivars compared to Fusarium susceptible cultivars. This was demonstrated in *Solanum lycopersicum*, *Pisum sativum*, and *Gossypium herbaceum* which have different degrees of resistance to FA due to different detoxification abilities of the plants (Curir *et al.*, 2000).

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Figure 2: Cell condition of *Dendrobium* hybrid PLBs untreated and treated with FA (scale bar= 20 µm); **A** (Control), **B** (PLB treated with 0.05mM FA), **C** (PLB treated with 0.10 mM FA), **D** (PLB treated with 0.15 mM FA), and **E** (PLB treated with 0.20 mM FA), ic (intact cells), dc (dense cellular material).

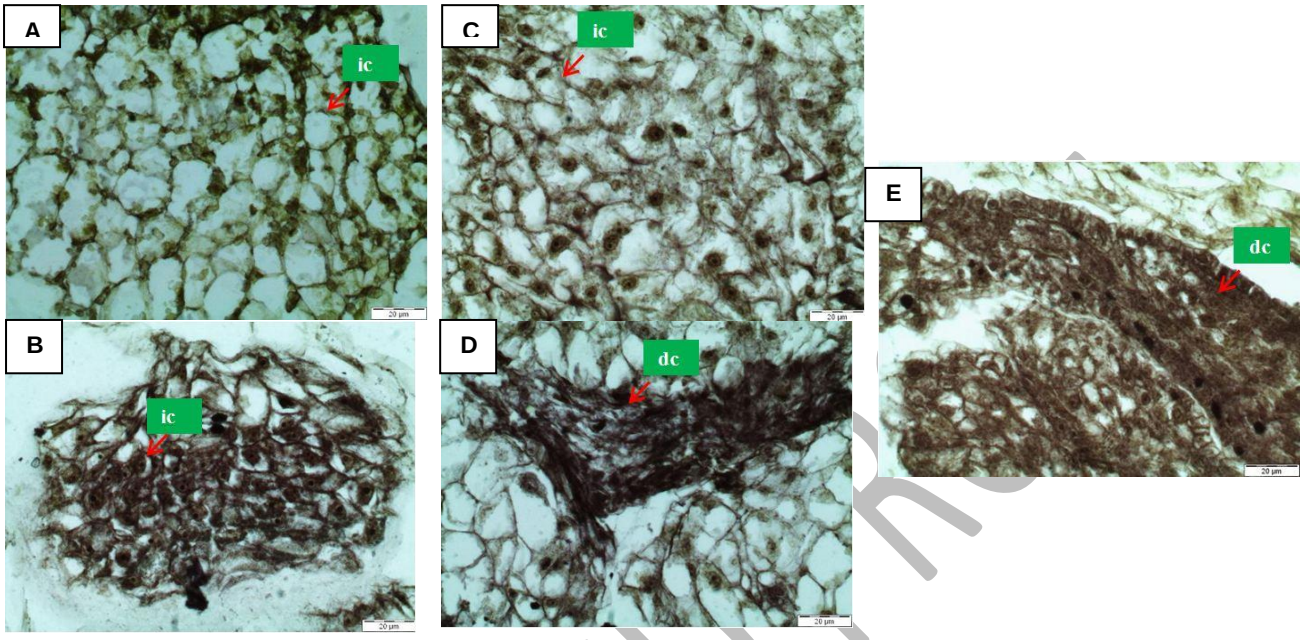

Figure 3: Stomata of FA treated *Dendrobium* hybrid PLBs (scale bar= 1.00 K×). **A** (Control), **B** (PLB treated with 0.05mM FA), **C** (PLB treated with 0.10 mM FA), **D** (PLB treated with 0.15 mM FA), and **E (**PLB treated with 0.20 mM FA).

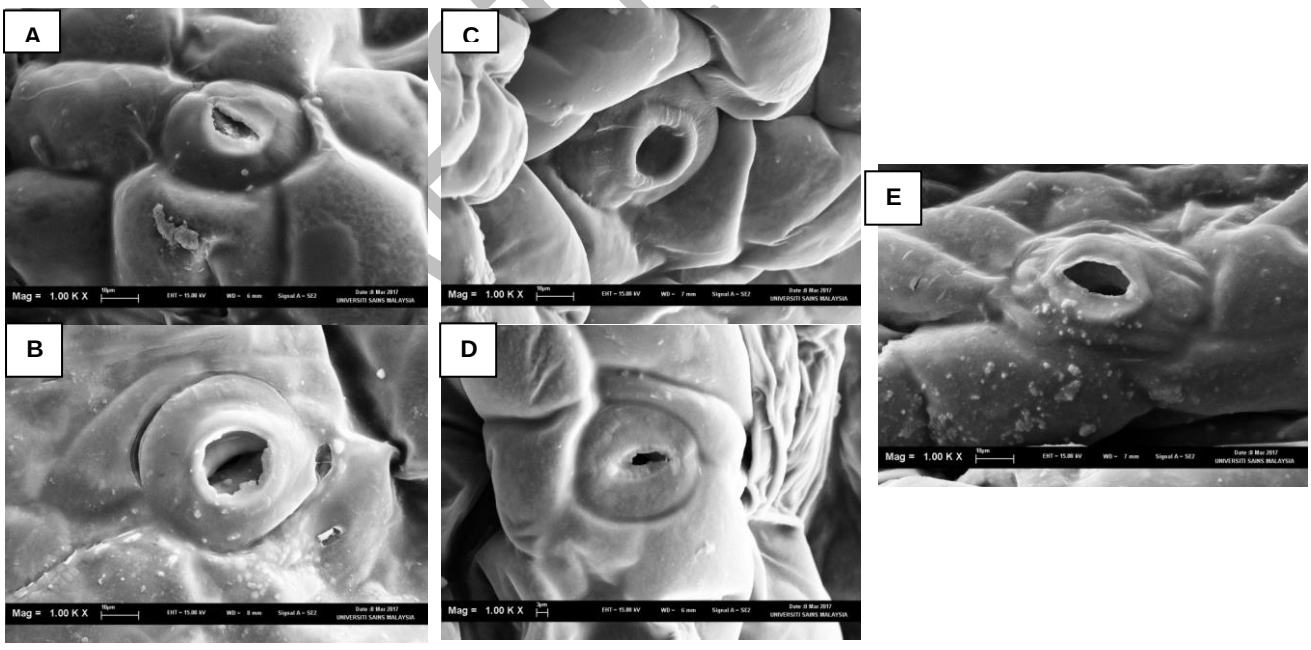

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Table 3: DAMD banding profiles of DNA samples obtained from FA treated PLBs

Primer	SI for control & 0.05 mM FA treated PLBs	SI for control & 0.10 mM FA treated PLBs	SI for control & 0.15 mM FA treated PLBs	SI for control & 0.20 mM FA treated PLBs
M13	0.80	0.8	0.73	0.75
6.2H (+)	0.70	0.75	0.71	0.67
URP6R	0.83	0.83	0.83	0.67
URP2F	0	0.35	0.70	0.50
HBV3	0.77	0.88	0.22	0.94
HBV5	0.67	0.86	0.71	0.77
URP13R	1.00	0.91	0.83	0.67
URP32F	0.50	0.40	0.25	0.20
URP30F	0.40	0.40	0.40	0.25
6.2H (-)	0.67	0.67	0.80	0.92

Table 4: Response of regenerated leaves from FA treated PLBs against *F. proliferatum* spore suspension (%).

Percentage of susceptibility response of regenerated leaves when tested with *F. proliferatum* spore suspension (%)			
FA concentration (mM)	Resistant	Intermediate	Susceptible
Control	0	0	100.0
0.05	0	33.3	66.7
0.10	0	0	100.0
0.15	0	0	100.0
0.20	0	0	100.0

Table 5: Response of regenerated leaves from FA treated PLBs against *F. oxysporum* spore suspension (%).

Percentage of susceptibility response of regenerated leaves when tested with *F. oxysporum* spore suspension (%)			
FA concentration (mM)	Resistant	Intermediate	Susceptible
Control	0	0	100.0
0.05	0	50.0	50.0
0.10	0	0	100.0
0.15	0	0	100.0
0.20	0	0	100.0

CONCLUSION

In this research, *in vitro* selection of disease resistant *Dendrobium* orchid PLBs using different concentrations of FA was determined and 0.05 mM FA was confirmed to be the most optimum concentration of FA in this study as PLBs treated in this concentration of FA showed least damage to cell and stomata and most resistance to the *Fusarium* species.. PLBs treated with 0 to 0.20 mM FA showed decline in survival and shoot regeneration rate as the concentration of FA increased. The highest survival rate was obtained in PLBs treated with 0.10 mM FA. Besides, histology analysis proved that severe changes in the cell morphology have occurred when the PLBs were treated with FA. These damages in the cell influence many processes and functions which are vital for plant survival. Evidence from SEM analysis also verified the significance of damage caused by high concentration of FA to the PLBs. The changes in surface morphology of the PLBs and decrease in the width of stomatal aperture could be related to the decline in survival rate of these PLBs. DAMD analysis showed that there was a high degree of polymorphism when the PLBs were treated with high concentration of FA. Apart from that, it was found that leaflets regenerated from FA treated PLBs showed intermediate resistance towards these fungi.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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AUTHORS' CONTRIBUTION STATEMENT

NMIMN, LZ and SS provided the materials and ideas for the study. SSS designed and carried out the experiments and wrote the article.

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