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Stress Effect and Development of Leaf-Bridge Bioassay of Dendrobium Sonia-28 Orchid Plantlets Resistant to Fusarium Proliferatum

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Abstract

Among horticultural and floral crops, orchids are outstanding in their diversity of shape, form and colour. *Dendrobium* sonia-28 is an important orchid hybrid in the Malaysian flower industry, but suffers from fungal diseases especially those caused by *Fusarium proliferatum*. Protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 were subjected to various doses of *Fusarium proliferatum* culture filtrate (CF), fusaric acid (FA) and gamma irradiation. Results showed that PLBs survival rate were inversely related to the treatment doses as well as experience time. Another main objective of this study was to evaluate degree of resistance among *Dendrobium* sonia-28 plantlets derived from PLBs treated with CF, FA and gamma irradiation methods. Plantlets obtained from the various treated PLBs were inoculated with a pathogen spore suspension of 10,000 spore/mL. Leaf-bridge bioassay results revealed that leaflets challenged with higher concentrations of CF, FA and gamma irradiation showed less disease symptoms after treatment, indicating that there is a strong relationship between *in vivo* and *in vitro* resistance of *Dendrobium* sonia-28 to infection to *Fusarium proliferatum*.

Keywords: Leaf bridge bioassay; *Dendrobium* sonia-28; *Fusarium proliferatum* culture filtrate; Fusaric acid; Gamma irradiation

Abbreviations: CF: Culture Filtrate; FA: Fusaric Acid; MS: Murashige & Skoog; PLBs: Protocorm-Like Bodies; Spore/mL: Spore per milliliter

Introduction

Orchids are one of the most important ornamental plants, occupying top position among all flowering plants in terms of economic value for cut flower production and potted plants [1,2]. Frenetic production of orchids increased by up to 15, 20 and 30% in 2006, 2007 and 2008, respectively [3]. *Dendrobium* sonia-28 is an important orchid hybrid in the Malaysian flower industry due to its flowering recurrence and dense inflorescences, but serious production problems are being encountered due to fungal diseases especially those caused by *Fusarium proliferatum* [4,5].

Fusarium is one of the major plant pathogenic fungi, which influences agricultural economics worldwide and contains more than one hundred species with a notable impact on the growth of various plants [6,7]. The difficulty in controlling Fusarium wilt has stimulated research into its biological control, enhanced by concerns for environmental protection [8,9]. Mutation, somaclonal variation and *in vitro* selection technologies offer opportunities to enhance genetic variability to improve agronomic traits such as disease resistance and crop yield against Fusarium wilt fungi, which may be better than conventional selective breeding methods, chemical application or plant breeding technologies [10].

Growing resistant plants is one of the cheapest and most efficient control measures of plant diseases [11]. Identification of resistant plants against *Fusarium* is a very efficient step in obtaining resistant plants to the pathogen [11,12].

Fusarium screening is an important means of selecting plants resistant to Fusarium wilt [13]. Reducing plant susceptibility to purified toxins or pathogen culture filtrate (CF) containing toxins may lead to decreased disease damage [14]. This may be achieved by employing these compounds in plant selection protocols that permit fungal growth without toxin-inflicted damage (tolerance) or those that inhibit proliferation of the fungus (resistance) [15].

In vitro selection relies on the availability of an efficient plant regeneration system, selective agents for selecting plant cells and *in vitro* cultured tissues, and a positive correlation between phenotypic expression at cellular and the whole plant levels [16]. Resistance in plants derived from *in vitro* selection based on the use of a purified toxin or CF is not always stable [17]. Resistance is a quantitative character and through selection, obtaining of more resistant varieties is possible [18].

As reported by Encheva, the stability of induced mutation in agronomic characters needs to be confirmed in the next generation [19]. Mutagenesis, physical or chemical was effective for induction of mutations in tissue cultures. It was established that induced mutagenesis is more effective when using embryos at an early stage of development as compared with air dry seeds [20]. *In vitro* selected variants should be finally field-tested to confirm genetic stability of the selected trait [21-23]. Recent studies have shown that there are some positive relationships between the pathogenicity of *Fusarium* and pathogenesis processes for plants grown under *in vivo* and *in vitro* conditions [24].

Here, we aimed to evaluate effect of CF, FA and gamma irradiation doses and experimental duration on *Dendrobium* sonia-28 PLBs survival rate as well as establish leaf bridge assay technique and evaluate disease resistance of *Dendrobium* sonia-28 plantlets obtained from treated PLBs using *Fusarium proliferatum* inoculation under *in vitro* condition.

Materials and Methods

In vitro propagation of PLBs

In vitro cultures of *Dendrobium* sonia-28 PLBs were used for this *in vitro* selection study. The PLBs were cultured and maintained in

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semi-solid half-strength MS medium conditions [25] supplemented with 2% (w/v) sucrose, 2.75 g/L GelriteTM (Duchefa, the Netherlands) and 1mg/L of benzylaminopurine (BAP; Duchefa, the Netherlands). The cultures were incubated at 25°C ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Philips TLD, 36W, 150 $\mu mol.m^{-2}/s^{-1}$). The pH value (CyberScan PC 510 pH/mV/Conductivity/TDS/°C/°F Bench Meter, Eutech Instruments, Singapore) was adjusted to 5.7-5.8 before autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan). The PLBs were then subcultured every four weeks.

CF treatment of *Dendrobium* sonia-28 PLBs

The method reported by Tripathi et al. was used for the preparation of *Fusarium proliferatum* CF. Fungal colonies of *Fusarium proliferatum* were transferred to 5 cm-diameter Petri dishes containing potato dextrose agar (PDA) and maintained in the dark at room temperature (27°C to 30°C) to establish pure cultures conditions [26]. Mycelium-covered cultures grown on PDA were sliced after one month. Sliced discs with the size of approximately 5 mm were transferred to fresh PDA using sterile no. 2 cork borer and maintained in the dark condition at room temperature. The contents were transferred to fresh media for two more times and maintained at the same condition. Ten to fifteen pieces of PDA containing mycelial were inoculated in 50 mL of liquid MS medium in 250 mL Erlenmeyer flask in order to maintain and provide nutrients. The Erlenmeyer flasks inoculated with *Fusarium proliferatum* were incubated at room temperature (27°C to 30°C) under dim light (40 $\mu mol.m^{-2}.s^{-1}$). After fifteen days, 50 mL liquid MS medium was divided into five equal parts to maintain fungal medium in uncrowded place and maintained under dim light at room temperature. Small mycelium balls were emerged in the mentioned medium after six weeks. Suspension then passed through filter paper number one (Whatman) followed by centrifugation at 10,000xg for 20 minutes. Supernatant was then sterilized via nitrocellulose membrane filter (0.22 µm) and stored at -20°C (Figure 1).

One hundred (100) PLBs (10 petri dish, each petri dish consisting 10 PLBs) from each of the two size ranges (1 mm to 2 mm and 3 mm to 4 mm) were inoculated on MS medium containing 5, 10, 15 and 20% CF and incubated for four weeks under a 16-hour photoperiod using cool white fluorescent lamps at 25°C ± 2°C. One hundred (100) additional PLBs (control) from each of the two size ranges were cultured in Petri dishes, and incubated under the same conditions.

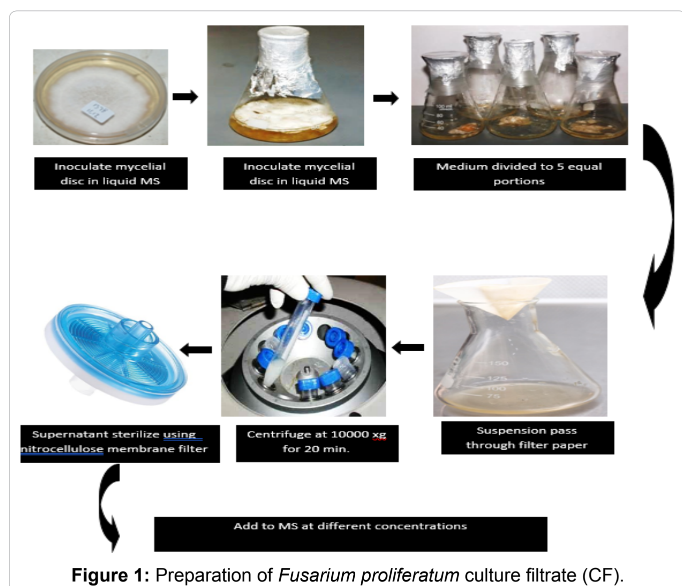

Figure 1: Preparation of *Fusarium proliferatum* culture filtrate (CF).

FA treatment of *Dendrobium* sonia-28 PLBs

PLBs in the size range of 1 mm to 2 mm and 3 mm to 4 mm were inoculated with different concentrations of FA (0, 0.05, 0.10, 0.15 and 0.20 mM). PLB samples incubated under above mentioned conditions.

Selection of CF and FA-tolerant PLBs

PLBs surviving post CF and FA treatments after four weeks and that were green in appearance were selected and subsequently transferred to MS medium supplemented with the same concentrations for additional treatment cycle (four-week) to produce resistant PLBs. At the end of the selection process, PLB's survivability was scored based on colour of PLBs. Survived PLBs remained green, while dead PLBs turned dark brown or white without green patches. The survival percentage of PLBs after selection process was calculated as follow:

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

For further propagation of tolerant PLBs and produce enough material for further experiments, survived PLBs were multiplied on MS medium in the absence of CF and FA.

Gamma radiation on *Dendrobium* sonia-28 PLBs

PLBs (3 mm to 5 mm) were irradiated with different doses of 0, 10, 20, 30, 40, 50, 60, 80, 100, 150 and 200 Gy. PLBs then transferred in new MS media and incubated under the same conditions. The first vegetative generation in which treatment was performed was referred to as M1V1. The survival percentage of irradiated PLBs was scored based on above mentioned formula. The study continued until the fourth generation (M1V4) to see the stability of the induced traits before selecting as mutated plants.

Evaluation of treated plantlets disease resistance

The leaf-bridge bioassay is a simple and easy to set-up method for testing plants against pathogens of interest in the laboratory. It is particularly useful if plant materials are limited and field trial is not possible conditions [27]. In this study, leaf-bridge bioassay was conducted with some modifications according to Abdullah conditions [27].

Evaluation of leaflet size and spore concentration suitable for disease expression: Leaflets of one (1) year-old *Dendrobium* sonia-28 plantlets grown from PLBs treated or untreated under *in vitro* selection were used for leaf-bridge assay. Firstly, experiments were carried out to ascertain the appropriate spore concentration and size of leaflets suitable for disease expression. The optimized leaflet length and spore concentration was then used for CF, FA and gamma irradiation-treated plantlets in a completely randomized design.

In order to challenge the leaflets with an appropriate pathogen density, the spore preparation was diluted to give a series of different concentrations of pathogen spores ranging from 10, 100, 1,000 and 10,000 spores per milliliter (spores/mL). The number of spore/mL of these suspensions was calculated using a haemacytometer. Ninety (90) plantlets of *Dendrobium* sonia-28 were tested for expression of disease symptoms, and then each concentration of pathogen was inoculated onto leaflets of differing group lengths which mostly can be found (1 cm to 1.5 cm, 1.5 cm to 2 cm, 2 cm to 2.5 cm), with ten replicates for each. Disease development and severity were monitored and measured over four weeks.

Establishment of the leaf-bridge assay: Leaflets were clean-cut with a sterile scalpel blade at the base of the petiole to avoid damaging the tissue according to Abdullah conditions [27]. Leaflets of identical size and position were taken from 1-year-old axenic *Dendrobium* sonia-28's plantlets growing in tissue culture. The sterile semi-solid half-strength MS-medium was poured into one compartment of the three-compartment Petri dish. Leaflets were arranged so that the end of the petiole was completely embedded in the solidified, half-strength MS media supplemented with 2% sucrose to maintain leaflet growth. Under these conditions, leaflets remained healthy for at least 3-4 weeks. The remaining portions of the leaflets were raised over the partition and were free from contact with the medium or plastic surfaces. This was important to avoid any growth and spread of pathogens between leaves and the plastic or agar surfaces. Pathogen inoculum in the form of fungal spore suspensions in one (1) mL were positioned at the end of the abaxial leaflet surfaces using cotton buds under laminar airflow in aseptic conditions. The arrangement of the remaining surfaces of the leaves in the leaf-bridge ensured that the pathogen entered the leaf tissues and progressed through the leaflets. Plates were sealed using parafilm.

Leaf-bridge bioassay of treated and untreated leaflets: Leaf-bridge bioassay was conducted according to Abdullah conditions [27]. Leaflets were carefully selected from a batch of various concentrations of CF, FA and gamma-irradiation treated and untreated plantlets (for each concentration, 20 leaflets) and positioned into the three-compartment Petri dish as described above. A completely randomized design was used for the plantlet bioassay using ten (10) Petri dish replicates for each treatment concentrations with each replicate containing two (2) leaflets. Petri dishes were sealed with parafilm and incubated in a refrigerated incubator at 15°C under an 18-h photoperiod (at 25 lE. m^{-2} s^{-1}).

After four weeks, untreated control leaflets with the size of 1 cm to 1.5 cm inoculated with 10,000 spore/mL displayed 99% disease incidence compared to 84% and 62% disease incidence in leaflets with the size of 1.5 cm to 2 cm and 2 cm to 2.5 cm, respectively which inoculated with 10,000 spore suspension. However, lower spore suspension showed less disease symptoms. Then leaflet size of 1 cm to 1.5 cm and spore suspension of 10,000 spore/mL have been selected for evaluation of disease resistance in treated leaflets. Therefore, two (2) treated leaflets (1 cm to 1.5 cm) were inoculated by rubbing the conidial suspension of 10,000 spore/mL on the tip of leaflet surfaces. Some leaflets were not inoculated and some were rubbed with distilled water to serve as positive and negative controls, respectively and to distinguish tolerance stability in treated plantlets. Three-compartment Petri dishes has been used for *Dendrobium* sonia-28 bioassay. Petri dishes were sealed with parafilm and incubated under the conditions as been described above. The appearance of disease symptoms was observed daily and the progress of infection was estimated every seven days for one month, after which all control plantlets showed full disease symptoms.

Evaluation of disease symptoms: Individual leaflets were inoculated at the tip with spore suspension and then incubated for disease development according to Abdullah conditions [27]. Disease progress was monitored by visual observation. Infected regions of leaflets were indicated by discolouration of the diseased tissues, beginning at the inoculation point, over the incubation period of 28 days. Observations were taken at approximately seven day intervals (7, 14, 21 and 28) of each leaflet to monitor the progress of disease symptoms and to measure the relative infected leaflet area.

Monitoring of disease progress in leaflets infected with fungal pathogens was done by dividing infected leaflets into five sections along the midrib and recording presence of disease symptoms for each section. Each leaf section was numbered to facilitate measurement of disease progress. Disease progress was scored using a 0-5 scale according to the absence or presence of fungal growth on each section, representing the linear progress of disease symptoms towards the inoculated end of the leaflets. Leaflets were deemed tolerant, intermediate and susceptible with scores of 0-2, 2-3 and >3, respectively.

Disease symptoms on leaflets were observed within 28 days of inoculation and the numbers of leaflets showing disease symptoms were recorded after the inoculation period. The final evaluation in the fourth week was based on the number of sections presenting fungal growth on each leaflet.

Disease incidence was calculated using the following formula conditions [27]**:**

$$\text{Disease Incidence} = \frac{\text{Number of leaflet sections with disease symptoms}}{\text{Total number of leaflet sections challenged}} \times 100$$

Statistical analysis

Statistical analysis was performed using predictive analytic software SPSS (SPSS 16.0, IBM, US). Data collected were analyzed for the variant using one-way and two-way ANOVA and the differences were contrasted using Tukey's multiple range test at 5% significance level.

Results

Effect of CF concentration on PLBs survival rate

CF at various concentrations showed a significant effect on survival rates of PLBs at both size ranges. Lower post-CF survival was obtained when PLBs were treated with higher concentrations of CF at the end of experiment. Mean survival rate percentage of untreated PLBs of 1 mm to 2 mm and 3 mm to 4 mm size ranges were 97% and 99%, respectively.

PLBs in the size range of 1 mm to 2 mm had 19, 14, 8 and 2% survival percentages rate at CF concentrations of 5, 10, 15 and 20%, respectively (Table 1). PLB size of 3 mm to 4 mm, displayed higher survival rate and optimum results compared to 1 mm to 2 mm, therefore chosen for future experiments. Results of Tukey's HSD test ($p<0.05$) indicated that CF concentrations showed more significant effects on PLBs survival rate compared to PLBs size (Table 1).

Effect of FA concentration on PLBs survival rate

Results indicated that at the end of experiment, mean survival rate percentage of untreated PLBs of the size of 3 mm to 4 mm and 1mm to 2 mm were 98% and 88% respectively. Increasing concentrations of FA significantly reduces the PLBs survivability for each PLB size. PLBs in the size range of 3 mm to 4 mm resulted 20, 11, 5 and 3% survival percentages rate at FA concentrations of 0.05, 0.1, 0.15 and 0.2 mM, respectively (Table 2). Results of Tukey's HSD test ($p<0.05$) indicated that FA concentrations showed more significant effects on PLBs survival rate compared to PLBs size (Table 2). Based on higher survival rate of treated PLBs with the size of 3 mm to 4 mm, this size was found optimal in order to obtain reproducible results compare to the size of 1 mm to 2 mm. PLBs resumed growth and proliferated at optimum rate once maintained in a medium devoid of FA.

Effects of different gamma radiation doses on PLBs

Irradiation with doses of 10 to 200 Gy produced significant effects

A				
CF (%)	**First week**	**Second week**	**Third week**	**Fourth week**
Control	100 ± 0.000^{a}	99 ± 3.077^{a}	99 ± 3.078^{a}	98 ± 4.104^{a}
5	33.5 ± 7.451^{b}	29 ± 8.522^{b}	25 ± 8.271^{b}	21 ± 11.821^{b}
10	32 ± 8.944^{b}	28 ± 10.340^{b}	23 ± 11.286^{b}	20 ± 13.377^{b}
15	26 ± 8.207^{c}	20 ± 9.177^{c}	14.5 ± 9.445^{c}	11.5 ± 10.340^{c}
20	19.5 ± 7.591^{d}	16 ± 5.871^{c}	10 ± 8.870^{c}	7 ± 8.645^{c}

B				
size	**First week**	**Second week**	**Third week**	**Fourth week**
(1-2) mm	39 ± 31.574	34.8 ± 32.654	30.6 ±35.074	28 ± 35.743
(3-4) mm	45.4 ± 28.941	42.4 ± 30.543	38.2 ± 32.744	35.2 ± 34.714

C					
CF Treatment		**Mean PLBs survival rate (%)**			
		First selection cycle		**Second selection cycle**	
		First two week	**Second two week**	**First two week**	**Second two week**
0	(1-2) mm	100 ± 0.000^{a}	98 ± 4.216^{a}	98 ± 4.216^{a}	97 ± 4.830^{a}
	(3-4) mm	100 ± 0.000^{a}	100 ± 0.000^{a}	100 ± 0.000^{a}	99 ± 3.162^{a}
5%	(1-2) mm	31 ± 5.676^{cd}	26 ± 5.164^{cd}	23 ± 9.486^{bc}	19 ± 7.378^{bc}
	(3-4) mm	36 ± 8.433^{bc}	32 ± 10.327^{bc}	27 ± 6.749^{b}	24 ± 15.055^{b}
10%	(1-2) mm	26 ± 5.163^{de}	22 ± 6.324^{de}	16 ± 5.164^{cde}	14 ± 5.164^{cd}
	(3-4) mm	38 ±7.888^{b}	35 ± 9.718^{b}	30 ± 11.547^{b}	26 ± 16.465^{b}
15%	(1-2) mm	23 ± 4.830^{e}	14 ± 5.164^{f}	10 ± 6.666^{ef}	8 ± 6.324^{de}
	(3-4) mm	29 ± 9.944^{de}	26 ± 8.433^{cd}	19 ± 9.944^{cd}	15 ± 12.692^{cd}
20%	(1-2) mm	15 ± 5.270^{f}	14 ± 5.164^{f}	6 ± 5.164^{f}	2 ± 4.216^{e}
	(3-4) mm	24 ± 6.699^{e}	19 ± 5.676^{ef}	15 ± 9.718^{de}	12 ± 9.189^{cd}

Table 1 (a,b,c): Effect of various concentrations of CF on the survival rate of *Dendrobium* sonia-28 orchid PLBs after two selection cycles. Letters a-f represent significant differences at p<0.05.

A				
FA conc. (mM)	**First week**	**Second week**	**Third week**	**Fourth week**
0	98.5 ± 3.663^{a}	97.5 ± 5.501^{a}	95 ± 6.882^{a}	93 ± 8.013^{a}
0.05	40 ± 22.118^{b}	30 ±13.765^{b}	26.5 ± 14.965^{b}	16 ± 9.947^{b}
0.1	24 ± 13.945^{c}	21.5 ± 9.333^{c}	17 ± 9.233^{c}	8.5 ± 8.750^{c}
0.15	20.5 ± 19.050^{c}	15 ± 14.680^{d}	13 ± 11.286^{c}	4 ± 5.982^{d}
0.2	10.5 ± 8.870^{d}	8 ± 6.156^{e}	5.5 ± 6.048^{d}	1.5 ± 3.663^{d}

B				
size	**First week**	**Second week**	**Third week**	**Fourth week**
(1-2) mm	31 ± 34.061	28.6 ± 34.346	25 ± 34.061	21.8 ± 34.148
(3-4) mm	46.8 ± 34.013	40.4 ± 33.133	37.8 ± 33.399	27.4 ± 36.967

C					
FA Treatment		**Mean PLBs survival rate (%)**			
		First selection cycle		**Second selection cycle**	
		First two week	Second two week	First two week	Second two week
Control	(1-2) mm	97 ± 4.830^{a}	95 ± 7.071^{a}	91 ± 7.378^{b}	88 ± 7.888^{b}
	(3-4) mm	100 ± 0.000^{a}	100 ± 0.000^{a}	99 ± 3.162^{a}	98 ± 4.216^{a}
0.05 m M	(1-2) mm	21 ± 5.676d^{e}	19 ± 5.676^{d}	15 ± 5.270^{ef}	12 ± 6.324^{d}
	(3-4) mm	60 ± 12.472^{b}	41 ± 9.944^{b}	38 ± 12.293^{c}	20 ± 11.547^{c}
0.1mM	(1-2) mm	17 ± 6.749^{ef}	15 ± 5.270^{de}	11 ± 5.676^{fg}	6 ± 6.992^{def}
	(3-4) mm	32 ± 15.491^{c}	28 ± 7.888^{c}	23 ± 8.233^{d}	11 ± 9.944^{de}
0.15 mM	(1-2) mm	11 ± 5.676^{ef}	9 ± 5.676^{ef}	6 ± 5.164^{gh}	3 ± 4.830^{f}
	(3-4) mm	30 ± 23.094^{de}	22 ± 18.135^{cd}	20 ± 11.547^{de}	5 ± 7.071^{ef}
0.2mM	(1-2) mm	9 ± 5.676^{f}	5 ± 5.270^{f}	2 ± 4.216^{h}	0 ± 0.000^{f}
	(3-4) mm	12 ± 11.353^{ef}	11 ± 5.676^{ef}	9 ± 5.676^{fg}	3 ± 4.830^{f}

Table 2 (a,b,c): Effect of various concentrations of FA on the survival rate of *Dendrobium* sonia-28 orchid PLBs after two selection cycles. Letters a-h represent significant differences at p<0.05.

Gamma radiation (Gy)	**Mean of PLBs survival rate**			
	First week	**Second week**	**Third week**	**Fourth week**
0	100 ± 0.000^{a}	100 ± 0.000^{a}	99 ± 3.162^{a}	98 ± 4.216^{a}
10	96 ± 5.163^{a}	92 ± 4.216^{ab}	80 ± 10.540^{b}	84 ± 9.661^{b}
20	99 ± 3.162^{a}	85 ± 5.270^{b}	76 ± 16.465^{bc}	82 ± 9.189^{b}
30	94 ± 6.992^{a}	74 ± 8.433^{c}	67 ± 9.486^{bc}	59 ± 7.379^{c}
40	90 ± 14.142^{ab}	71 ± 13.703^{c}	67 ± 14.944^{bc}	45 ± 21.213^{d}
50	96 ± 5.164^{a}	72 ± 16.865^{c}	67 ± 21.628^{bc}	48 ± 13.984^{d}
60	94 ± 5.164^{a}	69 ± 8.756^{c}	65 ± 10.801^{c}	42 ± 9.189^{d}
80	93 ± 12.517^{ab}	67 ± 14.181^{c}	47 ± 9.489^{d}	26 ± 10.749^{e}
100	89 ± 14.491^{ab}	65 ± 12.693^{c}	39 ± 13.703^{de}	23 ± 16.363^{e}
150	92 ± 10.328^{ab}	45 ± 10.801^{d}	29 ±15.239^{ef}	17 ± 12.516^{ef}
200	83 ± 20.575^{b}	39 ± 7.379^{d}	20 ± 14.142^{f}	7 ± 4.830^{f}

Table 3: Effect of various doses of gamma irradiation on the survival rate of *Dendrobium* sonia-28 orchid PLBs within four weeks. Letters a-f represent significant differences at p<0.05.

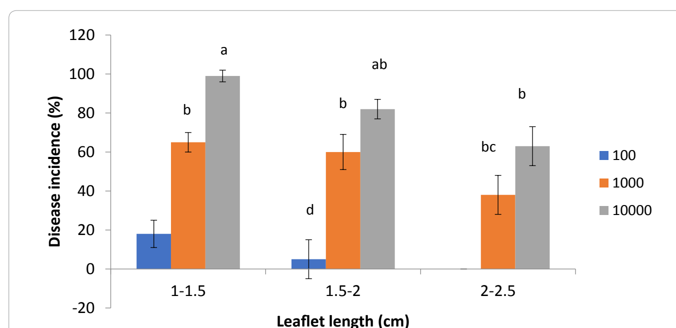

Figure 2: Occurrence of disease incidence after different concentrations of Fusarium proliferatum spore suspension in differing lengths of untreated Dendrobium sonia-28 leaflets within 28 days of incubation. Letters a-d represent significant differences at P<0.05 level.

on survival of PLBs after four weeks. Survival analyses demonstrated that treatment duration showed a significant effect on survival of PLBs. At the end of the experiment, there was a significant decrease of survival rate compare to first week. As per the results of the first week, the survival rate of PLBs irradiated at 10, 20, 30, 40, 50, 60, 80, 100, 150 and 200 Gy of gamma irradiation, were found to be 96, 99, 94, 90, 96, 94, 93, 89, 92 and 83% of survival rate percentage of control PLBs, respectively. Meanwhile, survival rate at the fourth week were recorded at 86, 84, 60, 46, 49, 43, 27, 23, 17 and 7% of survival rate in non-irradiated control PLBs, respectively (Table 3).

Effects of spore concentrations and different lengths of leaflets on disease symptom

Untreated leaflets of *Dendrobium* sonia-28 with different lengths were tested for symptom expression by treating with different concentrations of spore/mL. There were no detectable lesions with only a small and yellowish-brown discoloration at the inoculation point for leaflets infected with 100 spores per inoculation during the whole experimental period (Figure 2). Those treated with 1000 spore/mL produced mild symptom development relatively late in the experiment (e.g. between 2 and 3 weeks). While as early as 4 days, leaflets showed symptoms after inoculation with 10,000 spore/mL. These leaflets showed gradual chlorosis, turning yellow and wilting. Results obtained from this study demonstrated that the degree of disease symptoms differed depending on leaf size. Disease occurrence among leaflets of 1 cm to 1.5 cm challenged with 100, 1,000 and 10,000 spore/mL was 18%, 65% and 99%, respectively (Figure 2). Thus, a spore concentration

of 10,000 per mL seemed to be more effective to produce disease symptoms in leaflets of the range size of 1 cm to 1.5 cm.

Disease symptom levels on CF treated leaflets

Quantitative assessment of disease development as measured by the progress of discoloration of the CF-treated leaflets showed that the relationship between the severity of infection and incubation period was nearly linear when the 1 cm to 1.5 cm leaflets were challenged with 10,000 spore/mL for 30 days. The first visible lesions on the control leaflets appeared 4 days after inoculation, while 7, 9, 8 and 9 days were needed for the 5, 10, 15 and 20% treated plantlets, respectively. Disease symptoms in all CF-treated leaflets were reduced compared to the control, and those exposed to increasing CF concentrations shown milder disease symptoms (Figures 3 and 4).

Results indicated that the disease incidence percentage decreased down from 99 to 57, 52, 33 and 38% in leaflets obtained from 0, 5, 10, 15 and 20% treated CF PLBs, respectively (Figures 3 and 4). Further, the number of resistant plantlets regenerated from 5, 10, 15 and 20% CF-treated *in vitro*-selected PLBs ranged from 3, 4, 6 and 7, respectively (Table 4).

Disease symptom levels on FA-treated leaflets

The region of infected tissue was proportional to the incubation period. The first visible symptoms of spore inoculation appeared at day 4 (controls) and days 6, 6, 7 and 7 for plantlets obtained from 0.05, 0.1, 0.15 and 0.2 mM FA-treated PLBs, respectively. Disease incidence was 99, 36, 52, 48 and 19% for leaflets obtained from PLBs treated with 0, 0.05, 0.1, 0.15 and 0.2 mM FA, respectively (Figures 5 and 6). Resistant somaclones of FA-treated plantlets at concentrations of 0, 0.05, 0.1, 0.15 and 0.2 mM ranged from 0, 5, 4, 4 to 7, each out of ten screened plantlets, respectively (Table 4).

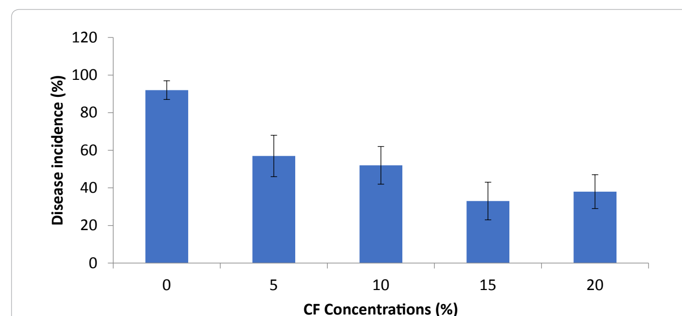

Figure 3: Disease incidence caused by Fusarium proliferatum spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of Dendrobium sonia-28 subjected to various concentrations of CF within 28 days of incubation. Letters a-b represent significant differences at P<0.05 level.

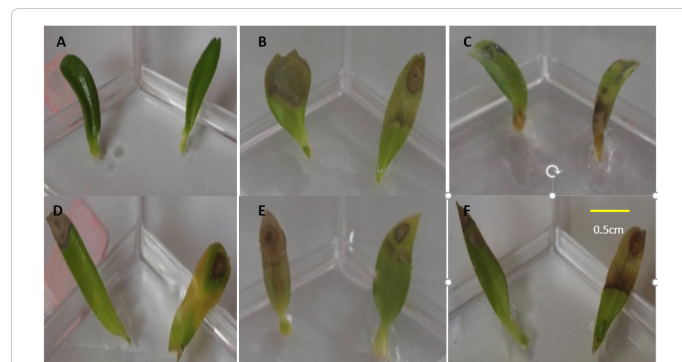

Figure 4: Disease incidence caused by Fusarium proliferatum spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of Dendrobium sonia-28 subjected to various concentrations of CF within 28 days of incubation. A (deionized H2O), B (non-inoculated), C (5% CF-treated leaflets), D (10% CF-treated leaflets), E (15% CF-treated leaflets), F (20% CF-treated leaflets).

Treatment	No. of plantlets screened	Resistant (0-2)	Intermediate (2-3)	Susceptible (>3)
Control	10	0	2	8
5% CF	10	3	2	5
10% CF	10	4	2	4
15% CF	10	6	2	2
20% CF	10	7	2	1
0.05 mM	10	5	3	2
0.1 mM	10	4	3	3
0.15 mM	10	4	4	2
0.2 mM	10	7	2	1
10 Gy	10	2	5	3
20 Gy	10	3	3	4
30 Gy	10	3	4	3
40 Gy	10	5	1	4
50 Gy	10	5	3	2
60 Gy	10	5	5	0
80 Gy	10	5	4	1
100 Gy	10	6	3	1
150 Gy	10	6	4	0
200 Gy	10	5	4	1

Table 4: Leaf-bridge assay analysis of somaclones divided into three disease reaction categories when inoculated with 10,000 spore/mL of *Fusarium proliferatum* for gamma irradiation treatment protocol.

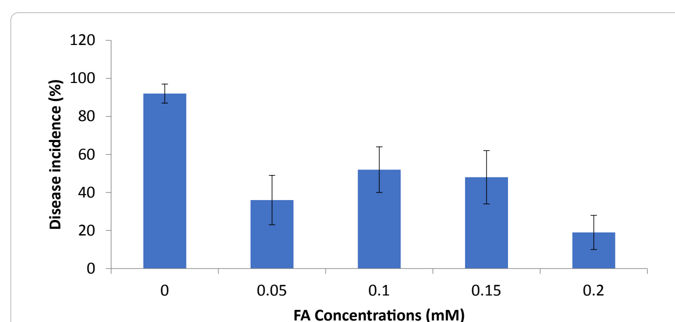

Figure 5: Disease incidence caused by *Fusarium proliferatum* spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of *Dendrobium* sonia-28 subjected to various concentrations of FA within 28 days of incubation. Letters a-d represent significant differences at P<0.05 level.

Disease symptom levels on gamma-irradiated leaflets

The first visible symptoms in leaflets obtained from irradiated PLBs normally arose between 4 to 8 days, depending on the gamma irradiation doses used. Our results revealed that at the end of the experiment, untreated leaflets presented 99% disease incidence, whereas disease incidence in leaflets obtained from 150 Gy-irradiated PLBs was 21% (Figures 7 and 8). Furthermore, results of Tukey's HSD (P<0.05) indicated that the difference in disease incidence values of gamma irradiated plantlets was statistically significant from un-irradiated plantlets (Figure 7). However, results indicated that different doses of irradiation were not correlated with disease incidence. Furthermore, plantlets obtained from irradiated PLBs showed higher levels of resistance than untreated plantlets (Table 4).

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Discussion

Exposure of sugarcane (*Saccharum* spp.) calli to *Fusarium sacchari* CF at either embryo maturation or germination stages resulted in callus necrosis which intensified with increasing CF levels in the media. When the embryogenic calli were subjected to CF stress by four weeks with nearly 100% of calli on all tested CF treatments shown necrotic symptom [14]. Similarly, Modgil et al. stated that different concentrations of fungal CF of *Dematophora necatrix* on calli and regenerates of apple rootstock Malling 7 were effective for the screening of potential induced resistant plantlets [28]. Frequency of surviving callus decreased with increasing CF concentration, and survived regenerates decreased more after six weeks of inoculation compare to week 2 and 4 post inoculation. Zhang et al. also reported that callus induction of garlic plants showed significant decreases after inoculation with *Sclerotium cepivorum* CF [29]. They also demonstrated that if pathogen CF concentration increased, then the garlic callus survival rate significantly reduced. Dallagnol et al. stated that brown spot severity caused by *Bipolaris oryzae* infection on rice leaves increased overtime (0-144 hours after infection) [30]. Inoculation with *Pythium ultimum* led to a steady increase in mortality of *Picea abies* seedlings and resulting in full loss of the seedling population 17 days after inoculation. Inoculation with less virulent pathogen led to a mortality about 30% of the seedlings [31]. Bani et al. also reported that *Fusarium oxysporum* CF displayed disease symptoms in pea cultivars, which disease severity increases after 48 hours compare to 24 hours' post inoculation [32]. Dori et al. stated that cell wall fragments, fungal enzymes and elicitors presented in spore free CF may cause some damages in plant cells [33]. Tripathi et al. inoculated banana plants with bacterial suspensions of *Xanthomonas*, and results showed that the symptoms produced in plants were very similar to the *in vitro* screening tests [26]. Furthermore, cultivars tested using *in vivo* plants were classified into the same groups of resistances as previously using *in vitro* plantlets.

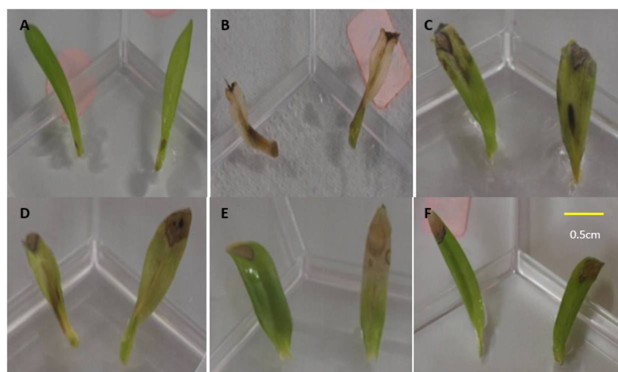

Figure 6: Disease incidence caused by *Fusarium proliferatum* spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of *Dendrobium* sonia-28 subjected to various concentrations of FA within 28 days of incubation. A (treated with deionized H_2O), B (non-inoculated), C (0.05 mM FA-treated leaflets), D (0.1 mM FA-treated leaflets), E (0.15 mM FA-treated leaflets), F (0.2 mM FA-treated leaflets).

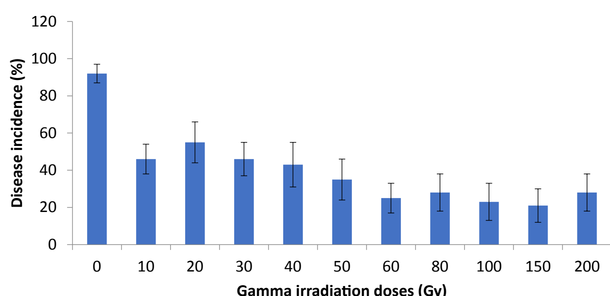

Figure 7: Disease incidence caused by *Fusarium proliferatum* spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of *Dendrobium* sonia-28 subjected to various doses of gamma irradiation within 28 days of incubation. Letters a-c represent significant differences at P<0.05 level.

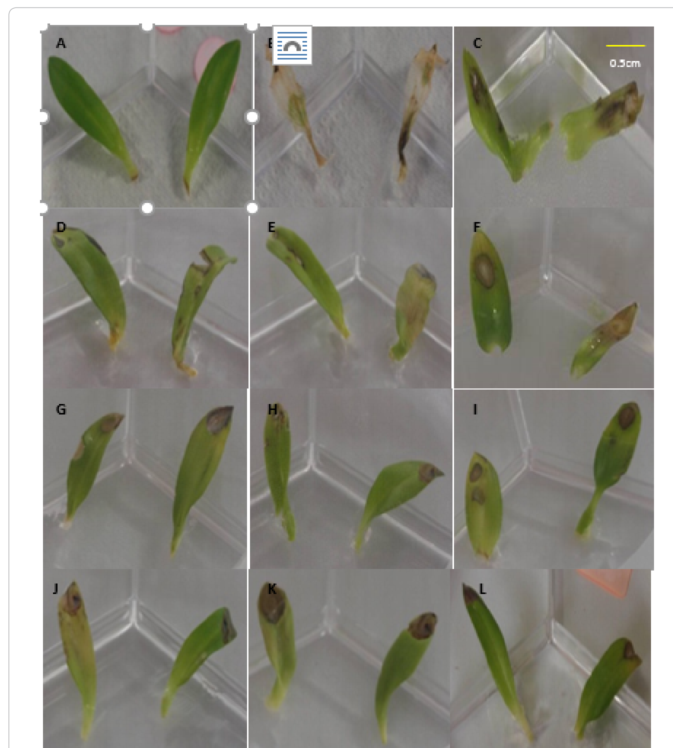

Figure 8: Disease incidence caused by *Fusarium proliferatum* spore suspension (10,000 spore/mL) from leaf-bridge assay observations of leaflets obtained from PLBs of *Dendrobium* sonia-28 treated with various doses of gamma irradiation within 28 days of incubation. A (treated with deionized H_2O), B (non-irradiated), C (10 Gy), D (20 Gy), E (30 Gy), F (40 Gy), G (50 Gy), H (60 Gy), I (80 Gy), J (100 Gy), K (150 Gy), L (200 Gy) treated leaflets.

Virk and Nagpal inoculated calli of *Citrus jambhiri* with different concentrations of *Phytophthora parasitica* culture filtrate [34]. Regenerated plants of survived calli, were subjected to infection under *in vivo* conditions by adding freshly prepared mycelial suspension. They concluded that under *in vivo* condition, total of 81% of the selected regenerated plantlets showed resistance to *Phytophthora parasitica* and none of the control plants displayed resistance level. Moreover, Purwati and Sudarsono selected *Fusarium oxysporum* culture filtrate insensitive variants of abaca (*Musa textilis* Nee) embryogenic calli [35]. Selected calli were grown and developed variants of abaca plantlets. Results indicated that untreated abaca were very susceptible to *Fusarium oxysporum* infection. Meanwhile, plants obtained from culture filtrate treated calli shown resistant against infection compare to the untreated plantlets.

Similar observation was reported by Kumar et al. who reported that about 30% of the plants regenerated from the resistant calluses of chrysanthemum (*Dendranthema grandiflorum Tzelev*) against *Septoria obesa* culture filtrate [36]. Similarly, Wilson et al. inoculated *in vitro* plants of potato cv [37]. *Russet Burbank* (Vancouver strain) with bacterial cultures of *Streptomyces scabiei*. They concluded that

glasshouse and field trials identified 51 potato variants with significantly reduced disease incidence and severity compared with the parent cultivar. Similarly, El-Bramawy et al. selected FA-resistant hybrids of maize, by using tissue culture system [38]. For examining the stability of resistance, the FA- resistant genotypes were selected and exposed again to FA. They concluded that resistant genotypes of maize were much less sensitive to toxin in field compare to untreated FA plants. Morpurgo et al. inoculated *Musa acuminata* Colla banana's shoot tips under different concentrations of FA and *Fusarium oxysporum sp. cubense* culture filtrate [39]. At the end of the experiment, they inoculated obtained explants with a conidial suspension *of Fusarium oxysporum sp. cubense* and assessed the correlation between *in vivo* and *in vitro* behaviour. Morpurgo et al. concluded that no clear linkage between *in vivo* and *in vitro* behaviour among susceptible and resistant banana explants was observed and suggested that the use of *Fusarium oxysporum sp. cubense* culture filtrate or FA in a screening programme for selecting a novel resistant genotype of banana is not feasible [39].

Jiao et al. indicated that the tobacco suspension cell death rate increased gradually as the concentration of FA was increased from 10 μM to 200 μM [40]. Results of their study also showed that the cell death rate was increased with increasing FA treatment duration. Samadi and Behbudi confirmed that FA caused cell necrosis in saffron plants. They also reported that increasing FA concentrations range from 0 μM to 200 μM resulted to increases of necrotic cells [41].

Wang et al. highlighted that cucumber seedling was seriously wilted when treated with FA for 24 hours [42]. A similar result was demonstrated by Abouzeid et al., who reported that complete wilting was observed in corn grains when treated with a mixture of two FA [43]. Damaged cell membrane by FA should resulted to increased water loss and leading to plant wilting [42]. Fakhouri et al. also demonstrated that tomato seedlings were wilted when treated with 100 ppm of FA [44]. They concluded that the mechanisms of wilting induced by fungal toxins may involve the reduction of the water movement, stomatal opening and the loss of membrane semi-permeability. Similarly, Li et al. also found FA caused banana plantlets to wilt, deteriorate and die *in vitro* with symptoms becoming more severe as concentrations were increased [45]. El-Hassan et al. reported that higher concentrations of FA increased significantly the infection percentage and disease severity of inoculated potatoes, while the lower concentration of FA was ineffective in comparative to the control [46].

Kumar et al. stated that gamma irradiation affects greatly on germination of nine popular rice varieties and their results indicated that increasing doses of gamma irradiation had significant effect on germination for the first seven days under laboratory conditions [47]. Increasing of gamma radiation doses resulted to a strict reduction in percentage of germination in irradiated seeds as compared to control.

Similarly, Marcu et al. stated that increasing of gamma irradiation doses resulted to decreasing germination of maize (*Zea mays*) [48]. They concluded that a statistically significant germination inhibition was noticed at higher doses of gamma radiation. Furthermore, Minis et al. reported that low doses of gamma radiation increased the germination percentage of seeds of *Moluccella laevis* when compared with control and the higher doses [49]. Moghaddam et al. also reported that *Centella asiatica* survival rate kept decreasing with increasing irradiation dosage for three weeks after irradiation [50]. A similar gradual reduction in the survival rate has been observed in X-ray irradiated wheat [51].

Mahlanza et al. pointed out that after inoculation of sugarcane (*Saccharum sp.*) with *Fusarium sacchari,* there is a correlation between tolerance to CF *in vitro* and that displayed by the organism [14]. Similarly, Sengar et al. stated that *in vitro* selection of sugarcane (*Saccharum sp.*) for red rot resistance using CF of *Colletotrichum falcatum* was effective and results showed that there is a positive association between *in vitro* and *in vivo* methods of selection plant for disease resistance [52].

Sengar et al. showed that plants regenerated from calli selected on higher CF concentrations exhibited higher levels of resistance to red rot than those screened at lower CF concentrations and controls [52]. They observed that the number of resistant plants differed based on different concentrations of CF on various sugarcane genotypes. The percentage of resistant plants regenerated from *in vitro* selected from first genotype sugarcane calli increased from 20% to 75% when CF concentration was increased from 0% to 15%. Resistant plants obtained from another sugarcane calli selected on CF at concentration varying from 0% to 15% increased from 0% to 87%.

Similarly, Jayasankar et al. inoculated masses of Chardonnay' (clone 02Ch) and grapevine (*Vitis vinifera L.*) to the CF of *Elsinoe ampelina*. Results showed that out of 40 *in vitro* selected plants from both resistant lines, 39 remained resistant even after several days [53]. Whereas, Yusnita et al. evaluated responses of the regenerated peanut against *Sclerotium rolfsii* after *in vitro* selection of insensitive somatic embryos and concluded that most of the pathogen CF-resistant peanut lines were not resistant against direct pathogen infection of leaf tissues.

Matsumoto et al. stated that *in vitro* selections of banana plants using FA and *Fusarium oxysporum sp. cubense* culture filtrate resulted to the tolerant regenerated plants against Fusarium wilt caused by the fungus *Fusarium oxysporum f. sp.* Cubense [54]. They concluded that plants obtained from *in vitro* selected FA and CF showed tolerance to the disease in the greenhouse, and some of the plants also showed resistance in the field although the level of tolerance observed was not sufficiently high.

Similar observation was reported by Flores et al. who screened seeds of passion fruit with different concentrations of either *Fusarium oxysporum f. sp. passiflorae* CF or FA [17]. Plants obtained from selected and non-selected seeds were inoculated with a conidial suspension of *Fusarium oxysporum sp. passiflorae* to assess correlation between *in vivo* and *in vitro* responses. They concluded that the percentage of surviving plants did not differ significantly between FA and CF selected and non-selected plants and there was not any strong correlation between *in vitro* selection and *in vivo* resistance in their study. Thakur et al. also reported that Carnation (*Dianthus caryophyllus L.*) calli inoculated with *Fusarium oxysporum f. sp.* dianthi culture filtrate and results from *in vivo* screening showed that about 32% of the plants regenerated from the resistant calli had acquired considerable resistance against the pathogen in the field [18]. Furthermore, Cassells and Walsh developed an *in vitro* selection of *Helianthus tuberosus* nodes against *Sclerotinia sclerotiorum* [55]. They concluded that there was a strong positive correlation between capacity of nodes to grow *in vitro* and field resistance.

Remotti et al. reported that cell suspension cultures of 'Peter Pears', a cultivar of *Gladiolus x grandiflorus* have been challenged with different concentrations of FA [56]. They reported that each regenerated plant was transferred to a medium supplemented with 0.35 mM FA to test the sensitivity for FA at plantlet level. Their results indicated that FA treated colonies, showed growth and survival rate between 0% to 100% compare to 0% survival rate of the untreated control callus.

Remotti and Löffler stated that Gladiolus genotypes were

challenged *in vitro* with various concentrations of FA [57]. However, they concluded that only two of the developed bioassays gave significantly coinciding results with the Fusarium-resistance assessed in a greenhouse experiment. They suggested that a part of the Fusarium resistance is based on FA insensitivity. Wenzel and Foroughi-Wehr also pointed out that different lines of barley were screened *in vitro* against FA [58]. In green house and field, selected and untreated control regenerated plants inoculated with FA. Results indicated that most greenhouse or field-grown plants differed in their level of susceptibility to FA compared to the level of the starting material, but these data were in no instance significant.

Saxena et al. inoculated callus cultures of rose-scented geranium (*Pelargonium graveolens*) with various concentrations of *Alternaria alternat* CF [59]. Resistant calli were selected and placed on regeneration medium. They indicated that pathogen resistance of the regenerants were confirmed by exposing their leaves to the same concentrations of CF. While the parental wild type demonstrated typical susceptibility, the leaves of putative resistant clones remained green.

Similarly, Nasir and Riazuddin stated that friable calli of *Gladiolus* inoculated with different concentrations of FA [60]. The regenerated of all *in vitro* selected cell lines were inoculated with a conidial suspension of the *Fusarium oxysporum*. Their results indicated that plantlets of all selected cell lines exhibited significant growth as compared with the control after application of conidia of the *F. oxysporum*.

Krishna et al. stated that the irradiated plants of banana, (*Musa paradisiaca L.*) acquired tolerance characteristics against pathogenic fungi [10]. Al-Safadi and Arabi applied gamma irradiation to improve resistance of potota (*Solanum tuberosum*) to late blight disease caused by *Phytophthora infestans* [61]. Gamma irradiated potato plantlets were later inoculated with sporangial suspension. Their results indicated that mutants tolerant to late blight disease appeared from irradiated explants compare to non-irradiated explants and the percentage of tolerant mutants increase with increasing gamma irradiation doses.

Mahlanza et al. applied a combination of *in vitro* culture and mutagenesis using ethyl methane sulfonate (EMS) followed by CF mediated selection to assess the effect of chemical mutagen on production of variant sugarcane plants tolerant to *Fusarium sacchari* [14]. Their results indicated that plants produced from EMS-treated calli displayed improved root regrowth in the presence of CF pressure compared with those from non-treated calli. In this regard, there are several reports on EMS treatment of calli, followed by appropriate screening protocols for the production of plants tolerant to diseases [35,54].

Kumar et al. applied 20 Gy gamma irradiation and 15% *Septoria obese* CF in *in vitro* selection process in order to obtain resistant calli of Chrysanthemum (*Dendranthema Grandiflora Tzelev*) against leaf pathogen of *Septoria obese* [62]. Their results indicated that plants obtained from selected resistant calli, showed 100% resistance against the pathogen in the pot in greenhouse. The plants showed no disease symptoms for four years. Similarly, Encheva et al. stated that sunflower (*Helianthus annuus L.*) zygotic embryos treated with ultrasound, showed 100% resistance to the parasite *Orobanche* in the field section [63]. Jain also reported that somatic embryogenic cell cultures of date palm were irradiated with gamma radiation, and regenerated plants in greenhouse were treated with toxin of *Fusarium oxysporum* [64]. Their results indicated that toxin tolerant plants in the field evaluation did not show any sign of disease. Furthermore, Saleem et al. pointed out that the application of gamma rays for basmati rice (*Oryza sativa L.)* callus was effective in obtaining resistant plants against salt stress [65].

Similar result was reported by Encheva who reported that immature sunflower embryos of sunflower were treated with gamma radiation. Their results showed that regenerated plants obtained from irradiated embryos possessed 100 % resistance to *Orobanche cumanaparasite* and stable inheritance in the next generations [19]. These results were confirmed during three years of evaluation.

Encheva et al. also reported similar observations after ultrasound application for the embryos of sunflower [20]. The mutant lines produced from embryos treated by ultrasound treatment showed 100% resistance to the local broomrape (*Orobanche cumana*) population. They concluded that the resistance of the mutant sunflower lines to *Orobanche cumana* occurred as a result of mutation of a single dominant gene.

Conclusion

The outcomes of the present investigations indicated that increasing concentrations of CF, FA and gamma radiation treatments resulted to higher mortality rates on *Dendrobium* sonia-28 PLBs. While, leaf bridge assay results have shown potential for obtaining resistant *Dendrobium* sonia-28 orchid plantlets against *Fusarium proliferatum*. Our results indicated that increasing treatment concentrations resulted to more resistant plantlets. Furthermore, FA treated plantlets seemed to show lower disease incidence and higher resistance compared to CF and gamma irradiation.

However, for practical use of these resistant plants, further glass house and field research should be done to improve the effectiveness of *in vitro* selection derived plantlets against fungal diseases.

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