



Fusarium proliferatum culture filtrate sensitivity of *Dendrobium sonia-28*'s PLBs derived regenerated plantlets

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Received 20 March 2014; Received in revised form 1 August 2014; Accepted 6 August 2014

ABSTRACT

Aims: *Dendrobium sonia-28* is an important orchid in the Malaysian flower industry and currently facing serious production problems due fungal diseases. To overcome this impediment, one of the strategies being pursued is by the production of new orchid mutants. In this prospect, the present study was aimed to determine effects of *Fusarium proliferatum* culture filtrate on the potential of producing somaclonal variants *Dendrobium sonia-28* plants tolerance to *F. proliferatum* disease infection.

Methodology and results: Here, we assessed *Fusarium proliferatum* pathogenicity on the survival and fresh weight responses of *Dendrobium sonia-28* PLBs using culture filtrate. PLBs death increased at the highest concentrations and resulted in reduced weight among survivors. There were appreciable variations in plantlet structure and morphology relative to increase fungal filtrate concentration. Plantlets infected with lower concentrations of the fungus had better development of the shoot and root as well as the foliage. Histology and SEM analysis disclosed severe cell wall damage and stomatal closure in protocorm-like bodies (PLBs) infected with the higher fungal filtrate concentrations.

Conclusion, significance and impact study: As well as giving insights into the clonal reproduction of *F. proliferatum*, these results emphasizes the significance of somaclonal variation plants production based on low fungal infection tolerance in floriculture technology. Hence, the ability of the *Dendrobium sonia-28* to survive after *F. proliferatum* infection opens new avenues for resistance gene discovery and future development of fungus resistant transgenic orchids.

Keywords: *Dendrobium sonia-28*, Somaclonal variation, PLBs, Culture filtrate.

INTRODUCTION

The Asia-Pacific region has the major share of the total world area under floriculture production (FAO, 2010). In Southeast Asia, this sector is profitable agri-business. Cut flowers and many other floricultural products are key export products for many countries. These include Malaysia, where the horticultural sector has recorded phenomenal growth over the past years especially in the floriculture industry which has seen a significant increase in land under cultivation from 3,370 ha in 2005 to 7,000 ha in 2010 (Hamir *et al.*, 2008). In particular, the cut flower industry has recorded a notable growth from mid-90s, with *Dendrobium* orchids being the main grown flower plants (Jong *et al.*, 1995; Khosravi *et al.*, 2009).

Members of this orchid genus possess medicinal properties against many diseases, including fever, diabetes, infection, cancer, and eye problems (Jong *et al.*, 1995). *Dendrobium* species have also nutritional values and their uptake can boost athletic performance (Jong *et al.*, 1995). However, they are best known for their

flowering floriferousness, broad range in flower colour, size, and shape with year-round availability as well as long post-harvest life span (Kuehnle, 2007; Khosravi *et al.*, 2009).

Dendrobium sonia-28 a hybrid resulting from the cross between *Dendrobium Caesar* and *Dendrobium Tomie Drake*, is a famous for its pink-coloured and good cut flowers. Through *in vitro* system, *Dendrobium sonia-28* reproduces based on regeneration of new plantlets through protocorms and protocorm-like bodies (PLBs) (Saiprasad and Polisetty, 2003). *Dendrobiums* are susceptible to several pathogens and the diseases they transmit (Samuels and Brayford, 1994; Hirooka *et al.*, 2005; Halleen *et al.*, 2006; Hirooka *et al.*, 2006). Such pathogens include fungi of the genus *Fusarium* which attack the root system causing wilting (Starr *et al.*, 1989, Rossman *et al.*, 2013), growth stunt, defoliation, chlorosis, failure vascular system, browning of the vascular system (Agrios, 1988) as well as discolouration of the rhizome and stem bases (Elena and Kranias, 1996). Most fungal pathogens infesting orchids exhibit the same general

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nature of breeding, principally, living as spores in the soil or as mycelium in diseased plant litter and are spreading with host propagation materials (Fantino and Fantuz, 1990; Doan and Carris, 1998).

F. proliferatum has increased potential for producing diverse mycotoxins and was regarded as a major pest of many crops (Kushiro *et al.*, 2012), including commercially cut-flower plants (Fattahi *et al.*, 2014). Attempts to fight these infections that can seriously reduce market ability rely heavily on the increased use of chemical insecticides, in particular fungicides. Although some such as chlorothalonil, azoxystrobin, fludioxonil and palladium worked well, the development of resistance has severely hindered success in such approach (Wedge and Elmer, 2008; Wedge *et al.*, 2013). To increase profits, there has been a great deal of research adopting new production technologies for short production cycles in commercial nurseries. However, this has led to serious productivity problems, in particular increased susceptibility to diseases (Wedge and Elmer, 2008). However, these technological successes were associated with productivity problems.

In support of this contention, it has been reported that *Dendrobium sonia-28*, an important ornamental orchid in Malaysia is experiencing a reduced germination pace and risks of producing unsought progenies (Poobathy *et al.*, 2013). Additionally, the use of pathogen toxin as selective agents at the tissue culture might be a good source of variability that could lead to selection of individuals with various levels of resistance to the pathogen (Svabova and Lebeda, 2005). In the case of antifungal compounds, a better understanding of orchid-fungus interactions is relevant and important.

The present work was carried out to determine the effect of *F. proliferatum* culture filtrate on *Dendrobium sonia-28* PLBs followed by the morphological studies of the regenerated plantlets.

MATERIALS AND METHODS

Plant material and culture condition

In vitro cultures of PLB of *Dendrobium sonia-28* were used for *in vitro* selection study. The PLBs were cultured/maintained in a half-strength semi-solid MS media (Murashige and Skoog, 1962) supplemented with 1 mg/L of Benzylaminopurine (BAP) and 2% sucrose. The pH was adjusted to 5.8 prior to autoclaving. The PLBs were grown at 25 °C under 16 h photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150 µmol/m².s). The PLBs were then subcultured for every four weeks. Newly developed healthy PLBs in range of 1-2 and 3-4 mm were used as explants for the following treatments.

Preparation of *Fusarium proliferatum* culture filtrate and selection media

For the preparation of *Fusarium proliferatum* culture filtrate, 5 mm size discs of PDA that contained 30 days old fungal cultures were transferred onto fresh PDA. After two

weeks of culture, ten (10) to fifteen (15) pieces of PDA containing fungal were inoculated in 50 mL of liquid MS medium in 250 mL Erlenmeyer flask. After two weeks of incubation, the medium was divided into five equal parts and maintained under dim light at 22 °C. Small mycelium balls that emerged in suspension after six weeks were then allowed to pass through a filter paper (Whatman), followed by centrifugation at 10,000xg for 20 min. Supernatant was then sterilized using a nitrocellulose filter (0.22 µm) and stored at -20 °C.

Culture filtrate treatment on *Dendrobium sonia-28* PLBs

Culture filtrate (CF) was added to the autoclaved MS medium after filter sterilization in different concentrations. One hundred (100) PLBs in two different sizes, each size were inoculated on MS medium containing 5, 10, 15 and 20% CF. Ten (10) pieces of PLBs were placed in a Petri dishes and incubated in the similar culture room condition as 25±2 °C under a 16 h photoperiod using cool white fluorescent lamps.

Selection and regeneration of PLBs tolerant to culture filtrate

After four weeks of incubation on MS medium containing 5, 10, 15 and 20% of CF, survived PLBs which appeared green were selected and further subcultured onto a fresh selection medium containing same CF concentration and incubated for another four weeks. Tissue culture response of the PLBs was recorded after four weeks as the (1) fresh weight of growing PLBs and after eight weeks as the (2) frequencies of PLBs survival rate (number of PLBs that formed expressed as a percentage of the number of explants tested).

Scanning electron microscope (SEM) of PLBs

Comparison of stomatal behaviour between inoculated and healthy PLBs were made using scanning electron microscope observation through the freeze drying method. Experiments were initiated at hour 3 of the inoculation, consequently, the treated and untreated PLBs were immediately removed from the recovery medium under light condition following they were dabbed dry with a piece of filter paper, they were neither treated nor coated preceding the viewing; thus, they were observed in their natural state. The VP SEM was initiated with a saturated humidity of 4 °C; the apparatus consisted of a Peltier cooling stage possessing a gas pressure of 500-700 Pa. The tension value was recorded at an extra high value of 15 kV. The samples produced were later viewed with the aid of a Leo Supra 50VP Field Emission scanning electron microscope (Carl Zeiss SMT, Germany). To facilitate measurement of stomatal closure, tow PLBs, each containing approximately 15 stomata, were taken for each treatment.

Histological observation of PLBs

The PLBs were also subjected to a histological study to observe differences in both resistant and sensitive PLBs of the *Dendrobium sonia-28*. Histological observations were carried out in PLBs at four weeks after CF treatment on five replicates per each concentration.

Morphological studies

The regenerating explants for control and plantlets of *Dendrobium sonia-28* that were selected upon exposure to culture filtrate were subcultured six times (each subculture, 28 days). Results were recorded after six months of regeneration for: (1) the number of offshoots per plantlet, (2) the length of offshoots per plantlet, (3) the number of fully opened leaves per plantlet, (4) the length of fully opened leaves per plantlet, (5) the number of roots (> 0.1 cm was considered for counting) and (6) the length of roots per plantlet. Three plantlets per replicate were subjected to the experiment with four replicates for each treatment.

Statistical analysis

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

RESULTS AND DISCUSSION

Effect of fungal culture filtrate concentration on PLBs of *Dendrobium sonia-28*

Interaction of various culture filtrate concentration indicated higher concentration of CF was found to have significant effect on surviving rate and fresh weight of PLBs in both different sizes. Venkatalalam *et al.* (1995) and Saxena *et al.* (2008) reported that the growth on the toxic media was dependent on the size of the callus. This is could be the reason of culture filtrate (CF) had a significant effect on small size of PLBs' survival rate (Figure 1). Highest survival percentages (24%) and lowest (12%) of 3-4 mm PLBs obtained from interactions of 5 and 20% of culture filtrates (Figure 1). Similar trend was obtained for determination of PLB's fresh weight. Increasing culture filtrate concentration to 20% showed a greater decrease of PLBs final fresh weight (0.11 g) as compared to PLBs inoculated with lower concentration of culture filtrate (Figure 2).

Number of previous studies reported that the toxin present in CF was able to inhibit cell growth (Selvapandiyan *et al.*, 1988). For instance, Tripathi *et al.* (2008) demonstrated in a study on *in vitro* selection for onion resistance against purple blotch disease *Alternaria porri*, embryos exposed to toxic culture filtrate at different periods of times. They have noted that continuous exposure resulted increased mortality rate. Conversely,

higher survival rate was recorded in calli inoculated with lower concentration of CF (below 7.5 mL/L). In addition, Kumar *et al.* (2008) reported similar findings regarding to the decrease of the callus weight compare to the control at the higher concentrations of culture filtrate in *Chrysanthemum (Dendranthemagran diflorum Tzelev)*. Furthermore, Hartman *et al.* (1986) also showed a correlation between growth of bean cultivars calli (fresh weight loss and necrosis) and pathogenicity of culture filtrate. Successive exposures to the CF during screening may lead to undesirable mutations which result in plants exhibiting susceptibilities to biotic and abiotic factors (Matsumoto *et al.*, 2010). This may explain that the high mortality observed in CF higher concentration. This regular decrease in PLBs survival rate with the toxic culture filtrate in the medium was probably due to the presence of toxic metabolites in the toxic culture filtrate and it may be used as an effective screening agent in a cell selection program.

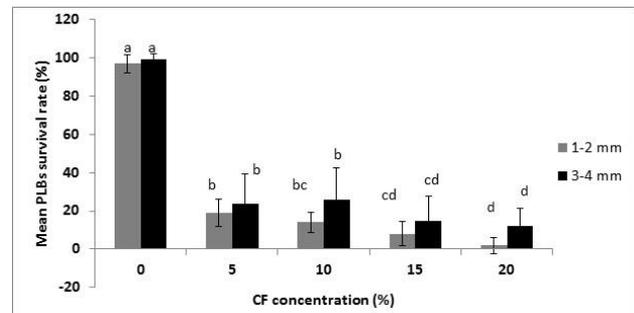


Figure 1: Effect of various concentrations of culture filtrate on the survival rate of *Dendrobium sonia-28* orchid's PLBs in two different sizes of 1-2 and 3-4 mm.

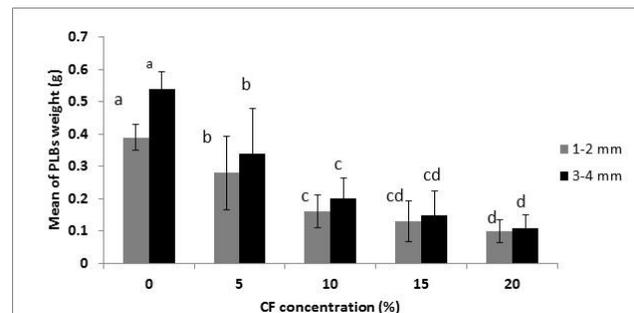


Figure 2: Effect of various concentrations of culture filtrate (CF) on the final fresh weight of *Dendrobium sonia-28* orchid's PLBs in two different sizes of 1-2 and 3-4 mm.

Histological observation of PLBs of *Dendrobium sonia-28*

Histological observation of untreated PLBs displayed intact cells with uniform polyhedral shapes and also revealed that the meristematic cells are densely arranged on the external layers. This epidermal meristematic cell is

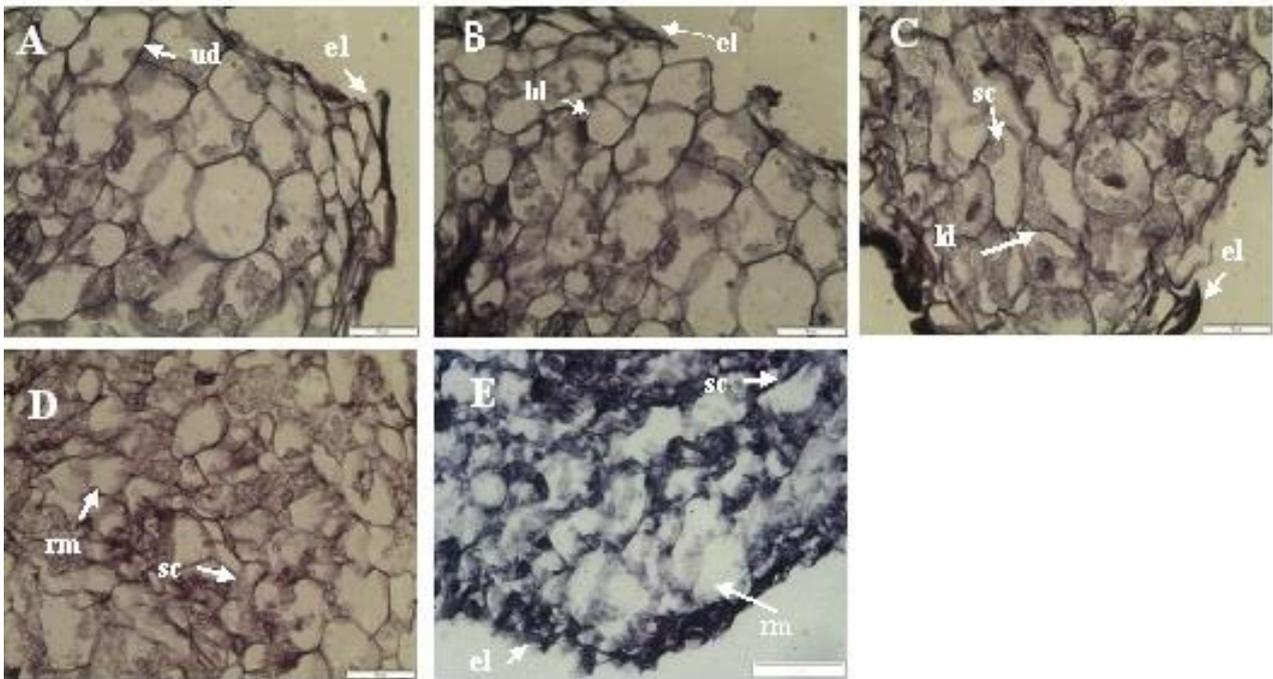


Figure 3: Histology observation on the PLBs of *Dendrobium sonia-28* (Scale bar=50 μ m). A, Untreated PLBs; B, PLBs treated with 5% culture filtrate; C, PLBs treated with 10% culture filtrate; D, PLBs treated with 15% culture filtrate; E, PLBs treated with 20% culture filtrate.

very thin, consist only one to three rows of small cell layers of thin-walled cells, one over the other, without intercellular spaces. Sections from treated PLBs by different concentrations of CF revealed that the epidermal cell was mainly restricted to the outermost cell layers. Hence, CF prevented epidermal formation in treated PLBs. It decreases number of epidermal layers from 3 ± 0.4 in non-treated PLBs to 2 ± 0.5 , 2 ± 0.4 , 1 ± 0.5 and 1 ± 0.4 in PLBs treated with 5, 10, 15 and 20% of CF (Figure 3). The extent of changes was much greater after treatment with higher concentrations of CF at a lower concentration. This may suggest that although metabolites of *Fusarium proliferatum* culture filtrate are capable of inducing alterations in cell structure, their effect strengthens with increasing concentrations. El-Hassan *et al.* (2007) demonstrated through histological analysis, it was observed that fusaric acid inoculation decreases number of periderm layers in potato tuber slices.

The inner cells consist of large vacuolated parenchymatic cells with less visible nucleus. In PLBs growing under CF treatments, the epidermal layers of the PLBs are thinner than in PLBs growing in control conditions. Our observations also indicated that cell shrinking could be associated to CF concentration since shrinking cells increased with increasing concentration of CF compare to control. In fact, cell damage was rapidly increased in PLBs developing at higher concentration of CF (15 and 20%). Similar observation was reported by

Diniz and Oliveira (2009) on *Zea mays* L. seedlings that parenchymal cell length decreased at increasing concentrations of fusaric acid.

Surface morphological observations of PLBs

The surface morphology of PLBs of *Dendrobium sonia-28* was observed under scanning electron microscope (SEM) and shown in Figure 4. Experiments were initiated at hour 3 of the inoculation under the light condition.

SEM studies of the control and treated PLBs indicated that CF damages exterior regions of the PLBs. Numerous stomatal were distributed around the untreated PLBs surface. All concentrations of CF increased the stomatal closure of PLBs and this was intensified with increasing concentrations of CF (0-20%). Results obtained from this study showed that 30 randomly selected stomatal in each of untreated PLBs. Virtually all stomatal were fully open (96%) in control treatment whilst on inoculated PLBs with high CF concentration (20%) with none was fully open, few were partially open (7%), and the majority was closed (93%). In inoculated PLBs with lower concentrations of CF (5, 10, 15%), stomatal behaviour was nearly identical to that of stomatal in PLBs inoculated with highest concentration of CF. In inoculated PLBs with 5, 10, 15% of CF concentration, many of the stomatal were closed and that the percentage were 76, 83 and 90%, respectively.

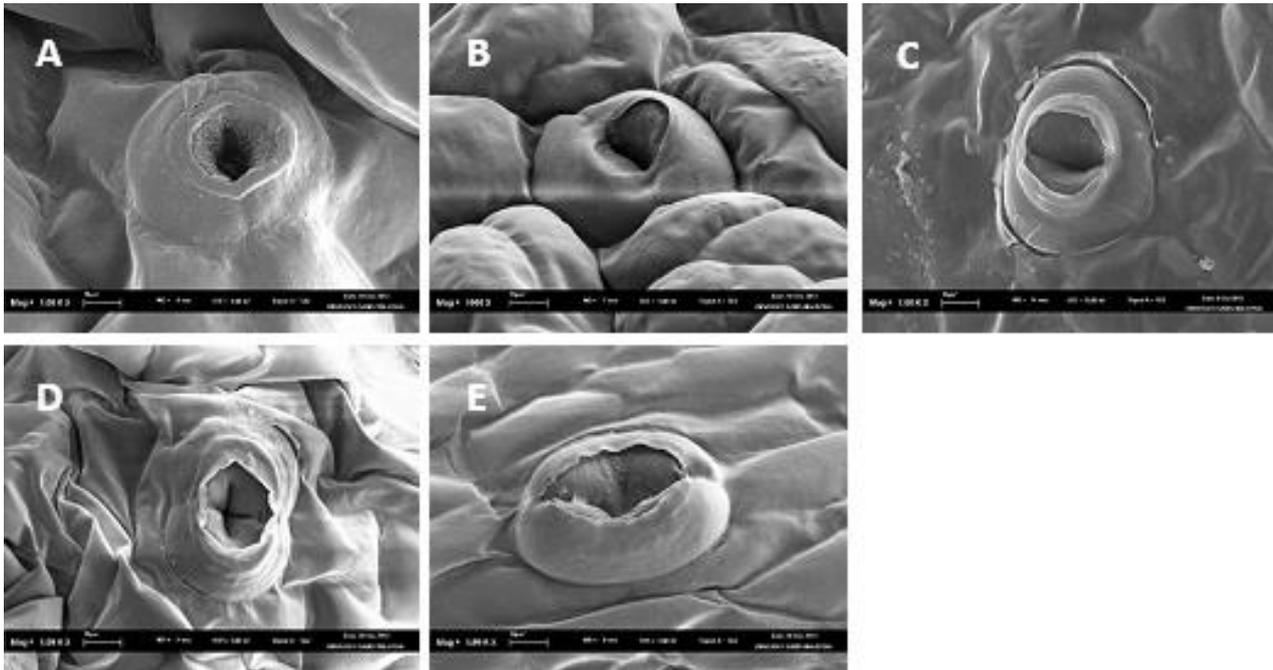


Figure 4: SEM of *Dendrobium sonia-28* PLB stomata (Scale bar = 1.00Kx). A, Untreated PLBs; B, The stomata of PLBs treated with 5% culture filtrate; C, Stomata of PLBs treated with 10% culture filtrate; D, Stomata of PLBs treated with 15% culture filtrate; E, Stomata of PLBs treated with 20% culture filtrate.

It is believed that stomatal play an important regulatory role in plant-environment interactions (Pei *et al.*, 2000; Garcia-Mata and Lamattina, 2001; Desikan *et al.*, 2002). Accordingly, stomatal function can be perturbed following by plant attack by a pathogen, which triggers the H₂O₂/NO in the defense process. Stomatal opening was necessary for fungal penetration (Pierre *et al.*, 1977). It has been found that abiotic factors such as water status and solar radiation are main effectors on stomatal conductance but foliar pathogens may also affect plants by penetrating from stomatal (Goodman *et al.* 1986; Lucas, 1998). Zeng *et al.* (2010) reviewed that stomatal represent a major route of pathogen invasion and stomatal closure appears to be part of plant immune response. Our results align well with previous studies by Majernik (1971), and Ayres and Zadoks (1979) reporting *Blumeria graminis* infection impair stomatal opening in Barley and Pea plants in light. Furthermore, Martin *et al.* (1975) showed reduced transpiration (indicating stomatal closure) by wheat seedlings within 3-6 h after inoculation with *B. graminis* conidia. Powdery mildew infection causes stomata closure in the light and its opening in the darkness (Majernik, 1971; Ayres, 1976). Moreover, Martin *et al.* (1975) reported that within 6 h of powdery mildew pathogen inoculation, wheat stomatal opening was inhibited.

Morphological characteristics of *in vitro* plantlets upon CF treatment

Incorporation of different concentrations of CF in MS media containing affected physiological conditions of the regenerated plants such as number of leaf, plantlet's height, and roots. Morphological changes on *in vitro* plantlets were more observed on plantlets derived from PLBs inoculated with higher CF concentrations (Table 1). Most of treated plantlets have narrow and pointed leaves as compared with broad leaves of the normal *Dendrobium sonia-28* plantlets. In addition, abnormal plantlets with stunted shoots were seen on each regenerated plantlets treated with higher CF concentrations. Similarly, Gonzalez *et al.* (2006) also indicated that *Pseudomonas syringae* pv. *Phaseolicola* culture filtrate was responsible for a lower bud shoot growth in a race-specific susceptible cultivar of bean explants.

Significant inhibition of root growth was observed at higher concentration of CF compared to untreated plantlets with the lengths reached significantly less values than those recorded when CF stress was imposed at higher concentrations (Table 1). The application of culture filtrates (CFs) of *Fusarium* on plants has been often associated with the inhibition of the growth of plant root system (Chen and Swart, 2002; Khan *et al.*, 2004). Our findings is correlated with Khan *et al.* (2004) which reported that the culture filtrates of *Fusarium oxysporum* at normal concentration and 50% diluted of CF reduced the root length of germinating seeds of both chickpea

Table 1: Effect of various concentrations of culture filtrate on the no. of offshoots, offshoot length (cm), no. of fully opened leaf, leaf length (cm), no. of roots and root lengths (cm) of *Dendrobium sonia*-28 orchid plantlets after 6 months.

CF Conc. (%)	No. of Offshoots	Off shoot length (cm)	No. of leaf	Leaf length (cm)	No. of roots	Root length (cm)
0	2±0.32 ^a	0.8±0.14 ^a	4±0.62 ^a	2.5 ±0.22 ^a	4 ±0.94 ^a	1.5 ±0.53 ^a
5	1±0.21 ^b	0.6±0.11 ^b	3±0.49 ^b	1.2±0.26 ^b	3±1.11 ^b	0.6±0.27 ^b
10	1±0.20 ^b	0.4±0.10 ^c	3±0.62 ^c	0.9±0.20 ^c	2±0.42 ^c	0.4±0.43 ^c
15	1±0.18 ^b	0.2±0.07 ^c	2±0.26 ^c	0.7±0.09 ^d	2±1.13 ^c	0.2±0.04 ^c
20	1±0.18 ^b	0.1±0.23 ^c	2±0.32 ^c	0.5±0.15 ^d	1±0.17 ^c	0.1±0.53 ^c

Values within the column followed by different letter(s) are significantly different at $p \leq 0.05$ by Tukey's multiple range test.

varieties compared to their control. Chen and Swart (2002) reported similar findings regarding to the significant inhibition of root growth in five varieties of *Amaranthus hybridus* exposing culture filtrate of *Fusarium oxysporum*.

CONCLUSION

It can be concluded from the present study that the effect of the CF was more severe when incorporated at the higher concentrations, as evidenced by the higher levels of PLBs necrosis at 20% CF. Differential effects of the CF occurring on the various physiological processes and resulting consequent decrease in plantlet shoot and root growth. Furthermore, histology and SEM observations were found to be a better indicator of the effect of CF on PLBs as more damages on cell walls and surface of PLBs were obtained amongst the higher concentration of CF treatment. In *Dendrobium sonia*-28 plants, for obtaining resistant plants by applying *in vitro* selection methods, it is necessary to pursue research on *in vivo* as well as *in vitro* resistant mechanisms to this wilt disease. *Dendrobium sonia*-28 PLBs tolerance to *Fusarium proliferatum* will be tested in the greenhouse and field tests will be carried out.

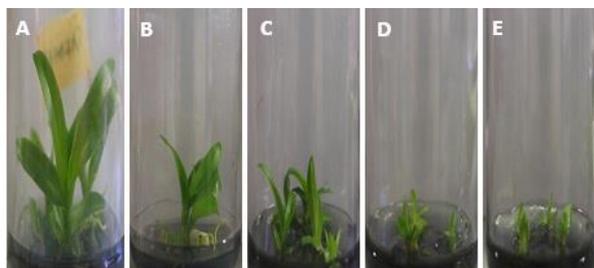


Figure 5: Effect of culture filtrate at different concentration on *Dendrobium sonia*-28 plantlets after 6 months of inoculation. A, Untreated plantlet; B, Plantlet treated with 5% culture filtrate; C, Plantlet treated with 10% culture filtrate; D, Plantlet treated with 15% culture filtrate; E, Plantlet treated with 20% culture filtrate. Scale bar = 1 cm.

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