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Synergistic Effect of PPFD and Mycorrhization for Efficient *in vitro* Propagation of *Dendrobium chrysanthum* Wall. ex Lindl.

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A B S T R A C T

Integration of optimal photosynthetic photon flux density (PPFD) and mycorrhization under *in vitro* conditions appears to have significant synergistic effect in enhancing the growth and survival of the medicinal orchid, *Dendrobium chrysanthum* under *in vitro* and *ex vitro* conditions. PPFD at 60 µmol $m^{-2}s^{-1}$ was found to be optimal for root colonization for mycorrhizal association by fungi (73% colonization). The growth of mycorrhizal plantlets was observed to be superior at 60 µmol $m^{-2}s^{-1}$ PPFD as compared to plantlets subjected to either lower or higher PPFD. The growth responses in terms of shoot length, leaf area, fresh weight (FW) and dry weight (DW) of mycorrhizal plantlets were consistently and significantly higher at 60 µmol $m^{-2}s^{-1}$ PPFD after 30, 60 and 90 days of treatment. PPFD affected development of stomata with increase in number of stomata per unit area of leaf with increasing light intensity. Maximum length of stomata was observed in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD but light intensity did not cause any substantial effect on width of stomata. After 120 days of hardening, highest survival percentage (97.33%) was obtained with mycorrhizal plantlets transferred from 60 µmol $m^{-2}s^{-1}$ PPFD. Growth parameters observed were also higher in mycorrhizal plantlets transferred from 60 µmol $m^{-2}s^{-1}$ PPFD. Mycorrhizal association *in vitro* at optimal PPFD produced the most marked growth response both under *in vitro* conditions and during *ex vitro* acclimatization.

Introduction

Orchids of the family Orchidaceae are one of the largest angiosperm families of the plant kingdom. Over the past 40 years orchids have been subjected to high commercial demand for their beautiful and vivid colored flowers, delicate scents and medicinal properties. However, increased biotic influences e.g., deforestation, socio-economic development and excessive exploitation for commercial purposes have led to a considerable depletion

of orchids in nature. Due to tremendous pressure on orchids in nature, the entire family "Orchidaceae" is listed in the Appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) for conservation. Many programs have been initiated for the conservation of orchids. *In vitro* culture via micropropagation is an important technique for *ex situ* conservation and management of plant genetic resources. Large quantities of uniform, high-value plants can be produced through micropropagation. However, conventional micropropagation is carried out under abnormal growth conditions such as high relative humidity, constant temperature, low photosynthetic photon flux density (PPFD), large diurnal fluctuation in CO_2 concentration, etc. (Debergh *et al.,*1992; Cui *et al.,* 2000). As a result, structural and functional abnormalities have been observed in micropropagated plants which make these plants susceptible to photoinhibition upon transfer to *ex vitro* environment (Capellades *et al.,* 1990; Zimmerman *et al.,* 1991). It is generally accepted that poor photosynthetic behavior is one of the most important abnormalities hindering *ex vitro* acclimatization of micropropagated plants. Abnormalities induced *in vitro* could be "corrected" by adjusting different environmental parameters *in vitro* (Debergh, 1991). Earlier studies have also shown that photosynthetic efficiency could be improved by raising PPFD during acclimatization (Amancio *et al.,* 1999).

The survival percentage and growth of micropropagated plants could also be improved by treatment with mycorrhizal fungi during acclimatization. Mycorrhizal association increased the resistance of plants to environmental stresses by stimulating growth regulators, increasing photosynthesis, and improving regulation of osmotic adjustment (Rabie and Almadani, 2005). The photosynthetic system is also known to be protected by associated mycorrhiza from over-reduction and photoinhibition. Contrary to mycorrhizal inoculation during acclimatization, induction of mycorrhizal association in plantlets under *in vitro* conditions provide an interesting model system to examine the hypothesis that colonization of roots *in vitro* would provide a certain degree of stress tolerance during acclimatization *ex vitro.* Studies on the physiology and behavior of these plants in controlled environments are also possible. *In vitro* systems have been extensively used as tools to discover and elucidate the nature of chemical dialogue between roots and fungi prior to and during the process of mycorrhization (Martin *et al.,* 2001; Pierleoni *et al.,* 2001).

Mycorrhizal association prior to transplantation has been suggested to be a better strategy for plants against the biotic and abiotic stresses that occur in greenhouses or in the field (Vestberg *et al.,* 2004). Furthermore, by introducing mycorrhizal fungi at an early stage, it would be possible to reduce the use of fertilizer and pesticides and allow the plants to grow in a more sustainable way (Cordier *et al.,* 2000).

The aim of the present study was to develop an effective protocol for maximum growth and survival of orchids by integrating optimal PPFD and mycorrhization *in vitro.*

Dendrobium chrysanthum (Fig. 1), being a medicinally important and endangered orchid of North-east India, has been selected as a model plant for the present study. The objectives of the investigation were designed to study the effect of optimal PPFD and mycorrhization (i) on the growth of *D. chrysanthum* plants *in vitro*, and (ii) establishment vis-a-vis growth of micropropagated plants post-transplantation.

Materials and Methods

Establishment of mycorrhizal association *in vitro* at different PPFDs

For mycorrhizal association *in vitro*, the procedures described by Hajong *et al.*, (2012, 2013) were followed. Plantlets measuring around 1-1.5 cm obtained from *in vitro* nodal explants of *D. chrysanthum* were used for the experiment. About 20 mL of oat meal agar medium (OMA-NC, modified from Rasmussen *et al.*, 1990) was poured into the Erlenmeyer flasks (Table 1). Five plantlets per flask were inoculated in the medium at uniform distances from each other in a circle. Simultaneously, blocks (1x1 mm^2) of the fungal isolate 4634 [isolated from *Ipomoea batatas* and obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India] were inoculated in the centre of the circle. The flasks without fungal inoculum were maintained as controls or non-mycorrhizal plantlets. A light gradient was established by placing the flasks at different PPFD (20, 40, 60 and 80 µmoles $m^{-2}s^{-1}$) supplied by cool white fluorescent lamps in BOD incubators. PPFD was measured (on top of the culture flasks) with a Lux meter (Lutron LX-101) and the value obtained was converted to µmol m^{-2} s^{-1} by multiplying with 0.0135 (conversion factor for cool white fluorescent lamps, Thimijan and Heins, 1982). The temperature of the BOD incubator was maintained at 25 ± 2 ºC, photoperiod at12 h and relative humidity at 70-80%.

Assessment of root colonization

Root colonization percentage was calculated from plants cultured at different light treatments. For assessment of root colonization, the slide method proposed by Giovannetti and Mosse (1998) was followed. A total of 30 root-segments (0.5 cm long), from each treatment, were selected at random from the stained samples and mounted on microscopic slide in groups of 10. Presence of infection was recorded in each of the 10 pieces, and percentage infection was calculated as:

% root colonization = (number of root segments colonized ÷ total number of root segments observed) X 100

Growth and total chlorophyll content of plantlets under in vitro conditions

The growth parameters namely, shoot length, number of leaves, leaf area, number of roots, root length, fresh weight (FW), dry weight (DW) and total chlorophyll content of *D. chrysanthum* grown at different PPFDs were measured after 30, 60 and 90 d of treatment. Additionally, stomata number and size were also observed after 90 d. To determine DW, plantlets were dried at 120 ºC for 20 min in a Sartorius moisture balance (MA-35) and their DW measured. Leaf area was measured with the help of a Leaf area meter (Systronics Leaf Area Meter 211).

Stomata number and size were determined by light microscope and Scanning Electron Microscope (SEM) observations of the lower epidermis of fully developed leaves. For SEM observation, segments of the fully developed leaves were cut into 3 x 3 mm^2 pieces and fixed in 3% glutaraldehyde for 2 h at 4 ºC. These leaf segments were washed twice in 0.1 M sodium cacodylate buffer at 4 ºC for 15 min each. Dehydration was carried out with different grades of acetone (30-100%). The segments were immersed in tetramethylsilane for 5-10 min. The process was repeated and then mounted on brass stubs and gold coated. Finally, the segments were observed under SEM.

For estimation of chlorophyll content, fresh leaf tissue (0.1 g) was crushed in 1 mL of pure

acetone (100%), centrifuged at 5,000g for 10 min at 4 ºC and quantified spectrophotometrically. Chlorophyll 'a' was determined at wavelength 661.6 nm and Chlorophyll 'b' at 644.8 nm (Lichtenthaler, 1987).

Survival, growth and total chlorophyll content of hardened mycorrhizal and non-mycorrhizal plantlets

Mycorrhizal and non-mycorrhizal plantlets of *D. chrysanthum* cultured *in vitro* at different PPFD for 90 d were taken for hardening.

Plantlets were taken out from the culture flasks by means of forceps and gently washed with sterile distilled water, then transferred to clean thermocol pots containing compost mix of approximately 1-1.5 cm^3 of brick pieces and charcoal chunks, chopped decaying litter and moss in a ratio of 1:1:1:1(Hajong *et al.,* 2013).

Prior to putting in the thermocol pots the compost mix was washed thoroughly with tap water, rinsed twice with sterile distilled water, air dried and autoclaved for 60 min at 121 ºC.

A single plantlet was transferred to each thermocol pot. Survival percentage and plant growth parameters *viz*., number of shoots, shoot length, number of leaves, leaf area, number of roots, root length, FW, DW and total chlorophyll content were measured after 120 d of transfer to the green house.

Statistical analysis

Twenty replicates were maintained for every treatment with each Erlenmeyer flask being one replication and the experiment was repeated thrice. Data was subjected to one-way analysis of variance (ANOVA) using Origin (ver. 8.0), and means were compared using least significant difference (LSD) at $p<0.05$ significance level.

Results and Discussion

Root colonization

The results obtained from the studies on percentage of root colonization at different PPFD treatments *in vitro* indicated that percentage of root colonization is related to PPFD. The highest percentage of root colonization (73%) by fungal isolate 4634 was recorded at 60 µmol $m^{-2}s^{-1}$ and the lowest percentage of root colonization was observed at 80 µmol $m^{-2}s^{-1}$ (Fig. 2). At 40 µmol $m^{-1}s^{-1}$ PPFD percentage of root colonization (44%) was lower than the percentage recorded at 60 µmol $m^{-2}s^{-1}$ while at 20 µmol $m^{-2}s^{-1}$ PPFD, percentage of root colonization (35.3%) was less than the percentage observed at 40 µmol $m^{-2}s^{-1}$. However, the lowest percentage of root colonization was observed at 80 µmol $m^{-2}s^{-1}$.

Growth and total chlorophyll content of mycorrhizal and non-mycorrhizal plantlets under *in vitro* conditions

The morphological features of mycorrhizal and non-mycorrhizal *D. chrysanthum* plantlets growing at different *in vitro* PPFD treatments were recorded. The mycorrhizal plantlets showed relatively better growth (with the exception of number of roots and root length) than non-mycorrhizal plantlets under all PPFD treatments. After 30 d, the shoot length (1.64 cm) attained by mycorrhizal plantlets cultured under 60 µmol $m^{-2}s^{-1}$ PPFD was significantly higher than the other treatments (Table 2). Number of leaves (5.20) of mycorrhizal plantlets was significantly higher than the non-mycorrhizal plantlets at the same PPFD, but was comparable to number of leaves (5.03) of mycorrhizal plantlets at 40 µmol $m^{-2}s^{-1}$ PPFD (Table 2). Leaf area (0.77 cm^2 $leaf^{-1}$), FW (0.079 g) and DW (0.0068 g) were observed to be highest in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD (Table 2). No significant difference was recorded in number of roots

and root length of plantlets at the different PPFDs (Table 2). Besides affecting the growth and morphological characteristics of plantlets, PPFD levels also influenced total chlorophyll content. Highest concentration of chlorophyll content was observed in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD (Table 2). After 60 d of culture, significant increases in shoot length (1.71 cm), leaf area (0.90 cm^2 $leaf^{-1}$), FW (0.084 g), DW (0.0072 g) and total chlorophyll content (0.56 mg/g FW) in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD were recorded (Table 3). A significant difference in the number of leaves was observed in mycorrhizal and non-mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD. However, the number of leaves of mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD did not increase significantly but was slightly higher than mycorrhizal plantlets at 40 µmol $m^{-2}s^{-1}$ PPFD (Table 3). No significant difference was recorded in number of roots and root length of plantlets at the different PPFD treatments (Table 3). Similar pattern of growth response was also observed after 90 d of culture. Shoot length (1.75 cm), leaf area (1.13 cm^2 $leaf^{-1}$), FW (0.090 g) and total chlorophyll content (0.59 mg/g FW) of mycorrhizal plantlets increased significantly at 60 µmol m^{-2} s^{-1} PPFD (Table 4, Fig. 3). Number of leaves (6.33) and DW (0.0078 g) in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD were significantly higher than the non-mycorrhizal plantlets at the same PPFD (Table 4). Number of leaves and DW in mycorrhizal plantlets at 40 and 60 µmol $m^{-2}s^{-1}$ PPFD were, however, not significantly different (Table 4). Number of roots and root length did not undergo any significant change at the different PPFDs at 90 d of treatment (Table 4). Light intensity also appeared to affect development of stomata. An increase in number of stomata per unit area of leaf at 90 d of treatment was recorded with increasing light intensity (Table 4, Fig. 4). SEM examinations revealed that length of stomata was found to be maximum in mycorrhizal plantlets at 60 µmol $m^{-2}s^{-1}$ PPFD but light intensity did not cause any substantial effect on width of stomata (Table 4, Fig. 4).

Effect of *in vitro* PPFD treatment on the survival, growth and total chlorophyll content of hardened mycorrhizal and non-mycorrhizal plantlets

Mycorrhizal as well as non-mycorrhizal plantlets of *D. chrysanthum* cultured *in vitro* at different PPFD treatments were taken for hardening. Observations on survival percentage; growth parameters and total chlorophyll content were made after 120 d of transfer to the greenhouse. Highest percentage of survival (97.33%) was obtained with mycorrhizal plantlets transferred from 60 µmol $m^{-2}s^{-1}$ PPFD (Fig. 5). The growth characteristics *viz*., number of shoots (1.33), shoot length (3.72 cm), number of leaves (6.20), leaf area (3.52 cm^2 $leaf^{-1}$), number of roots (7), root length (5.30 cm), FW (0.369 g), DW (0.0371 g) and total chlorophyll content (0.55 mg/g FW) of mycorrhizal plantlets transferred from 60 µmol $m^{-2}s^{-1}$ PPFD were recorded to be highest as compared to the others (Table 5, Fig. 6).

The interactions between efficiency of mycorrhiza and light are complex as light not only influences the plant growth through photosynthesis, but also through its effects on other factors (Fitter and Garbaye, 1994). Associated mycorrhizal fungi enhance the ability of plants to overcome low availability of nutrient resources by improving the uptake of low-mobility nutrients. The fungi in return receive an estimated 4-20% of the net photosynthates for its growth and maintenance (Fitter, 1991; Smith and Read, 1997). In the present study, PPFD treatment of 60 µmol $m^{-2}s^{-1}$ was found to be optimum for root colonization (73%) by the fungal isolate 4634. The amount of light received by the host plant

is believed to directly influence the development of mycorrhiza. Concentration of root exudates and soluble carbohydrates in the roots are considered to be important for the establishment of mycorrhizal infection both of which are known to decrease at low irradiance (Ferguson and Menge, 1982; Johnson *et al.,* 1982). The reduced mycorrhizal colonization under low irradiance has also been hypothesized to be due to competition between the host plant and fungus for carbon (Tester *et al.,* 1985).

Higher irradiance on the other hand, has been reported to stimulate mycorrhizal infections in plants (Peyronel, 1940; Daft and El-Giahmi, 1978). The low percentage of fungal root colonization observed at 80 µmol $m^{-2}s^{-1}$ in the study could be due to competition for limited resources between the fungi and the plant as high light irradiance is known to cause photoinhibition which impairs the photosynthetic ability of the plant (Powles 1984).

The growth of mycorrhizal plantlets was observed to be better at 60 µmol $m^{-2}s^{-1}$ PPFD as compared to plantlets subjected to either lower or higher PPFD. Earlier, reports indicated that highest level of mycorrhizal colonization and hence greater photosynthesis and better growth of mycorrhizal plants occurred under the light conditions that were most favorable for photosynthesis and growth of the host plants (Prajadinata and Santoso, 1993; Bereau *et al.,* 2000). Under *in vitro* conditions, the growth responses in terms of shoot length, leaf area, FW and DW of mycorrhizal plantlets were consistently and significantly higher at 60 µmol $m^{-2}s^{-1}$ PPFD. Johnson *et al.,* (1982) observed greater growth, photosynthesis and stomatal conductance of mycorrhizal plants of sweet orange at higher light intensity. Increased plant growth under favorable light and temperature had also been reported as a result of mycorrhizal association in onions (Hayman, 1974). In the present study, number of roots and root length did not undergo any significant change at the different PPFD treatments, however, it was observed that at lower PPFD, the number and length of roots were slightly reduced.

Lower PPFD reduced the growth rate of root length probably by reducing the rate of initiation of lateral roots, and to a smaller extent by reducing the average rate of root tip extension. In this study, though, direct photosynthesis and stomatal conductance were not measured but significant increase in total chlorophyll content and number of stomata in mycorrhizal plantlets was observed at 60 µmol $m^{-2}s^{-1}$. Chlorophyll content has been suggested to be a good indicator of the photosynthetic apparatus status (Alvarez *et al.,* 2012). Jo *et al.,* (2008) observed that PPFD level of 30 µmol $m^{-2}s^{-1}$ was found to support maximum concentrations of chlorophyll "a", chlorophyll "b" and carotenoids in *Alocasia amzonica*.

Earlier, Jeon *et al.,* (2005) also reported similar increase in a CAM orchid, *Doritaenopsis* which had highest concentration of total chlorophyll at low and intermediate PPFD level compared to those grown at high PPFD levels. According to Demmig-Adams and Adams III (1992), at high PPFD of 90 µmol $m^{-2}s^{-1}$, decrease in chlorophyll and carotenoid contents could be correlated to accelerated degeneration of these pigments.

Additionally, in this study, the development of mycorrhizal association might have led to increased growth, photosynthetic pigments and photosynthesis of host plants by enhancing the availability of mineral nutrition. Mycorrhizal association was observed to enhance chlorophyll content in *Malus pruniolia* (Guan, 2007).

Table.1 Composition of oat meal agar medium (OMA-NC, modified from [15]) used for mycorrhizal association experiment

Constituents	mM
NH_4NO_3	1.0
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.4
KCl	0.9
Cellulose	2.0 g
Yeast Extract	0.1 g
Oat Meal Powder	3.0 g
Agar	6.0 g
pH	5.8

Table.2 Effect of PPFD on growth and total chlorophyll content in non-mycorrhizal and mycorrhizal plantlets of *Dendrobium chrysanthum* after 30 days of culture

Parameters	PPFD (µmol $m^{-2}s^{-1}$)							
	20		40		60		80	
	NM	M	NM	M	NM	M	NM	M
Shoot Length (cm)	1.35±0.03^{cd}	1.42±0.03^{bcd}	1.42±0.03^{bcd}	1.49±0.02^{b}	1.44±0.03^{bc}	1.64±0.02^{a}	1.24±0.04^{e}	1.33±0.03^{de}
No. of Leaves	3.75±0.13^{b}	3.93±0.15^{b}	4.00±0.14^{b}	5.03±0.12^{a}	4.06±0.14^{b}	5.20±0.17^{a}	3.63±0.12^{b}	3.73±0.12^{b}
Leaf Area cm^2 $leaf^{-1}$	0.47±0.02^{cd}	0.48±0.02^{cd}	0.48±0.01^{cd}	0.50±0.02^{bc}	0.55±0.02^{b}	0.77±0.03^{a}	0.41±0.01^{e}	0.44±0.01^{de}
No. of Roots	2.23±0.09^{b}	2.43±0.09^{ab}	2.33±0.09^{ab}	2.43±0.03^{ab}	2.36±0.09^{ab}	2.53±0.09^{a}	2.33±0.12^{ab}	2.36±0.11^{ab}
Root Length (cm)	0.55±0.01^{b}	0.56±0.01^{b}	0.73±0.03^{a}	0.76±0.03^{a}	0.72±0.03^{a}	0.79±0.04^{a}	0.55±0.01^{a}	0.56±0.01^{a}
Fresh Weight (g)	0.057±0de	0.065±0^{c}	0.061±0^{cd}	0.071±0^{b}	0.065±0^{c}	0.079±0^{a}	0.054±0^{e}	0.058±0de
Dry weight (g)	0.0042±0^{d}	0.005±0^{c}	0.005±0^{d}	0.0064±0^{ab}	0.0062±0^{bc}	0.0068±0^{a}	0.005±0^{d}	0.006±0^{bc}
Total Chlorophyll (mg/g fr. wt.)	0.37±0^{e}	0.41±0.01^{d}	0.41±0.3^{d}	0.45±0^{c}	0.47±0^{b}	0.49±0^{a}	0.26±0^{g}	0.31±0.01^{f}

NM-non-mycorrhizal; M-mycorrhizal; values are mean ± SE; means followed by different letters in the same row are significantly different at $p<0.05$

Table.3 Effect of PPFD on growth and total chlorophyll content in non-mycorrhizal and mycorrhizal plantlets of *Dendrobium chrysanthum* after 60 days of culture

Parameters	PPFD (µmolm^{-2}s^{-1})							
	20		40		60		80	
	NM	M	NM	M	NM	M	NM	M
Shoot Length (cm)	1.36±0.03^{d}	1.41±0.02^{cd}	1.46±0.02^{c}	1.57±0.03^{b}	1.46±0.03^{c}	1.71±0.04^{a}	1.24±0.04^{e}	1.33±0.03^{d}
No. of Leaves	4.03±0.15^{c}	3.96±0.14^{c}	4.60±0.16^{b}	6.03±0.13^{a}	4.83±0.17^{b}	6.13±0.21^{a}	3.63±0.12^{c}	3.73±0.12^{c}
Leaf Area cm^{2} leaf^{-1}	0.49±0.03^{de}	0.49±0.03^{de}	0.50±0.04^{cd}	0.55±0.02^{bc}	0.60±0.02^{b}	0.90±0.02^{a}	0.43±0.01^{f}	0.44±0.01^{ef}
No. of Roots	2.30±0.09^{b}	2.56±0.09^{ab}	2.46±0.09^{ab}	2.53±0.09^{ab}	2.50±0.09^{ab}	2.60±0.09^{a}	2.33±0.12^{ab}	2.36±0.11^{ab}
Root Length (cm)	0.56±0.01^{b}	0.58±0.01^{b}	0.73±0.03^{a}	0.79±0.03^{a}	0.74±0.03^{a}	0.80±0.03^{a}	0.55±0.01^{b}	0.56±0.02^{b}
Fresh Weight (g)	0.059±0^{d}	0.067±0^{bc}	0.065±0^{c}	0.073±0^{b}	0.070±0^{bc}	0.084±0^{a}	0.051±0^{e}	0.057±0^{d}
Dry weight (g)	0.0042±0^{f}	0.0059±0^{bc}	0.0053±0de	0.0064±0^{b}	0.0063±0^{bc}	0.0072±0^{a}	0.0049±0^{e}	0.0058±0^{bcd}
Total Chlorophyll (mg/g fr. wt.)	0.40±0^{e}	0.42±0.01^{de}	0.46±0^{cd}	0.49±0^{bc}	0.52±0.01^{b}	0.56±0.01^{a}	0.26±0.01^{g}	0.31±0.02^{f}

NM-non-mycorrhizal; M-mycorrhizal; values are mean ± SE; means followed by different letters in the same row are significantly different at $p<0.05$

Table.4 Effect of PPFD on growth, total chlorophyll content and stomatal attributes in non-mycorrhizal and mycorrhizal plantlets of *Dendrobium chrysanthum* after 90 days of culture

Parameters	PPFD (µmolm^{-2}s^{-1})							
	20		40		60		80	
	NM	M	NM	M	NM	M	NM	M
Shoot Length (cm)	1.39±0.03^{de}	1.45±0.01^{cd}	1.50±0.02^{c}	1.62±0.03^{b}	1.50±0.02^{c}	1.75±0.04^{a}	1.24±0.04^{f}	1.33±0.03^{e}
No. of Leaves	4.23±0.16^{c}	4.50±0.13^{bc}	4.73±0.14^{b}	6.26±0.14^{a}	4.96±0.21^{b}	6.33±0.22^{a}	3.63±0.12^{d}	3.73±0.12^{d}
Leaf Area cm^{2} leaf^{-1}	0.61±0.03^{c}	0.62±0.03^{bc}	0.62±0.03^{bc}	0.65±0.02^{bc}	0.71±0.03^{b}	1.13±0.06^{a}	0.43±0.01^{d}	0.44±0.01^{d}
No. of Roots	2.40±0.09^{cd}	2.73±0.08^{ab}	2.56±0.09^{bcd}	2.63±0.09^{abc}	2.66±0.09^{ab}	2.83±0.07^{a}	2.33±0.12^{d}	2.36±0.11^{d}
Root Length (cm)	0.57±0.01^{b}	0.59±0.01^{b}	0.75±0.02^{a}	0.80±0.03^{a}	0.75±0.03^{a}	0.83±0.03^{a}	0.55±0.01^{b}	0.56±0.01^{b}
Fresh Weight (g)	0.062±0de	0.068±0^{cd}	0.066±0^{d}	0.076±0^{b}	0.075±0^{bc}	0.090±0^{a}	0.049±0^{f}	0.056±0^{ef}
Dry Weight (g)	0.0054±0^{cd}	0.006±0^{c}	0.0062±0^{bc}	0.0071±0^{ab}	0.0064±0^{bc}	0.0078±0^{a}	0.0046±0^{d}	0.0054±0^{cd}
Total Chlorophyll (mg/g fr. wt.)	0.42±0 ^{e}	0.44±0^{d}	0.48±0.01^{c}	0.53±0.01^{b}	0.54±0.01^{b}	0.59±0^{a}	0.13±0.01^{g}	0.21±0.01^{f}
No. of Stomata (mm^{-2})	48.87±0.62^{g}	67.60±0.61^{f}	71.00±0.58^{e}	93.30±0.95^{d}	97.17±0.54^{c}	108.30±0.54^{b}	123.73±0.53^{a}	125.67±0.15^{a}
Length of Stomata (µm)	3.15±0.05^{c}	3.23±0.06^{bc}	3.20±0.05^{bc}	2.95±0.06^{d}	3.31±0.05^{b}	3.46±0.05^{a}	2.78±0.05^{e}	2.80±0.05^{de}
Width of Stomata (µm)	2.28±0.06^{cd}	2.26±0.05^{d}	2.44±0.07^{bc}	2.51±0.08^{b}	2.57±0.07^{ab}	2.71±0.06^{a}	2.10±0.04^{e}	2.20±0.05^{de}

NM-non-mycorrhizal; M-mycorrhizal; values are mean ± SE; means followed by different letters in the same row are significantly different at $p<0.05$

Table.5 Effect of *in vitro* PPFD on growth and total chlorophyll content in non-mycorrhizal and mycorrhizal plantlets of *Dendrobiumchrysanthum* after 120 d of hardening

Parameters	PPFD (µmolm^{-2}s^{-1})					
	20		40		60	
	NM	M	NM	M	NM	M
No. of shoots	1.00 ± 0^{b}	1.00 ± 0^{b}	1.00 ± 0^{b}	1.00 ± 0^{b}	1.00 ± 0^{b}	1.33 ± 0.09^{a}
Shoot Length (cm)	1.46 ± 0.02^{d}	1.73 ± 0.03^{c}	1.59 ± 0.01^{d}	1.84 ± 0.06^{c}	2.11 ± 0.04^{b}	3.72 ± 0.07^{a}
No. of Leaves	2.33 ± 0.09^{d}	2.50 ± 0.09^{d}	3.47 ± 0.1^{c}	3.77 ± 0.13^{c}	4.20 ± 0.14^{b}	6.20 ± 0.11^{a}
Leaf Area cm^{2} leaf^{-1}	0.76 ± 0.01^{c}	0.82 ± 0.02^{c}	0.82 ± 0.02^{c}	0.91 ± 0.02^{c}	1.20 ± 0.06^{b}	3.52 ± 0.12^{a}
No. of Roots	1.40 ± 0.09^{e}	2.00 ± 0.15^{d}	2.60 ± 0.09^{c}	2.90 ± 0.12^{c}	3.26 ± 0.16^{b}	7.00 ± 0.15^{a}
Root Length (cm)	0.52 ± 0.01^{d}	0.53 ± 0.02^{d}	0.68 ± 0.02^{cd}	0.88 ± 0.03^{c}	1.95 ± 0.06^{b}	5.30 ± 0.21^{a}
Fresh Weight (g)	0.045 ± 0^{d}	0.049 ± 0^{d}	0.060 ± 0^{cd}	0.068 ± 0.02^{bc}	0.081 ± 0^{b}	0.369 ± 0.01^{a}
Dry Weight (g)	0.0032 ± 0^{d}	0.0039 ± 0^{d}	0.0051 ± 0^{c}	0.0057 ± 0^{c}	0.007 ± 0^{b}	0.0371 ± 0^{a}
Total Chlorophyll (mg/g fr. wt.)	0.25 ± 0^{d}	0.26 ± 0^{cd}	0.27 ± 0^{cd}	0.30 ± 0^{bc}	0.32 ± 0^{b}	0.55 ± 0.03^{a}

NM-non-mycorrhizal; M-mycorrhizal; values are mean ± SE; means followed by different letters in the same row are significantly different at p<0.05

Fig.1 *Dendrobium chrysanthum* blooming in natural habitat

Fig.2 Percentage of root colonization under different *in vitro* PPFD treatments. Bars represent mean ± SE; bars with different letters are significantly different at $p<0.05$

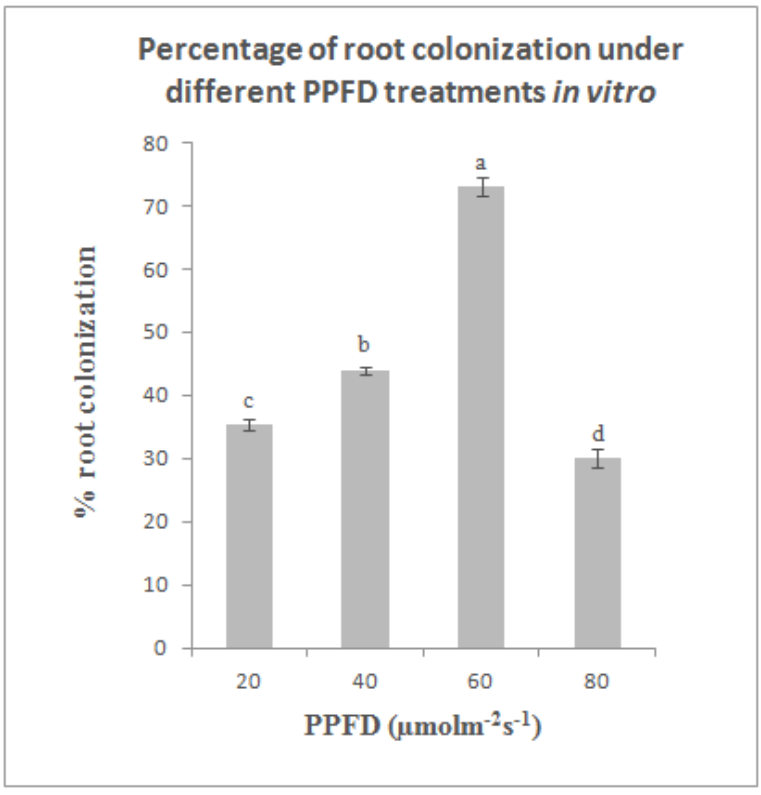


Fig.3 *In vitro* cultures of *Dendrobium chrysanthum*under 20, 40, 60 and 80 µmol $m^{-2}s^{-1}$ PPFD treatments after 90 d. Left – NM, non-mycorrhizal; right- M, mycorrhizal. Scale bars = 5 mm

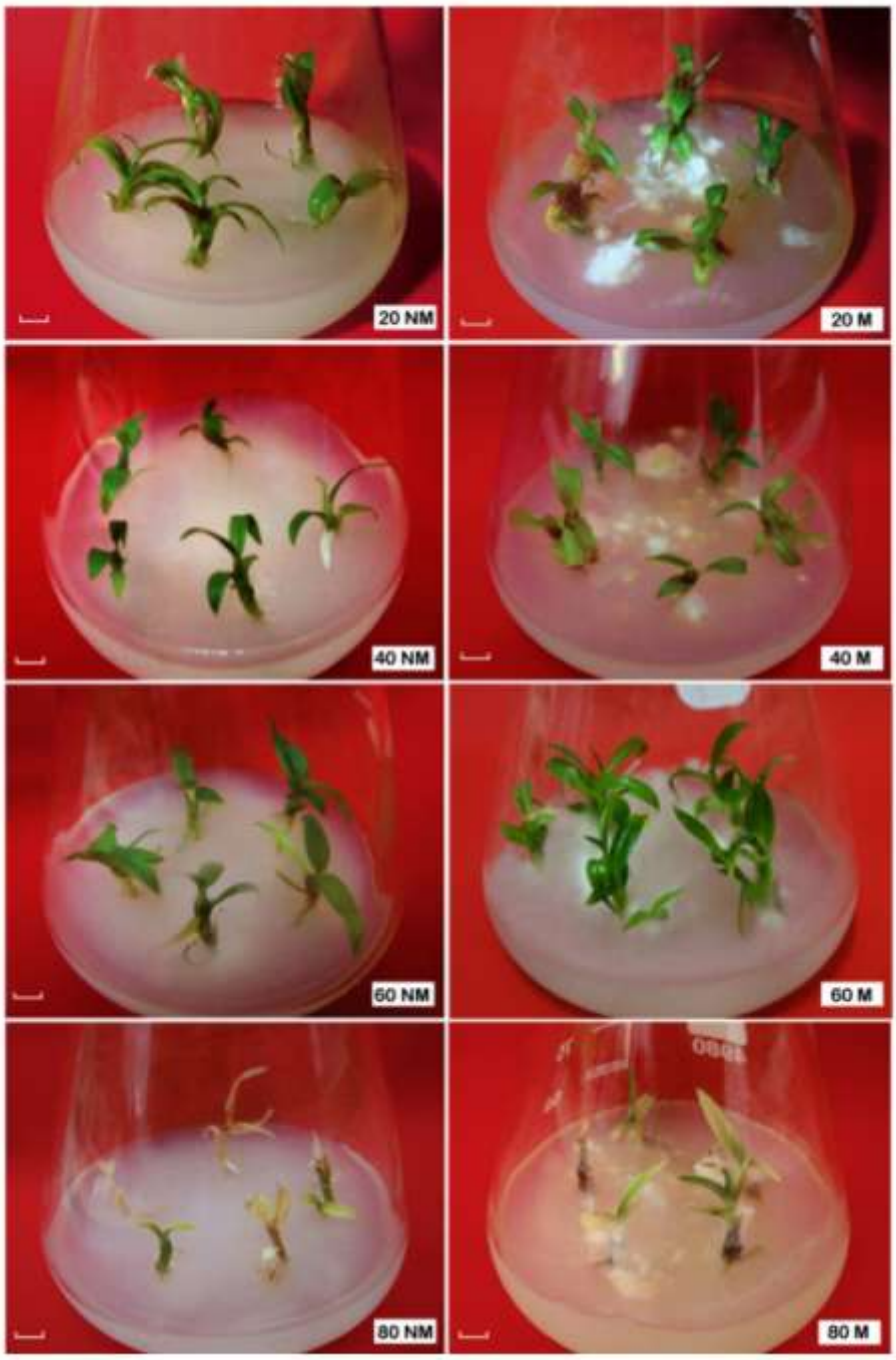


Fig.4 Scanning electron micrographs of lower epidermis of *Dendrobium chrysanthum* leaves after 90 d of *in vitro* culture under different PPFDs showing stomata. Left – NM, non-mycorrhizal; right- M, mycorrhizal

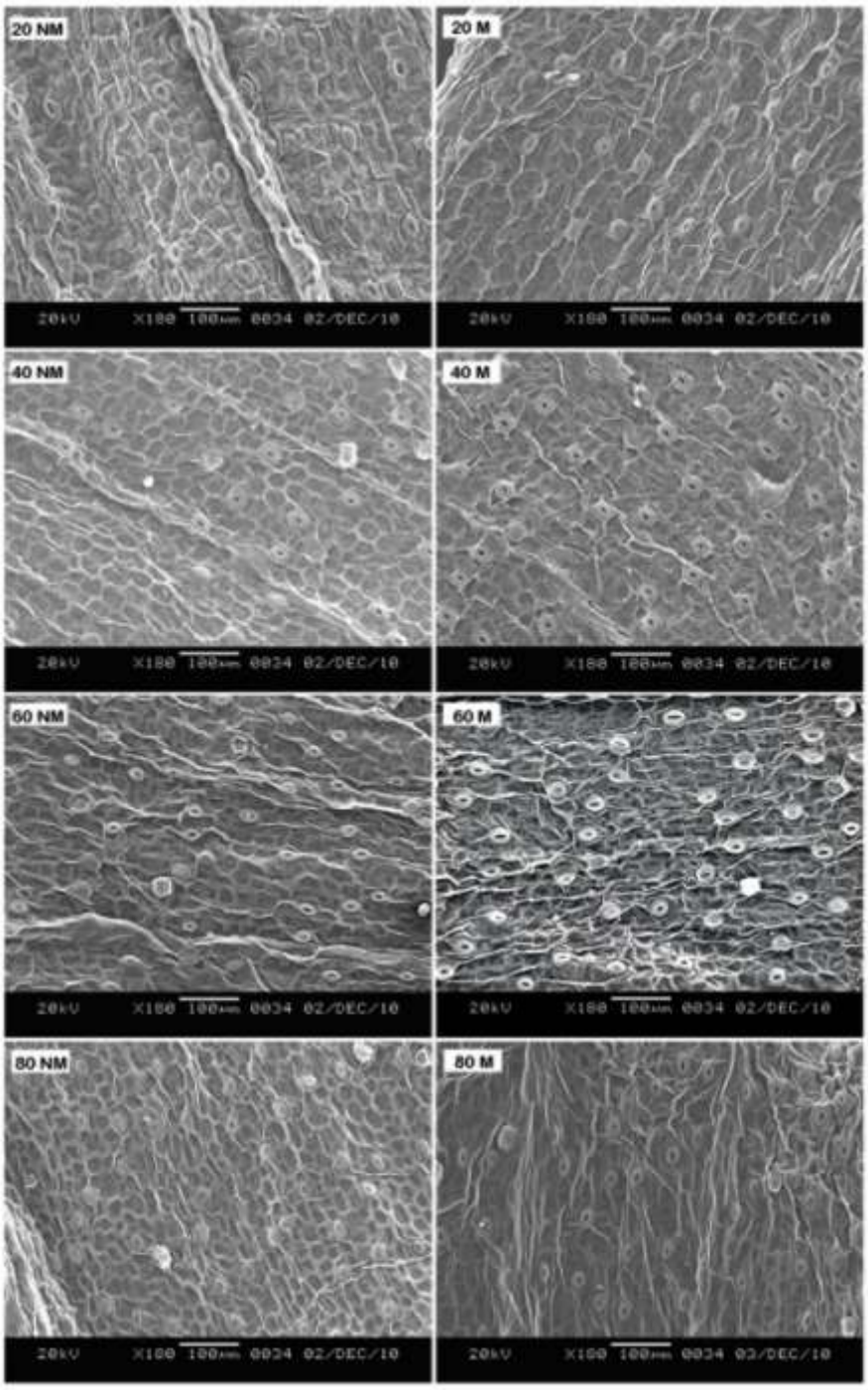


Fig.5 Effect of PPFD on survivability of non-mycorrhizal and mycorrhizal *Dendrobium chrysanthum* plantlets after 120 d of hardening. Bars represent mean ± SE; bars with different letters are significantly different at $p<0.05$. NM, non-mycorrhizal; M, mycorrhizal

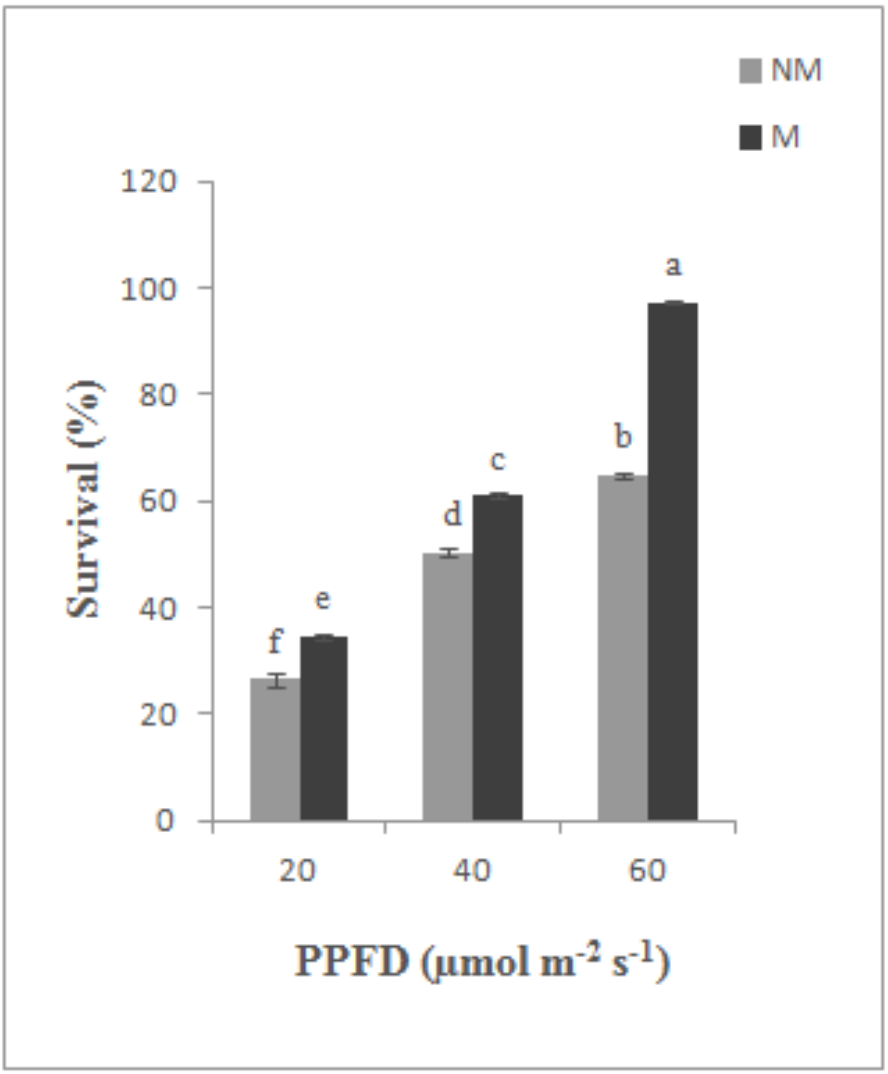
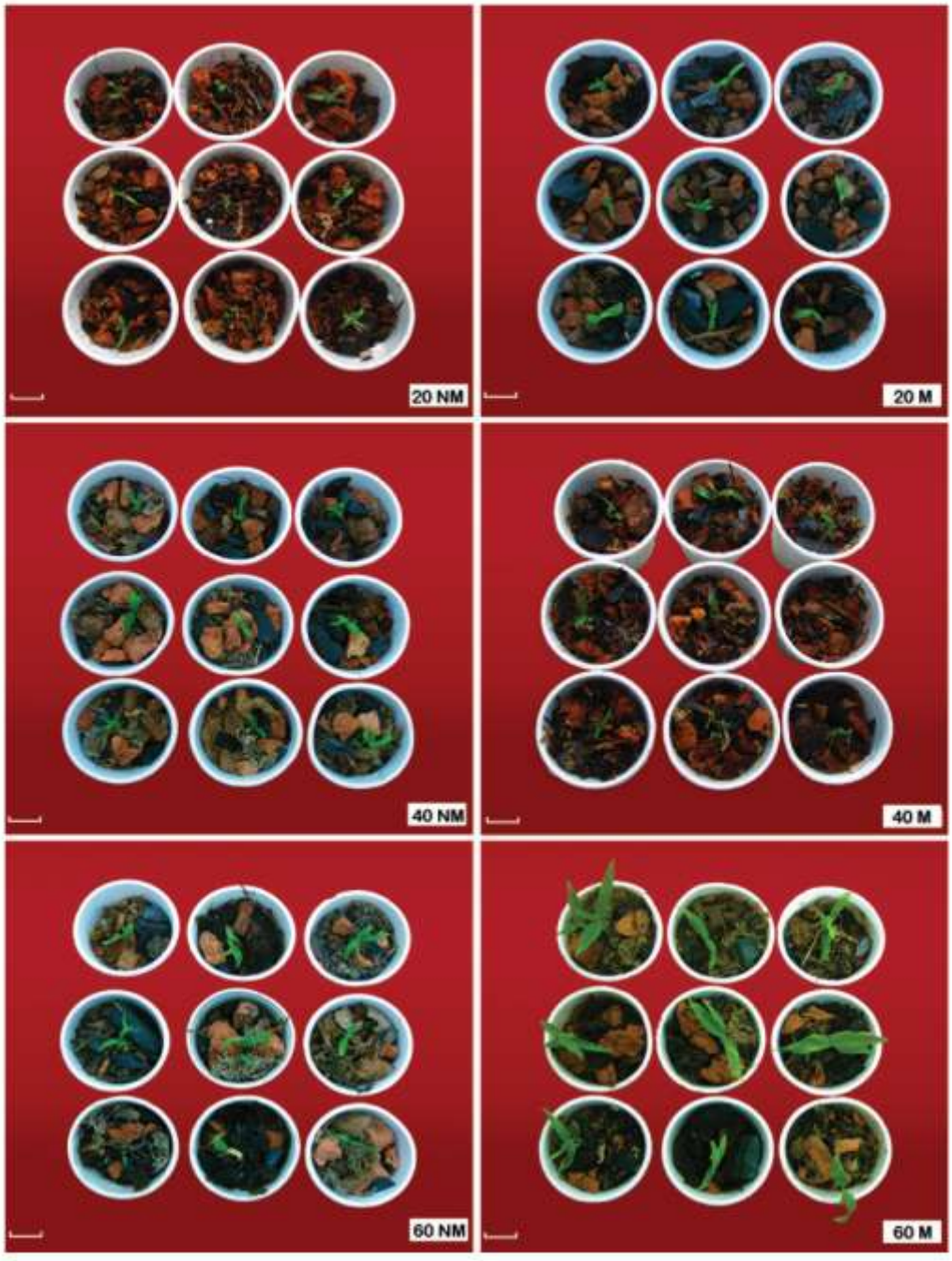


Fig.6 Plantlets after 120 d of hardening treated *in vitro* at 20, 40 and 60 µmol $m^{-2}s^{-1}$ PPFD. Left – NM, non-mycorrhizal; right- M, mycorrhizal. Scale bars = 5 mm



Contrary to the assumption that light intensity affects both the quantity and quality of stomata, in this study increasing light intensities only resulted in an observable increase in number of stomata per unit area of leaf and length of stomata while there was no significant effect on the width of stomata. Lee *et al.,* (2007) reported a progressive increase in number of stomata per unit area of leaf with increasing light intensities in *Withania somnifera*. In the present study, both width as well as length of stomata decreased slightly at 90 µmol $m^{-2}s^{-1}$ PPFD suggesting that the quantity of the stomata and not the quality is specifically under the influence of moderate light intensity.

For micropropagated plantlets, the acclimatization phase represents one of the most critical phases of micropropagation. Due to their unique characteristics, the *in vitro* plantlets could be subjected to high level of stress during this phase. In the present study, it was observed that inoculation of mycorrhizal fungi *in vitro* at optimal PPFD increased the survivability of micropropagated plantlets during acclimatization and enhanced their overall growth. Light intensity is one of the most important factors affecting *ex vitro* acclimatization of *in vitro* tissue culture plantlets (Hasegawa *et al.,* 1973). Alvarez *et al.,* (2012) showed that right amount of *in vitro* light treatment improved photochemical efficiency, developed mechanisms for excess light dissipation and improved morphological characteristics of *Gevuina avellana*. These characteristics accomplished during the *in vitro* process could help the plants endure stress during *ex vitro* transfer. The shock reaction to the abrupt change in irradiance during *ex vitro* transfer was lower in plants grown under higher PPFD *in vitro* (Kadlecek *et al.,* 2001). Further, in our study, the formation of mycorrhizal fungi might have been advantageous as orchid mycorrhizal fungi have been suggested to increase *ex vitro* survivability of orchid seedlings, enhance vegetative and morphological growth, induce early flowering, improve flower quality, and reduce disease infection (Chang 2008). Higher survival and growth rates of micropropagated mycorrhizal plants could be attributed to enhanced translocation of nutrients between root and shoot of the mycorrhizal plants (Osonubi *et al.,* 1991). Besides orchid mycorrhizal fungi, *Glomus etunicatum*, arbuscular mycorrhizal fungi have been reported to benefit acclimatization of several plants *viz*., anthurium, chrysanthemum and *Tapeinochilos ananassae* (Sohn *et al.,* 2003; Stancato and da Silveria, 2006; Oliveira *et al.,* 2011). *Ex vitro* shoot height and number of leaves were observed to be higher in arbuscular mycorrhizal fungi inoculated plantlets as compared to the non-inoculated plantlets of *Psidium guajava* and *Sesbania sesban* (Subhan *et al.,* 1998; Estrada-Luna *et al.,* 2000). Healthier and stronger roots were observed in micropropagted mycorrhizal plants of *Leucaena leucocephala* as compared to the non-mycorrhizal plants (Puthur *et al.,* 1998). In the present study, the growth characteristics of *D. chrysanthum* mycorrhizal plantlets transferred from 60 µmol $m^{-2}s^{-1}$ PPFD measured after 120 d of transfer were recorded to be highest. This seems to support the generally accepted presupposition that higher/optimal PPFD *in vitro* results in increased growth and better survival of micropropagated plantlets *ex vitro.*

To the best of our knowledge, this is the first report dealing with mycorrhizal association of *D. chrysanthum in vitro* at optimal PPFD which significantly produced the most marked growth response both under *in vitro* conditions and also during acclimatization under *ex vitro* environment. The integration of optimal PPFD and mycorrhizal association *in vitro* had an observable positive effect on the propagation of the medicinally important orchid *D. chrysanthum* both *in vitro* and *ex vitro*.

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Abbreviations

DW, dry weight; FW, fresh weight; M, mycorrhizal; NM, non-mycorrhizal; OMA-NC, oat meal agar medium; PPFD, photosynthetic photon flux density; SEM, Scanning Electron Microscope.

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