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Physiological, Photosynthesis, Biochemical Studies in Response to Different Photosynthetic Photon Flux Density During Acclimatization of Azalea Microshoots

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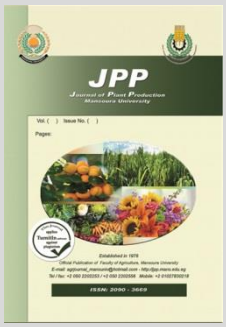

ABSTRACT

Depending on the importance of the acclimatization step during micropropagation of shrubs, the *ex-vitro* acclimatization of two azalea cultivars was investigated about the impacts of 50, 100, and 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). To investigate the influence of PPFD on azalea plants during *ex vitro* acclimatization, the morphological growth traits, photosynthetic indices, antioxidant enzymes, (reactive oxygen species) ROS, and Malondialdehyde (MDA) were studied. However, fresh and dry microshoot weight, plant height, and root length were recorded the better levels when treated with the highest PPFD (150 µmol m^{-2} s^{-1}). Also, microshoots cultivated in the highest levels of PPFD also had the best levels of net photosynthesis rate (NET), chlorophyll, and carotenoid with a decline in Fv/Fm values. *Ex-vitro formed* leaves had significantly higher pigment (chlorophyll and carotenoids) content than *in vitro*-grown leaves. Throughout the acclimatization phase, superoxide dismutase (SOD) activity increased. Similarly, increased activity of the enzymes catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) were also noted. These findings show that a level of PPFD at the highest level was appropriate for *Rhododendron* genus which includes azalea shrubs. The obtained results demonstrated that the capacity of plants to form an enzyme defense system that functions as an antioxidant, protecting them from oxidative stress and limiting the production of free radicals.

Keywords: Acclimatization, Azalea, PPFD, Light intensity, and ROS.

INTRODUCTION

The azalea shrubs, which belong to the family *Ericaceae,* have around 1000 officially documented cultivars, with numerous additional varieties in industrial circulation. (Meijón *et al.* 2011; Elmongy *et al.* 2020). China is currently increasing its commercial azalea production area to about 2500 hectares, serving landscape and home gardening needs (Zhou 2010). However, more than 300 cultivars are currently conserved across several nurseries in China (Zhou *et al.* 2013). Micropropagation has grown in popularity as a method for commercially mass-producing azaleas and rhododendrons (Elmongy *et al.* 2018). Advances in nodal explants and acclimatization breakthroughs in root generation under tissue culture conditions are credited by (Eeckhaut *et al.* 2010; Hsia and Korban 1997). To reduce the higher expenses associated with *in vitro* micropropagation in commercial plant production, Extensive attempts have been made to develop successful methods and media (Lei *et al.* 2015).

There are ~~found~~ a lot of factors that participated in the ornamental plant's development during *in vitro* and *ex-vitro* conditions, this factors including air condition, temperature, relative humidity, and net photosystem rate (Ahmed *et al.* 2020; Tian *et al.* 2014). Understanding the minimum, optimal, and ~~to~~ control in temperatures and light intensity can help the plants to grow faster and healthier more than changes in conditions (Kwon *et al.* 2018), because these variables affect which plant species may be produced productively in a certain place. This understanding becomes essential for optimizing crop production and selecting suitable plant species for specific environments. Furthermore, by understanding the function of these aspects, which belong a biotic stress, we can correctly identify plant problems (Elmongy *et al.* 2020).

To understand the factors that control plant development, it is necessary to know that light is essential in the environment since it is the primary source of energy for photosynthesis and other physiological functions in plants (Bian *et al.* 2015). Beyond photosynthesis, plants rely on light for regulating growth, directing development, and synthesizing chemical compounds (Li and Kubota 2009; Ahmed *et al.* 2020). Moreover, light management in horticultural plant development in vitro is a significant strategy for enhancing development, improving plant quality, and maximizing light usage efficiency. (Wang *et al.* 2016; Zhang *et al.* 2018). In this context, key factors influencing plant growth and development under *in vitro* conditions include light intensity, light quality, and photoperiod (Bantis *et al.* 2018; Bayat *et al.* 2018). Light intensity plays a crucial role in influencing various metabolic changes that are related to development and the photosynthesis process that converts CO_2 into carbohydrates, regulating biosynthesis in plants (Bayat *et al.* 2018). On the same trend, Light intensity directly impacts the transport of CO_2 and H_2O throughout both photosynthesis and transpiration operations. (Ahmed *et al.* 2020). PPFD had a positive effect on the radiation-use performance of plants by impacting the photosynthetic rate (Jayalath and van Iersel 2021), photosynthetic distribution

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(He *et al.* 2019), and chlorophyll content (LIU *et al.* 2014).

Previous data showed that the NET, CO_2 concentration during photosynthetic, stomatal conductance, and dehydrins (DHN) in lettuce and *Cucumis sativus*, increased with increasing light intensity (Ahmed *et al.* 2020; Hogewoning *et al.* 2010). According to Sago (2016), the total biomass of shoot, metabolic changes, morphological traits, and physiological parameters demonstrated an increase with the escalation of light intensity. Moreover, PPFD is one essential element that may be managed during the acclimatization stage since it can lead to a decrease in photosynthetic efficiency (photoinhibition), primarily due to oxidative damage to the photosystem II during natural conditions (Zhou *et al.* 2011). On the same trend, the content of carbohydrates and ascorbic acid was increased in plants treated with high levels of light intensity (Chen *et al.* 2016).

On the other hand, the shoots in micropropagation that are subjected to a biotic stress such as low or high light intensity beyond the optimal level for each plant, lead to notable morphological and physiological changes (Ahmed and Anis 2014b). The adaptation of plants *in vitro* requires swift adjustments in the function of antioxidant enzymes and compounds, closely correlated with phenotypic and environmental variations. During the adaptation process, plant cells rely on antioxidant metabolites which the antioxidant enzymes counteract the harmful effects of ROS (Kayihan *et al.* 2012; Xu *et al.* 2012).

Research has indicated significant fluctuations in the antioxidant systems throughout the *ex vitro* condition of Phalaenopsis, *Rauvolfia tetraphylla*, and *Tecomella undulate ex vitro* (Ali *et al.* 2005; Faisal and Anis 2009; Varshney and Anis 2012). Additionally, it has been observed that superoxide dismutase action initially stimulated in plant *Vitex trifolia* during the acclimatization stage, but gradually decreased with extended acclimatization time. SOD serves as a primary defense mechanism against reactive oxygen species, a crucial function given its ability to neutralize potentially damaging superoxide radicals (Ahmed and Anis 2014b).

Accordingly, this experiment was conducted to investigate two azalea varieties of microshoot that responded to various light intensity levels during the acclimatization stage. The morphological, physiological, photosynthetic, and biochemical effects of these azalea microshoots under various light conditions were investigated to determine which light intensity level was optimal and to comprehend how light exposure affected the growth and development of the azalea microshoots.

MATERIALS AND METHODS

Material for planting and growing conditions.

Azalea seeds (Zihudie and Mingchao cultivars) belong to the *Rhododendron genus* and are native to China and were germinated on half of Anderson's *Rhododendron* medium (Anderson, 1984). The nodal explants were taken after one month and transferred to multiplication media supplemented with 0.5 mg L^{-1} IAA with 1 mg L^{-1} Zeatin (Figure 1A). Shoots were rooted on a medium supplemented with 2 mg L^{-1} Indole-3-butyric acid for 8 weeks (Elmongy *et al.* 2018) (Figure 1B). For *ex vitro* acclimatization, azalea-rooted plants were transferred were put into plant pots with peat moss and perlite that were mixed according to volume (3:1). (Figure 1C). The time of this experiment was for 8 weeks with three different photosynthetic photon flux density levels (low PPFD 50 (LP), medium PPFD 100 (MP), and high PPFD 150 µmol m^{-2} s^{-1} (HP) with 16/8 h light–dark conditions). The air temperature in the experiment room was adapted to 26 ± 1 C and the humidity (RH) was adjusted for 98 % during 1st week, after that, it gradually declined by 7 %. During the acclimatization period, plants were irrigated every 4 days. This experiment was conducted at the Laboratory of the Ornamental Plants and Tissue Culture, Department of Horticulture, Zhejiang University, Hangzhou, China during the period between 2019-2020.

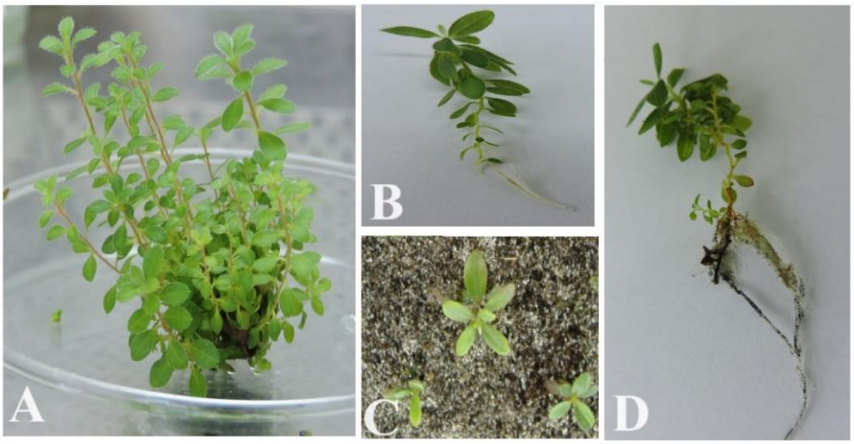

Fig. 1. The induction of multiplication azalea microshoots by nodal explants that transferred to media supplemented with 0.5 mg L^{-1} IAA with 1 mg L^{-1} Zeatin (A). The rooted microshoots after 8 weeks of rooted media supplemented 2 mg L^{-1} Indole-3-butyric acid (B-D). The peat moss/ perlite mixture (3:1, by volume) that used for *ex vitro* in azalea rooted plants (C).

To test the morphological parameters, after 56 days of transfer to the growth chamber, it was measured total fresh weight, dry weight, shoot length (cm), number of leaves/microshoot, and root length. It was collected leaves at (day 0, control) and 1, 2, 3, 4, 5, 6, and 7 weeks from transferring to culture media for the determination of different physiological, metabolic, and biochemical parameters.

Photosynthetic Chlorophyll quantitation

The chlorophyll fluorescence ~~in~~ on the abaxial side of newly cut-off leaf discs was determined, and for half an hour,

it was left the plants before calculation. Chlorophyll determination was made after incubating the plantlets in the dark for 30 min. The minimal fluorescence (F_0) was calculated after incubated in the dark at incident light of <0.1 µmol m^{-2} s^{-1}; maximal fluorescence (F_m) was determined after a 1-s saturating pulse (>3,500 µmol m^{-2} s^{-1}) in the same leaves. Maximal variable fluorescence (F_v=F_m−$_{F0}$) and photochemical efficiency of photosystem II (PSII) (Fv/Fm) were measured at adapted leaves after incubation in the dark (El-Mahrouk *et al.* 2016).

Net photosynthesis rate

The CO_2 levels were determined using a gas chromatograph (GC-2014, Shimadzu, Japan) using a heat conductivity sensor. A Poraplot Q capillary column (25 m × 0.53 mm, Hewlett Packard Co.) was used and Helium was employed as the transporting gas with a speed of at 13 mL min^{-1}. It was calculated the NET in the treated microshoots by the method of Shin *et al*. (2014).

Antioxidant enzymes extraction

Approximately 0.5 g of fully fresh tissues were collected every 7 days during the whole period of transfer to the acclimatization treatments. The plant material was homogenized in 4 ml of 0.1 M phosphate buffer (contained 2 mM EDTA + 1% PVP + pH 7.0 + at 4°C). Then it was centrifuged at 12000×g rpm for 10 min with 4°C temperature. The supernatant was stored at 4°C to use for measure enzyme activity. Three biological replicates were used to measure all enzymes.

Catalase (CAT, EC. 1.11.1.6) activity was tested using Góth (1991) technique. The reaction mixture had a total volume of 3 mL, which included 0.1 mL enzyme extract, 0.1 mL H_2O_2 (0.4%), and 2.8 mL phosphate buffer. The decrease in absorbance at 240 nm was measured when the level of inhibition H_2O_2 decreased.

According to Sheteiwy *et al*. (2017), superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity was assessed by assessing its suppression of the quantity of nitro blue tetrazolium (NBT). The reaction mixture had a total volume of 3.1 mL, which included 0.1 mL of enzyme extract and 3 mL of NBT solution. After adding 2mol L1 riboflavin, place the reaction tubes under 15 W fluorescent lamps for 15 minutes. The reaction mixture without any enzyme extract served as the control treatment. The volume of extract that induced 50% inhibition of NBT reduction was used to calculate one unit of SOD. Nitro-blue tetrazolium photoreduction was detected at 560 nm.

Glutathione reductase (GR, EC 1.6.4.2) activity was calculated using Rao (1992) technique. The measurement was at 340 nm depending on the oxidation of nicotinamide adenine dinucleotide phosphate NADPH. The substances of the reaction consisted of 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM NADPH, and 0.5 mM glutathione disulfide (GSSG). To start the reaction, it was using 0.1 mL extraction of enzyme then the mixture was left for 5 min at 25°C.

GPX antioxidant enzyme was measured using Tappel (1978) technique. The reaction mixture was formed of 9.2 mL of buffer (5 mM potassium HEPES, containing 1 mM EDTA with 1 mM NADPH). Then it was added 100 µL of 100 U mL^{-1} glutathione reductase, 50 µL of 200 mM glutathione (GSH), and 95 µL of 10 mM potassium cyanide. To start the reaction, it was using 5 µL of 0.042 % (w/w) H_2O_2 solution to each well. The absorbance changes were measured at 340 nm (ten minutes). The GPX enzyme activity was determined in terms of µmol mL^{-1} min^{-1}.

Estimation of Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents

To measure hydrogen peroxide (H_2O_2) contents, the extraction of microshoots (0.5 g each) was by 5.0 mL of 0.1 % TCA, then samples were centrifuged at 12,000 xg for 20 minutes (Perveen and Anis 2015). The reaction mixture (0.5 mL) was mixed with 10 mM potassium phosphate buffer (0.5 mL+ pH 7.0) by adding 1 mL of 1 mM KI. It was used 390 nm to calculate the supernatant, and it was using the standard curve to calculate the H_2O_2 levels. The extra-cellular hydroxyl radical's concentration was measured according to Halliwell *et al.* (1987) protocol, in which microshoots (0.5 g each) were incubated for two hours at 37 °C by adding 15 mM of 2-deoxy-D-ribose (pH 7.5). After that, the supernatant (0.7 mL) consisted of 3 mL of reaction substances which have 0.5 % *(w/v)* thiobarbituric acid (TBA, 1 % stock solution made in 5 mM NaOH) and 1 mL glacial acetic acid. Finally, the measurement was taken in which all mixed samples were heated at 99 °C by immersion for half an hour then the mixture was cooled down to 40 °C for 10 min

MDA levels were estimated as 2-thiobarbituric acid (TBA) reactive metabolites according to the method of Heath and Packer (1968) where 1.5 mL of extract solution was added to 2.5 mL of 5% TBA made in 5% trichloroacetic acid (TCA). After that, it was used a water bath at 99 °C for 20 minutes to mix the reaction substances, then, it was cooled down to 30 °C. The calculation of MDA was at 532 nm following the supernatant was centrifuged at 6,000 xg for 12 minutes. The measuring of correction of nonspecific turbidity was made at 600 nm.

Statistical analyses

The experiments were achieved as a factorial experiment in CRD. About 30 microshoots per application were set up, in addition, the experiments were done twice. The obtained results were analyzed by analysis of variance (ANOVA) way using SPSS version 16 (SPSS Inc., Chicago, USA). Duncan's multiple range test was used to test the significance of differences between means (< 0.05). The results were presented as the mean of two experiments ± SD.

RESULTS AND DISCUSSION

Results

Effect of light intensity on the morphological parameters of *in vitro* of two different Azalea cultivars during acclimatization

The data presented in Table 1 showed that increasing PPFD treatments generally results in increased fresh and dry biomass, micro-shoot length leaves numbers/shoot, and root length for both cultivars. The cultivar 1 showed a more significant increase in most parameters compared to cultivar 2 as the PPFD treatment increased. It is also notable that in the highest PPFD application (150 µmol m^{-2} s^{-1}), cultivar 1 generally outperforms cultivar 2 for most parameters, particularly in terms of all morphological traits that were tested. Overall, these results suggest that increasing PPFD treatments has a positive impact on the morphological parameters of both cultivars, with cultivar 1 exhibiting more pronounced responses to higher light intensities compared to cultivar 2.

Table 1. The effects of different PPFD treatments on the morphological parameters of *in vitro* of two different Azalea cultivars during acclimatization.

Azalea cultivar	PPFD treatment (µmol m^{-2} s^{-1})	Fresh weight/plantlets (g)	Dry weight/plantlets (g)	Shoot length (cm)	Leaves number /plant	Root length (cm)
Cultivar 1	50	3.76 bc	0.53 bc	4.66 c	12.66 b	7.22 bc
	100	3.95 b	0.71 ab	5.22 b	11.66 bc	8.34 b
	150	4.32 a	0.78 a	5.42 ab	14.33 ab	9.13 a
Cultivar 2	50	3.55 c	0.47 c	4.33 d	11.00 c	6.28 cd
	100	3.67bc	0.52bc	4.76 c	11.66 bc	6.87 c
	150	3.95 b	0.67 b	5.12 bc	15.33 a	7.14 bc

Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at P ≤ 0.05

PPFD effects on photosynthetic chlorophyll quantitation and carotenoids of *in vitro* of two different Azalea cultivars during acclimatization stage

According to data provided in Table 2, it was demonstrated that the different levels of PPFD have distinct effects on the chlorophyll pigment, and carotenoids, in addition, the highest levels of photosystem II (Fv/Fm) within the two Azalea cultivars during the acclimatization process. For both cultivar 1 and cultivar 2, it can be observed that higher PPFD levels correspond to increased chlorophyll and carotenoid content, as well as an elevated Fv/Fm ratio. This suggests that higher light intensity positively influences the photosynthetic pigments and efficiency in Azalea plants during acclimatization.

Table 2. The effects of PPFD application on chlorophyll pigment, carotenoids, and photosystem II (Fv/Fm) of *ex vitro* of two Azalea cultivars during acclimatization.

Azalea cultivar	PPFD treatment (µmol m^{-2} s^{-1})	Chlorophyll content (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Fv/Fm
Cultivar 1	50	1.89 c	0.44 ab	0.92 a
	100	1.99 b	0.37 bc	0.89 ab
	150	2.33 a	0.49 a	0.81 b
Cultivar 2	50	1.24 e	0.32 c	0.82 b
	100	1.77 d	0.22 d	0.74 c
	150	1.98 b	0.33 c	0.68 cd

Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at P ≤ 0.05

In addition, it showed significant differences in both azalea cultivars in their response to different levels of PPFD. It was noticed that under the highest PPFD application cultivar 1 tends to outperform cultivar 2 in terms of chlorophyll content and carotenoids, but the Fv/Fm ratio was recorded as the best content under the low PPFD treatment. These distinctions are crucial to consider in practical applications and cultivation practices for optimal productivity and plant health. Generally, the results emphasize the importance of light intensity management during the acclimatization of Azalea plants and highlight the significance of cultivar-specific responses in optimizing growth and physiological attributes.

PPFD effects on net photosynthesis rate of two different Azalea cultivars during acclimatization

The data presented in Figure 2 indicated clear variations in the net photosynthesis rate of the two Azalea cultivars during the acclimatization process under different levels of PPFD. Both cultivar 1 and cultivar 2 exhibit a noticeable trend wherein the lowest PPFD levels (50 µmol m^{-2} s^{-1}) are associated with an elevated net photosynthesis rate.

PPFD treatment at the lowest application (50 µmol m^{-2} s^{-1}) had differing effects on the two cultivars. Cultivar 1 initially experienced a decrease in net photosynthesis rate at 7 days, followed by an increase at 14 days, a subsequent decline reaching its lowest point at 28 days, and then a gradual rise to the maximum at 56 days. On the other hand, cultivar 2 showed a reduction in net photosynthesis rate at the low PPFD treatment at 21 days, followed by a gradual decline reaching its peak at 56 days.

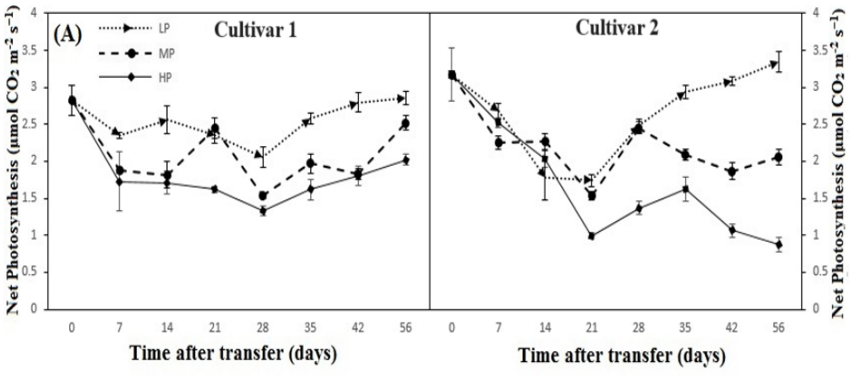

Fig 2. The effects of different PPFD treatments on net photosynthesis rate of *in vitro* of two different Azalea cultivars during acclimatization. Values represent mean ± standard deviation.

At the medium PPFD treatment (100 µmol m^{-2} s^{-1}), cultivar 1 demonstrated that a gradual decrease at 14 days, followed by an increase to its highest value at 21 days, a subsequent decrease reaching its minimum at 28 days, and a gradual increase to the maximum at 56 days.

Similarly, cultivar 2 at the medium PPFD treatment showed a gradual decline, reaching its lowest value at 21 days, followed by an increase to its highest value at 28 days, and then a gradual decrease until 42 days before a slight increase at 56 days. Furthermore, under the high PPFD treatment (150

µmol m^{-2} s^{-1}), cultivar 1 showed a gradual decline, reaching its lowest value at 28 days, followed by a gradual increase to the maximum at 56 days. In cultivar 2, the high PPFD treatment also led to a gradual decrease, reaching its lowest value at 21 days, followed by a gradual increase at 35 days, and then a subsequent gradual decrease until reaching its lowest value at 56 days.In general, the data presented indicates that both cultivar 1 and cultivar 2 exhibited significant responses to variations in light intensity over 56 days. However, the low PPFD treatment demonstrated the best results in net photosynthesis rate compared to the other treatments.

PPFD effects on antioxidant enzymes of *in vitro* of two different Azalea cultivars during acclimatization

Based on the results presented in (Fig. 3A-D), in both cultivars, all the treatments resulted in a gradual increase in both enzymatic antioxidants (CAT and SOD) and non-enzymatic antioxidants (GR and GPX) over 56 days. In both cultivar 1 and cultivar 2, the highest catalase (CAT) values were observed in response to the high PPFD treatment particularly at 56 and 42 days, respectively (Fig. 3A). Conversely, the lowest CAT value for both cultivars was recorded when the plants treated with PPFD at the lowest level at 7 days. Additionally, the medium PPFD treatment (MP level) for both cultivars showed significant differences from the other treatments at 35, 42, and 56 days. In terms of both cultivars, medium PPFD treatment and the low PPFD treatment affected the CAT activity at 7, 14, 21, and 28 days there without any significant effects, except for the interval at 21 days for cultivar 2.

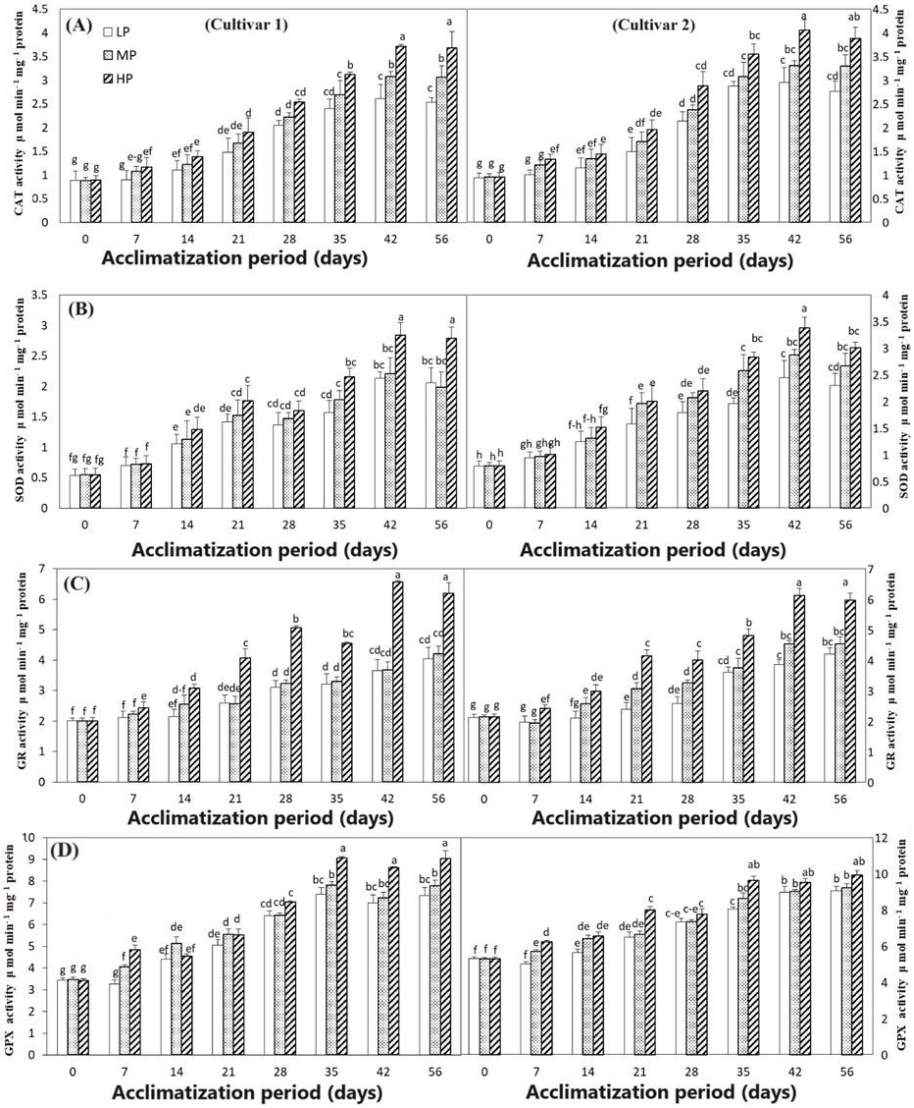

Fig 3. The effects of different PPFD treatments on catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPX) of two different Azalea cultivars during *in vitro* acclimatization. Values represent mean ± standard deviation. Lowercase letters represent significant differences among treatments ($p<0.05$).

Based on the results obtained over a 56-day experimental period, the treatment with the highest PPFD (HP level) yielded the highest level in superoxide dismutase (SOD) for both cultivars, followed by the medium PPFD (MP) and then the low PPFD (LP) treatments, respectively (Fig. 3B). In cultivar 1, no significant effect in SOD levels recorded among the medium PPFD and low PPFD treatments at all intervals, except at the intervals of 21 and 35 within the 56-days. Similarly, in cultivar 2, there between the medium PPFD and low PPFD treatments affected the SOD levels at 7 and 14 days, while significant differences were observed at the remaining time points. These results indicated that cultivar

1 exhibited greater responsiveness to different PPFD levels compared to cultivar 2.

The different PPFD levels resulted in a gradual increase in glutathione reductase (GR) levels throughout all measured periods (Fig. 3C). However, the high PPFD treatment (150 µmol m-2 s-1) induced the highest significant difference of GR compared to the medium (MP level) and low PPFD (LP level) treatments, respectively, around the entire experimental duration. No statistically significant difference was observed between the high PPFD treatments for both cultivars at 21, 42, and 56 days. Similarly, no significant difference was detected between the medium PPFD and low PPFD treatments in cultivar 1 across all periods, except for the 14-day interval. Contrastingly, cultivar 2 displayed significant differences in all periods, except at 7 and 56 days, between the medium PPFD and low PPFD treatments. The 14-day interval, however, exhibited a significant difference between the medium PPFD and low PPFD treatments.

According to the results presented in (Fig. 3D), different levels of PPFD had noticeable progressive glutathione peroxidase (GPX) levels during the study period. Plants treated with the highest PPFD (HP level) showed the highest GPX levels within both cultivars, followed by the medium PPFD (MP level) and the low PPFD (LP level) treatments, correspondingly throughout study. Additionally, a statistically significant difference was observed in the response range of the two cultivars to the applied light levels, with cultivar 1 showing a more pronounced response in increasing GPX levels compared to cultivar 2. Conversely, treated plants with medium PPFD and low PPFD levels at 28 and 42 days in cultivar 1 and at 21, 28, 42, and 56 days in cultivar 2 during the study period without any significant differences among means

In total, during the 56-days, the findings indicated that the applied levels of PPFD had a significant effect on both enzymatic antioxidants (CAT and SOD) and non-enzymatic antioxidants (GR and GPX) levels, under-treated with PPFD treatment (HP level) consistently affecting the best response, especially cultivar 1. Furthermore, the observed differential response between the plant varieties emphasizes the importance of considering species-specific reactions when studying the influence of light intensity on biochemical processes.

Effect of light intensity on hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents of *in vitro* of two different Azalea cultivars during acclimatization

As depicted in (Fig. 4A), the malondialdehyde (MDA) content for all treatments showed a progressive increase until reaching its peak at 14 days, followed by a gradual decline to reach the end of tested days in both cultivars. However, the high PPFD (LP level) treatment recorded low MDA content at 42 days of the entire period in cultivar 1. In both cultivars, the high PPFD treatment recorded the lowest MDA content as compared with the medium PPFD (MP level) and low PPFD (LP level) treatments over the 56-days. Conversely, there was no significant difference between the medium PPFD and low PPFD at all intervals, except at 35 days in cultivar 1, and at 7 and 56 days in cultivar 2 throughout the entire study period.

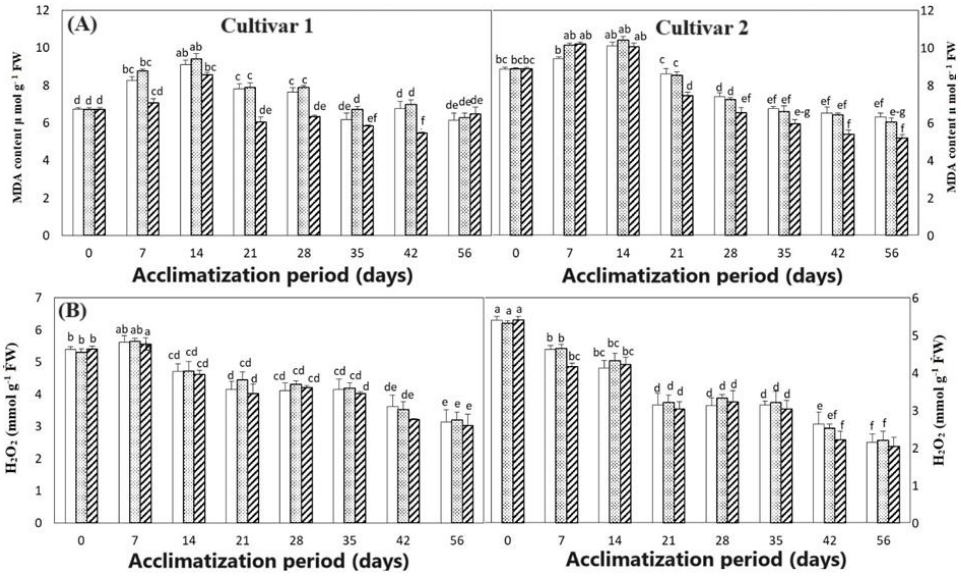

Figure 4. The effects of different PPFD treatments on Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents of *in vitro* of two different Azalea cultivars during acclimatization. Values represent mean ± standard deviation. Letters indicated the significant differences among treatments ($p<0.05$).

As for hydrogen peroxide (H_2O_2), it followed a similar pattern to MDA content, with all treatments leading to an increase in H_2O_2 content until reaching its peak at 7 days, followed by a gradual decrease to its lowest value at 56 days in cultivar 1 (Fig. 4B). Conversely, cultivar 2 exhibited the highest H_2O_2 content at day 0, which then gradually decreased until stabilizing at 21, 28, and 35 days, followed by a further decline towards its lowest value at the end of the entire period. All treatments at 14, 28, and 56 days in cultivar 1 affected H_2O_2 without any significant difference among means. Similarly, there was no significant difference between all treatments in cultivar 2, except at 7 and 42 days during the entire study period. However, there was a significant difference between the high PPFD (HP level) treatment and the other applications, as the high PPFD treatment yielded lower contents of H_2O_2 compared to the other treatments throughout the entire period in both cultivars. Overall, these findings underscored the influence of light intensity on oxidative stress markers in plant tissues, with potential implications for plant physiological responses.

Discussion

In micropropagation, the step of transplanting microshoots from tissue culture media to acclimatization condition is critical step in most plants (Ahmed and Anis 2014a), because this operation requires time and expense which makes the commercial production of tissue culture not easy (Fila *et al.* 1998). During the acclimatization process, the plants were adapted to external environment stresses (Ahmed *et al.* 2020). In addition, many morphological and physiological changes were in relationship with water content in microshoots during the *ex-vitro* process, in addition, controlling the photosynthetic system is an important factor during the acclimatization condition (Aragón *et al.* 2014). It was found in several plants growing in tissue culture that newly generated leaves replace *in vitro*-made leaves that cannot continue developing in *ex-vitro* environments (Perveen and Anis 2015). When comparing the micropropagated microshoots in *Nicotiana tabacum* to acclimated shoots, it was found that the *ex vitro* microshoots had an effective morphological trait like an increase in tall, a higher dry mass, and the largest leaf numbers. However, if the acclimatization process of microshoots is effective, their growth can increase significantly(Pospóšilová *et al.* 1999). Knowing the best temperatures as well as light intensity needed for plant growth and development can be helpful because these parameters are an indicator for plants that can be acclimated successfully (Kwon *et al.* 2018; Håkansson *et al.* 2002). It was reported that light intensity has a great effect on growth parameters during ex vitro of several species (Ali *et al.* 2005; Bantis *et al.* 2018; Bayat *et al.* 2018; Hogewoning *et al.* 2010; Jayalath and van Iersel 2021; Li and Kubota 2009). This result was in line with what we currently found as the increase in PPFD treatments enhanced the morphological parameters of both azalea cultivars.

The plant microshoots grown under *in vitro* condition has a low chlorophyll content Thus, ex Transplanting low-irradiance plants is recommended to ensure proper acclimatization of *in vitro-produced* plants (Perveen and Anis 2015). However, the process of acclimatization is genotype-dependent. After being transplanted *in vitro*, the chlorophyll content of several plant species has gradually increased (Ashrita *et al.* 2023). It was reported in many plants that plantlets that were exposed to high levels of radiation after transplanting resulted in photoinhibition, in addition to the pigments' photo-bleaching (El-Mahrouk *et al.* 2016). In addition, the microshoots treated with high levels of PPFD recorded a rise in photosynthetic activity. However, this poses a risk of ROS generation if the collected energy cannot be replaced chemically. (Matysiak 2004).in our results, it was observed that the high levels of PPFD were about the high chlorophyll content. Previous studies demonstrated that low PPFD is caused by photoinhibition after transplanting the micropropagated plant(Zhou *et al.* 2004). During the acclimatization process, it was also observed similar declines in Fv/Fm with rising PPFD in rhododendron (Matysiak 2004), *Dieffenbachia* (El-Mahrouk *et al.* 2016), *Albizia lebbeck* (Perveen and Anis 2015) and *Cassia alata* (Ahmed and Anis 2014a). It has been suggested that this result is due to weakly differentiated chloroplasts of *in vitro* obtained microshoots (Lee *et al.* 1985). Our results showed a similar trend in which The Fv/Fm was highest with low PPFD (50 µmol m^{-2} s^{-1}) on both azalea cultivars and Fv/Fm values declined with increased PPFD (Table 2).

Acclimatization of micropropagated plants concerns an increase in the action of the antioxidant enzymes, which directly affects the plants' life and performance by preserving the equilibrium between the production and scavenging of ROS (de Souza *et al.* 2021; Gonçalves *et al.* 2017). Free radicals (ROS) are scavenged by plants using enzymatic and antioxidant scavenging systems like SOD, CAT, APX, and GR as a defense against extremely harmful free radicals (Xu *et al.* 2012). The growth of microshoots during acclimatization was also regulated depending on these mechanisms. Previous studies indicated that ROS increased in chloroplasts within a biotic stress (high LIGHTING) (Mishra *et al.* 1995). Plants will activate a light defensive mechanism and different antioxidant enzymes, including SOD, CAT, and APX, when ROS formed in cells, this was corroborated with our study, where higher PPFD was linked to higher SOD, CAT, and GPX levels in both azalea cultivars (Figure 3). Additionally, during the early period of acclimatization progress, the CAT activity increased which supports that CAT could scavenge H_2O_2 and turn it into O_2 and H_2O in peroxisomes (Zhao *et al.* 2006). We can suggest that microshoots of azalea plants might be able to protect themselves against oxidative stress by raising the CAT activity (figure 3A). Similar observations on elevated SOD activity during the acclimatization period have been reported by de Souza *et al.* (2021). In addition, with high levels of PPFD, the enhanced CAT, SOD, CAT, and GPX activity has been documented which they able to reduce ROS in several plants Dieffenbachia cultivars (Figueiredo *et al.* 2021; Ahmed *et al.* 2020; Ahmed and Anis 2014b, a; Perveen and Anis 2015). Our data demonstrate that effective photosynthetic machinery developed in acclimated microshoots significantly decreased oxidative stress throughout the acclimatization period.

CONCLUSION

Treated the microshoots with high concentrations of PPFD (150 µmol m^{-2} s^{-1}), which had an impact on the plant biomass, height, root length, carotenoids, and chlorophyll content. Furthermore, the antioxidant system of platelets during acclimatization was thoroughly examined, and our findings show that oxidative stress is caused by increased PPFD. The first line of protection from ROS is thought to be SOD. According to reports, superoxide converts ferric ions to ferrous ions, which subsequently combine with H_2O_2 to produce. As a result, it contributes to the resistance and survival of microshoots by maintaining membrane integrity, inhibiting ROS and MDA, and preventing oxidative damage throughout the acclimatization process. All things considered, the data suggested that the azalea plants' effective field micropropagation system was the consequence of physiological changes brought on by a slow process of environmental adaptation. As a result, the acclimatization process outlined here along with the previously published micropropagation strategy offers a viable method for decreasing reliance on natural plant stands for medicinal applications while simultaneously contributing to plant conservation. During acclimatization, the tissue-cultured plantlets also formed a functioning photosynthetic apparatus and an antioxidant enzymatic protection mechanism. This strategy could also serve as a springboard for acclimating other economically and medicinally significant plants before establishing them in the field.

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الاستجابة في التغيرات الفسيولوجية والضوئيه والبيوكيمائيه تحت مستويات شده الاضاءه المختلفه اثناء عمليه تاقلم نباتات الازاليا

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الملخص

استنادا إلي أهمية خطوة الأقلمه أثناء عمليه الإكثار الدقيق لشجيرات الزينه، تمت دراسة عمليه الاقلمه لصنفين من الأزاليا التابعه لجنس الرودودينرون وذلك تحت مستويات مختلفه من شده الاضاءه (50 و100 و150 ميكرومول /م^2 / ثانيه كثافة الفوتون الضوئي) . لدراسه تأثير مستويات شده الاضاءه المختلفه على نباتات الأزاليا أثناء عمليه الاقلمه، تمت دراسة سمات النمو المورفولوجية ومؤشرات التمثيل الضوئي والإنزيمات المضادة للأكسدة ونواتج الشوارد الحره. في ظل أعلى مستوي من شده الفوتونات الضوئيه (150 ميكرومول م^2 ث$^{-1}$)، تم الحصول على اعلي مستويات من وزن للنباتات الطازجة والجافة، وارتفاع النباتات، وطول الجذور في كلا الصنفين. في ظل ظروف الاقلمه انتجت النباتات أيضًا أعلى مستويات لصبغات البناء الضوئي (الكلوروفيل، وتركيزات الكاروتينويد مع انخفاض في قيم Fv/Fm) وذلك عند مقارنتها بالأوراق المزروعة في ظروف معمل زراعه الانسجه، حيث كان محتوى الصبغات (الكلوروفيل والكاروتينات) في الأوراق الماقلمه أعلى بكثير. لوحظ ايضا خلال عمليه الاقلمه، زياده نشاط الانزيمات المضاده للتاكسد حيث زاد نشاط انزيم الديسميوتيز (SOD) . وبالمثل، لوحظ أيضًا زيادة نشاط إنزيم الكتاليز(CAT) ، وبيروكسيداز الأسكوربات(APX) ، والجلوتاثيون المختزل (GR) . اظهرت هذه التجربه ان مستوي شده الاضاءه الاعلي كان مناسبًا لجنس الأزاليا والرودودندرون. وأظهرت النتائج التي تم الحصول عليها قدرة النباتات على تكوين نظام دفاع إنزيمي يعمل كمضاد للأكسدة، ويحميها من الإجهاد التأكسدي ويحد من إنتاج الشوارد الحرة.

الكلمات الداله: الاقلمه, الازاليا , تدفق الفوتون الضوئي, شده الاضاءه, الشوارد الحره