

Full Length Research Paper

Biochemical and histological changes in *Dendrobium sonia*-28 protocorm-like bodies (PLBs) at various low temperatures and under mineral oil layer

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The study was conducted on the storage of *Dendrobium sonia*-28 protocorm-like bodies (PLBs) at various temperatures (5, 10, 15, 20, and 25°C) and under mineral oil layer. Biochemical analyses were conducted on the PLBs by determining the chlorophyll, total soluble protein and peroxidase activities. In addition, histological analyses of cell structure, rate of survival and shoot growth after the recovery process were carried out. This study was performed in two conditions; three (3) weeks and twelve (12) days of growth recovery period after 3 weeks of storage treatment. The PLBs were transferred to half strength Murashige and Skoog (MS) medium containing 1.0 mg/L benzylaminopurine (BAP) and kept at 25±2°C for twelve (12) days during recovery period. Overall, PLBs stored under 5°C and mineral oil layer produced the lowest values in chlorophyll analysis, rate of survival and shoot growth. However, total soluble protein content was increased during twelve (12) days of growth recovery period. Histological analysis has shown that the cell structure of these PLBs sustained a high degree of damage due to the treatment. PLBs stored under 5°C and mineral oil layer recorded the highest peroxidase activity attributed to higher stress conditions.

Key words: *Dendrobium sonia*-28, PLBs, low temperature, mineral oil, biochemical analysis.

INTRODUCTION

The Orchidaceae family, comprising about 25,000 species is considered as ornamentals plants and as such, they have been successfully and commercially grown globally as cut flowers and pot plants (Martin and Madassery 2006). *Dendrobium* occupies an important position in ornamental orchid cut flower industry since it produces high number of flowers per inflorescence and recurrent flowering. *Dendrobium* is usually propagated sexually by seeds and asexually by shoot division (Martin and Madassery, 2006). The production cycle from seedlings to full bloom of *Dendrobium* orchids is short, spanning approximately several weeks. They bloom

several times a year without any seasonal dependence and therefore can be easily obtained all year round and are well suited to be used as cut flowers for flower settings. Recently, the need for tropical orchids to be conserved has risen sharply, primarily due to extinction caused by extensive disturbance of their natural habitat and constant harvesting (Thammasiri 2005). There are some problems in the micropropagation of orchids especially when dealing with explants isolated from mature plants. Cells of orchids in tissue culture exude a large number of phenolics that become toxic to the cell when oxidized.

Generally, temperature plays an important role in plant growth and is a natural regulator in the morphogenesis of *in vitro* plants. Reducing culture temperature promotes storage corm induction in *Watsonia vanderspuyiae* plant (Ascough et al. 2008). Similarly, low temperature conditions (below 15°C) are effective in other plants species for micropropagation purposes such as 4°C for *Hyacinthus* (Bach et al. 1992), 10°C for *Lachenalia*

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Abbreviations: BAP, Benzylaminopurine; MS, Murashige and Skoog; PLB, protocorm-like bodies; TBA, thiobarbituric acid; NaOH, sodium hydroxide

(Slabbert and Niederwieser 1999) and banana (Hassan 2004). The main objective of the present study was to determine the effects of low temperature and mineral oil layer conditions on the *in vitro* growth of *Dendrobium sonia*-28 PLBs and their relationship by analyzing the levels of total soluble protein, chlorophyll, peroxidase activity and growth and survival rates supported by histological analysis.

MATERIALS AND METHODS

Plant materials

In vitro cultures of *Dendrobium sonia*-28 protocorm-like bodies (PLBs) were randomly selected for this study. PLBs stock cultures were grown on half strength Murashige and Skoog (1962) semi-solid medium supplemented with 1.0 mg/L benzylaminopurine (BAP), 20 g/L sucrose and 2.75 g/L gelrite. The pH was adjusted at 5.7 to 5.8 prior to autoclaving. Cultures were incubated at 25±2°C under 16 h photoperiod cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol m⁻²s⁻¹.

Experimental design

In this study, there are two main parameters. The variables are storage of *Dendrobium sonia*-28 PLBs at various temperatures and under mineral oil layer. Single PLBs ranging in size from 3 to 4 mm were precultured in half strength MS semi-solid medium without BAP. They were then stored at different temperatures (5, 10, 15, 20 and 25°C). After three weeks, PLBs were transferred to growth recovery semi-solid medium containing half strength MS basal salts with 1.0 mg/L BAP for 12 days. Biochemical analyses were conducted immediately after three (3) weeks of storage and again after 12 days of growth recovery period.

Mineral oil layer studies were conducted by culturing 3 to 4 mm PLBs of *Dendrobium sonia*-28 in half strength MS basal medium and covered with mineral oil layer (15 ml). PLBs in half strength MS semi-solid medium without mineral oil layer were used as control. All the PLBs were stored at 25±2°C with 16 h photoperiod for 3 weeks before transferring to growth recovery medium for 12 days following in which the biochemical analyses were conducted. Three (3) replicates containing 10 PLBs each were selected to determine the percentage of survival and shoot growth.

Biochemical analysis of the PLBs at different temperatures and mineral oil layer

The chlorophyll extraction and measurement was determined using Harborne method (1973). Enzyme extractions was carried out by grinding treated and control PLBs with protein extraction buffer with the ratio of PLB weight to buffer volume at 1:3 using a mortar and pestle placed in an ice bath. The protein extraction buffer consisted of 0.1 M Tris-hydrochloric acid, 1.0 mM EDTA and 0.1% mercaptoethanol dissolved in autoclaved distilled water. The pH of the buffer was adjusted to 8.0 with 0.1 M NaOH. The extract was then centrifuged at 12,000 rpm at 4°C for 20 min. The supernatants were used for the determination of total soluble protein and peroxidase activity (Flocco and Guilietti, 2003). The total soluble protein content determination was based on Bradford method (Bradford, 1976) while peroxidase activity was based on Flocco and Guilietti (2003). The biochemical analyses of the PLBs were assessed using variance (ANOVA) with SSPS software (version 16.0). All data

were subjected to the independent sample t-test and one-way ANOVA, and the differences in the means were compared using the Tukey HSD test.

Histological analysis

Preparations of histological slides of PLBs involve few important steps. First, the PLBs were dehydrated by transferring them into a series of alcohol thiobarbituric acid (TBA) at various concentrations (50 to 100%). They were treated three times with TBA and left overnight. Then, PLBs were exposed to xylene and wax I, II, and III. Specimens were then blocked and sliced using 6 micron microtome (Leica RM 2135) and stained with safranin and fast green. Slides were observed using light microscope (Olympus BX41).

RESULTS AND DISCUSSION

Chlorophyll analysis of PLBs at different temperatures

All the treatments possess relatively high total chlorophyll content, chlorophyll *a* and *b* except for those stored at 5°C after recovery period of 12 days (Figures 1 and 2). For temperature at 10°C, lower absorbance value for chlorophyll *b* was determined compared to chlorophyll *a* after growth recovery. According to one way ANOVA analysis, the data shown for both after treatment and growth recovery period for chlorophyll *b* was not significant ($P>0.05$). Meanwhile, both chlorophyll *a* and total chlorophyll content were significantly different ($P<0.05$). The results obtained were similar to Wilson et al. (1998) who reported that regardless of media composition, *in vitro* storage of broccoli seedlings at low temperatures for more than eight weeks showed dramatic losses after recovery as well as reduced photosynthetic ability.

Total soluble protein analysis of PLBs

Total soluble protein content was affected by low temperature treatments. PLBs cultured at 25°C produced the highest total soluble protein after treatments and growth recovery (Figures 3 and 4). However, growth recovery period increased the protein content in all treated PLBs. According to ANOVA analysis, the data obtained were not significantly different ($P>0.05$). Therefore, temperatures have less effect against the total soluble protein content. Bonjoch and Tamayo (2001) reported that normal metabolic activities decreased followed by reduction of growth when plants were subjected to any abiotic stress factors. Hence, the protein synthesis negatively affected anabolic process (Bohnert and Jensen, 1996) together with photosynthesis, transport of metabolic process and uptake and translocation of ions (Hsiao, 1973; Lichtenthaler, 1996). In addition, most plants activate their stress coping mechanisms during unfavorable conditions.

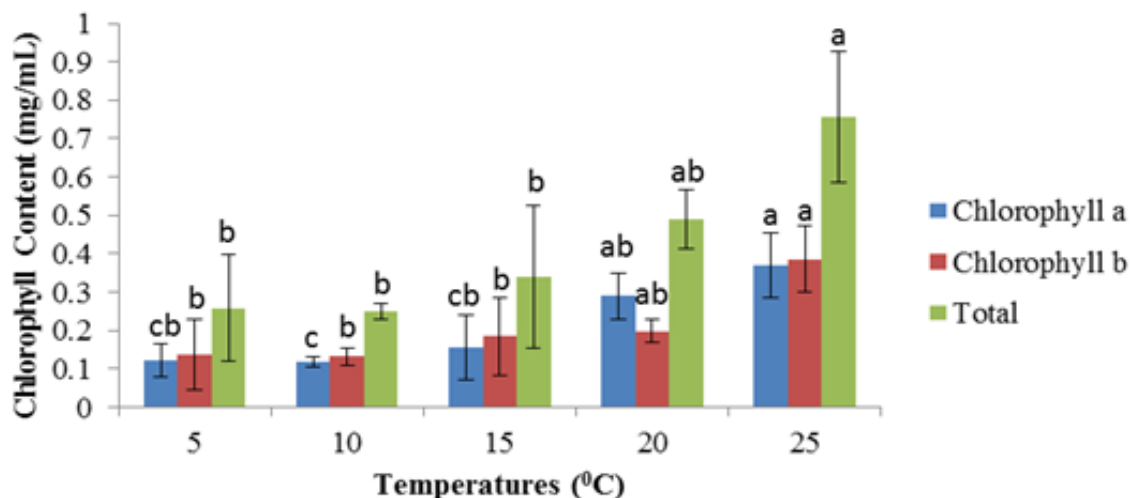


Figure 1. Chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations (mg/mL) of PLBs stored at different temperatures after 3 weeks. Error bars show mean \pm 0.5 of standard error.

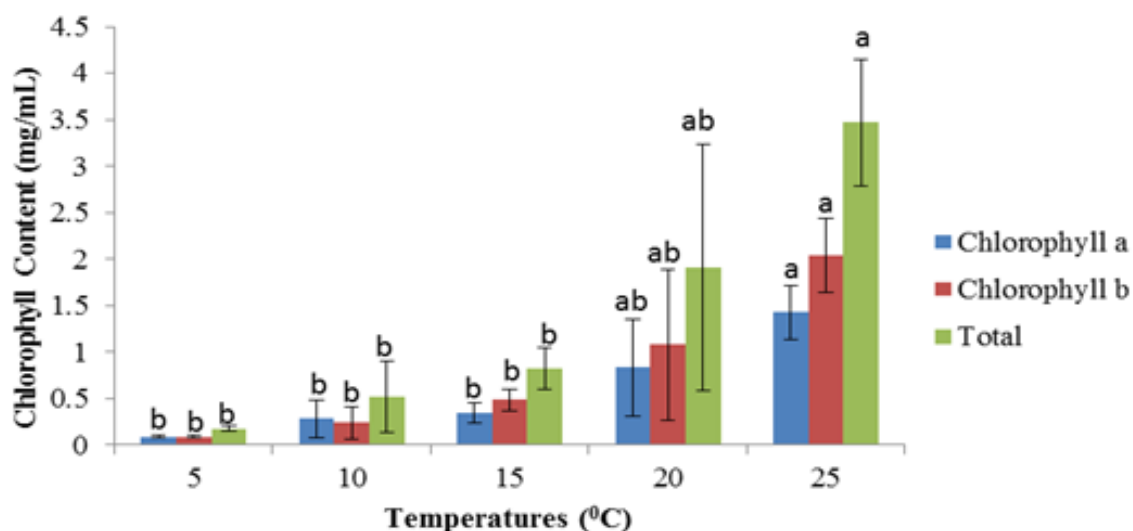


Figure 2. Chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations (mg/mL) of PLBs obtained from different temperatures after 12 days of growth recovery period. Error bars show mean \pm 0.5 of standard error.

Peroxidase activity

Peroxidase activity was determined after growth recovery and the highest peroxidase activity was detected at 5°C (Figure 5). PLBs cultured at low temperature (5°C) causes stress to the cells due to the osmotic flow of water out from the cells. Consequently, PLBs secrete a higher amount of peroxidases under stress. This could be due to the need for lignification process of the plant cells in order to avoid unnecessary out flow of water (Lee, 2007). Data obtained for the peroxidase activity was significantly different by using one-way ANOVA analysis. Therefore, the different temperatures affected the peroxidase activity of PLBs.

The survival rate and shoot growth of PLBs

The percentage of survival and shoot growth were determined after 12 days of growth recovery period. PLBs at 25°C recorded the highest percentage of shoot growth and survival rate which reduces as the temperature is lowered to 5°C (Figure 6). According to ANOVA analysis, the data obtained for both percentage of shoot growth and survival of PLBs was significantly different ($P < 0.05$). Lavieville et al. (1991) reported that the survival rate of callus cultures were more than 90% when cultured at low temperatures. However, the capacity for alkaloid synthesis rate declined tremendously after recovery. Fortes and Pereira (2001) reported that the growth rate of

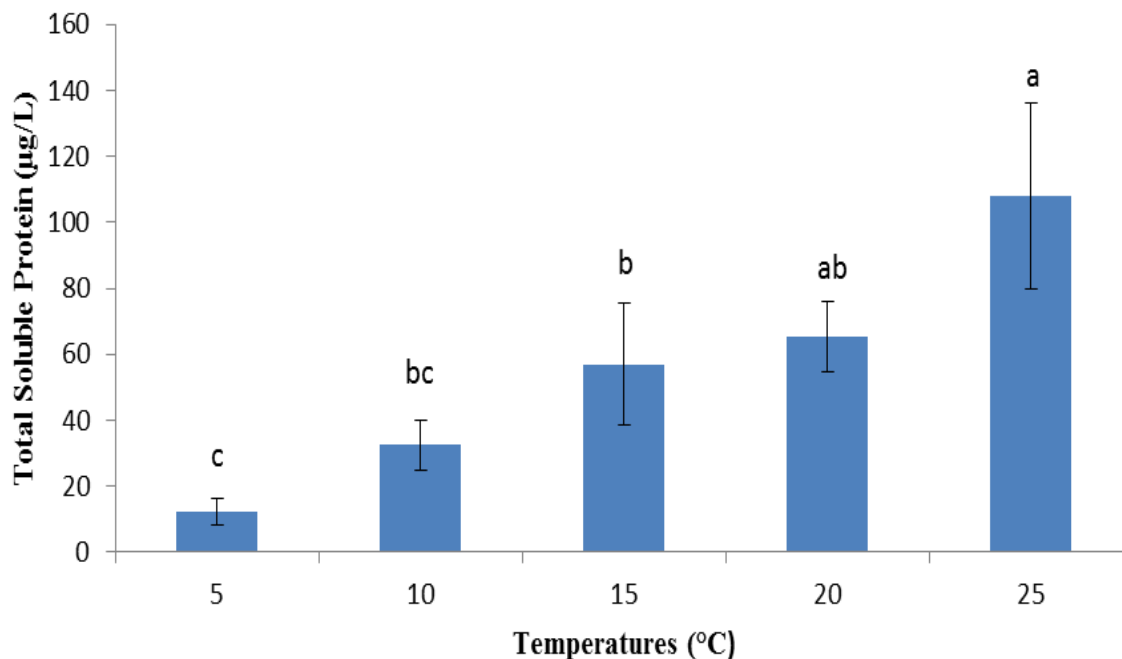


Figure 3. Total soluble protein concentrations ($\mu\text{g/L}$) of PLBs stored at different temperatures after 3 weeks. Different letters represent statistical differences at $P < 0.05$ according to the Tukey's test. Error bars show mean ± 0.5 of standard error.

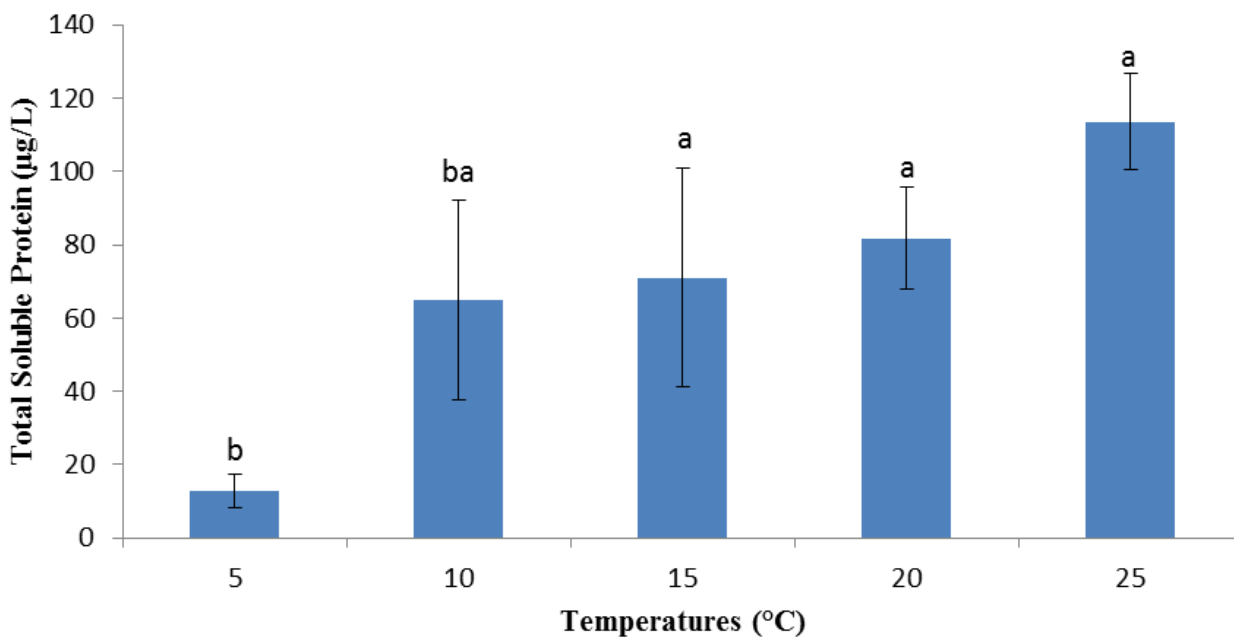


Figure 4. Total soluble protein concentrations ($\mu\text{g/L}$) of PLBs obtained from different temperatures after 12 days of growth recovery period. Different letters represent statistical differences at $P < 0.05$ according to the Tukey's test. Error bars show mean ± 0.5 of standard error.

in vitro asparagus shoots maintained at 6°C for four months was 15.6% and the rate of survival after nine months without treatment was 25%. Ascough et al. (2008)

reported that reduced culture temperature (10°C) together with gibberellic acid (GA_3) act synergistically for the corm induction promotion and growth in *Watsonia*

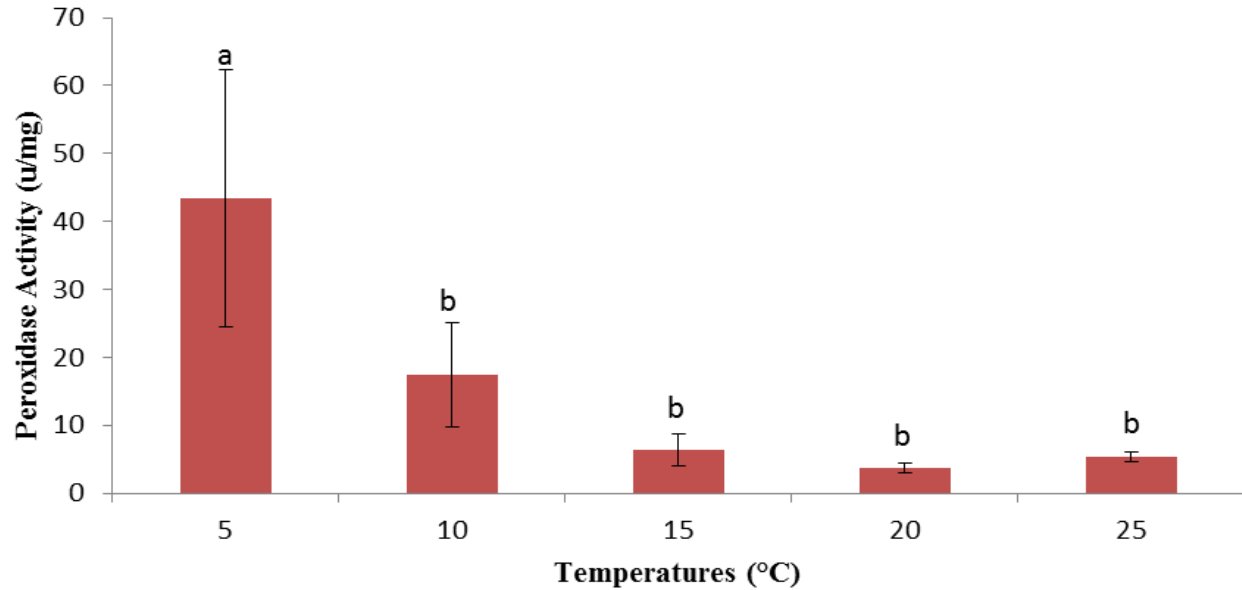


Figure 5. Peroxidase activity ($\mu\text{m/g}$) of PLBs obtained from different temperatures after 12 days of growth recovery period. Different letters represent statistical differences at $P<0.05$ according to the Tukey's test. Error bars show mean ± 0.05 of standard error.

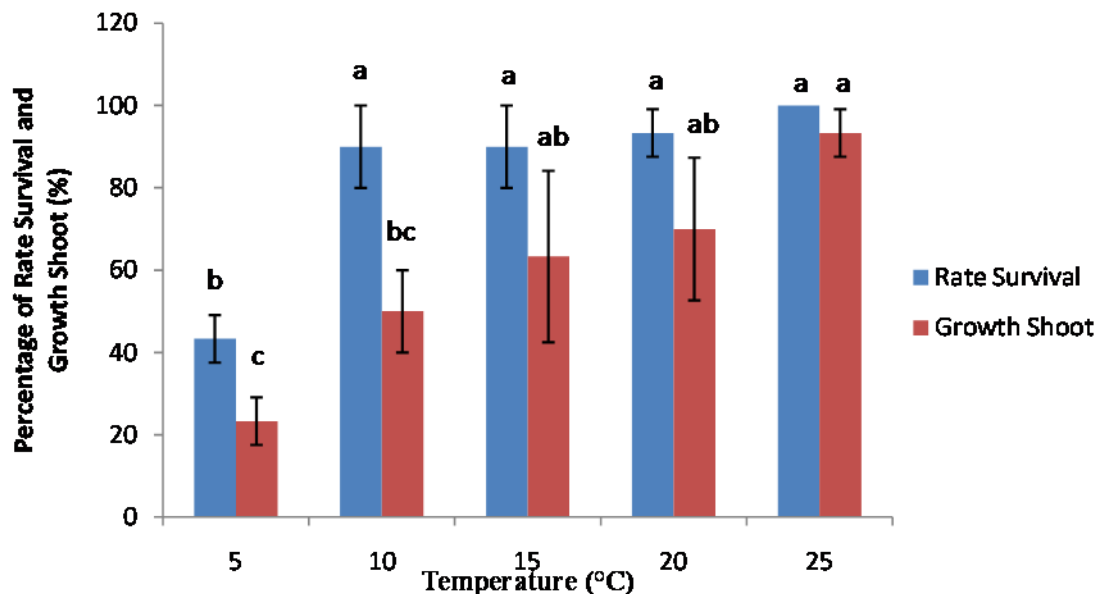


Figure 6. Survival rate and percentage of shoot growth of PLBs obtained from different temperatures after 12 days of growth recovery period. Different letters represent statistical difference at $P<0.05$ according to the Tukey's test. Error bars show mean ± 0.5 of standard error.

vanderspuyiae species.

Histological analysis of PLBs

After 12 days of recovery from storage under different temperatures, the PLBs were used for histological

observation. All the figures show the structure of nucleus, cytoplasm and cell wall clearly. The structure of the PLBs stored at 5°C indicated that they sustained more damages than other treatments (Figure 7). At this treatment, the cell walls and nucleus were damaged while other organelles were destroyed. The nucleus of PLBs stored at 20 and 25°C was not destroyed because it was

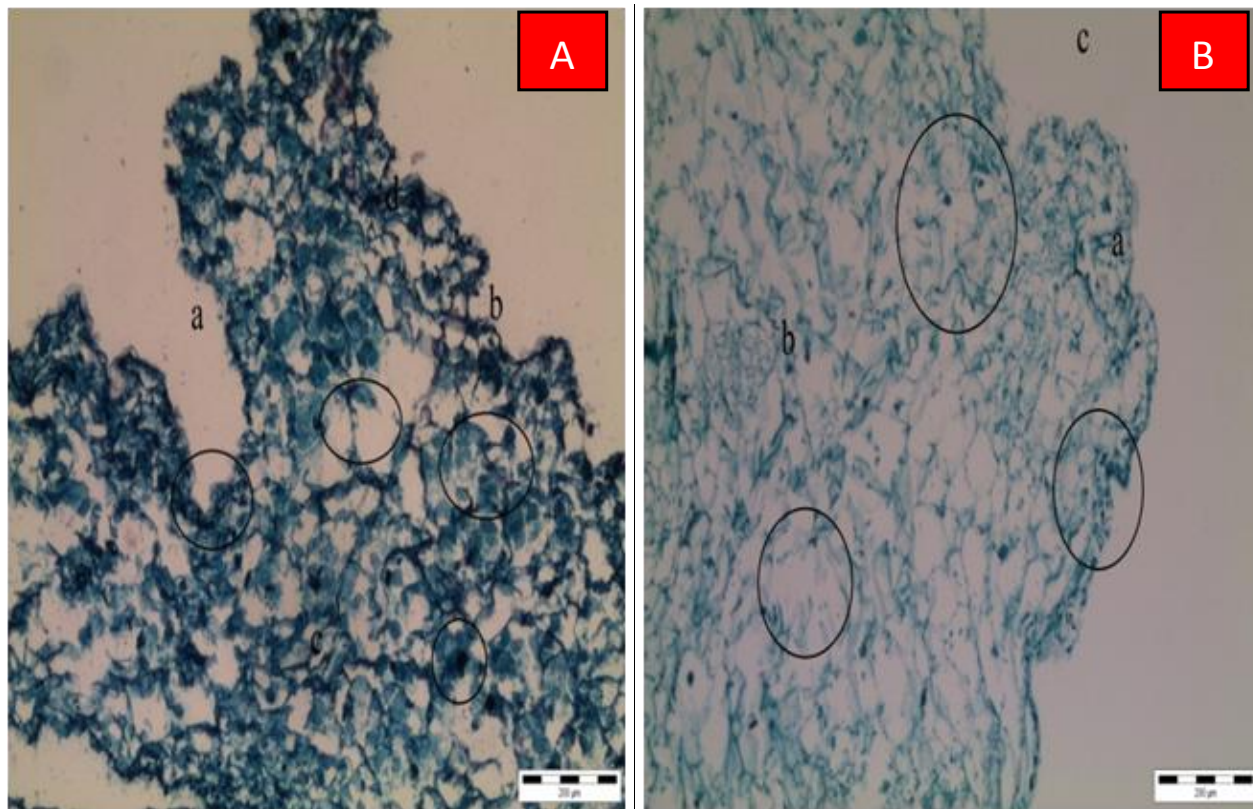


Figure 7. A and B represent cell structures of PLBs after treatment at 5°C. Histological section of *Dendrobium sonia-28* PLBs. (a) Border of cell surface, (b) cell wall, (c) nucleus, and (d) cytoplasm.

protected by an intact cell wall (Figures 8 and 9). It was observed that some of the cells showed an active cell division process.

Chlorophyll analysis of PLBs under mineral oil layer condition

PLBs cultured under mineral oil treatment recorded lower chlorophyll *a*, *b* and total chlorophyll content compared to untreated PLBs in the absence of mineral oil (Figures 10 and 11). According to one-way ANOVA statistical analysis, the data obtained for chlorophyll *a*, chlorophyll *b* and total chlorophyll contents were not significantly different. Zamora et al. (1999) reported that mineral oil proved to be an effective storage technique to prevent loss of moisture and to slow down the growth of bamboo node cultures for twelve weeks.

Total soluble protein analysis of PLBs

The highest total soluble protein concentration was obtained for the control PLBs in the absence of mineral oil layer both after treatment and after recovery (Figures 12 and 13). The level of total soluble protein was

increased after 12 days of growth recovery period. According to ANOVA ($P < 0.05$) analysis, the total soluble protein content of PLBs after treatment was significant. PLBs cultured under mineral oil layer could experience stress due to changes in metabolic pathways. Caplin (1959) suggested that mineral oil reduces oxygen transfer and the tissues show a slackening of the metabolism. In addition, in plants that have low or no stress tolerance mechanisms, acute damage occur rapidly. Apart from the loss of chlorophyll, other cellular pigments are hydrolyzed and exported through phloem followed by hydrolysis of mitochondrial protein and vascular tissues (Pell and Dann 1991; Gan and Amasino, 1997). Low protein concentration in the plants could be interpreted as a clear symptom of stress damage and plants undergo readjustment of metabolic fluxes and changes in biomass partitioning (Lambers et al., 1998).

Peroxidase activity

The PLBs cultured under mineral oil condition produced the highest peroxidase activity (Figure 14). However, the data was not significantly different according to one-way ANOVA analysis ($P > 0.05$). This suggested that mineral oil layer does not affect the peroxidase activity of PLBs.

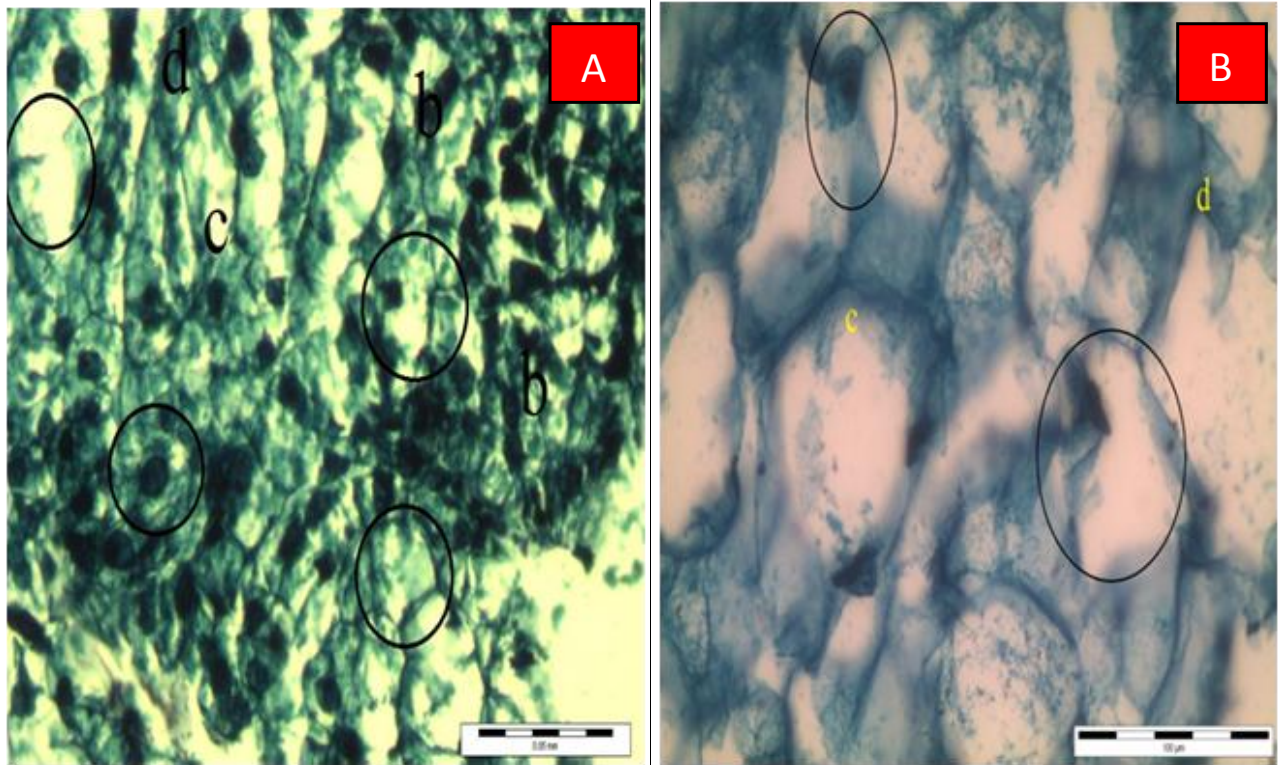


Figure 8. A and B represent cell structure of PLBs after treatment at 20°C. Histological section of *Dendrobium sonia-28* PLBs. (a) Border of cell surface, (b) cell wall, (c) nucleus, and (d) cytoplasm.

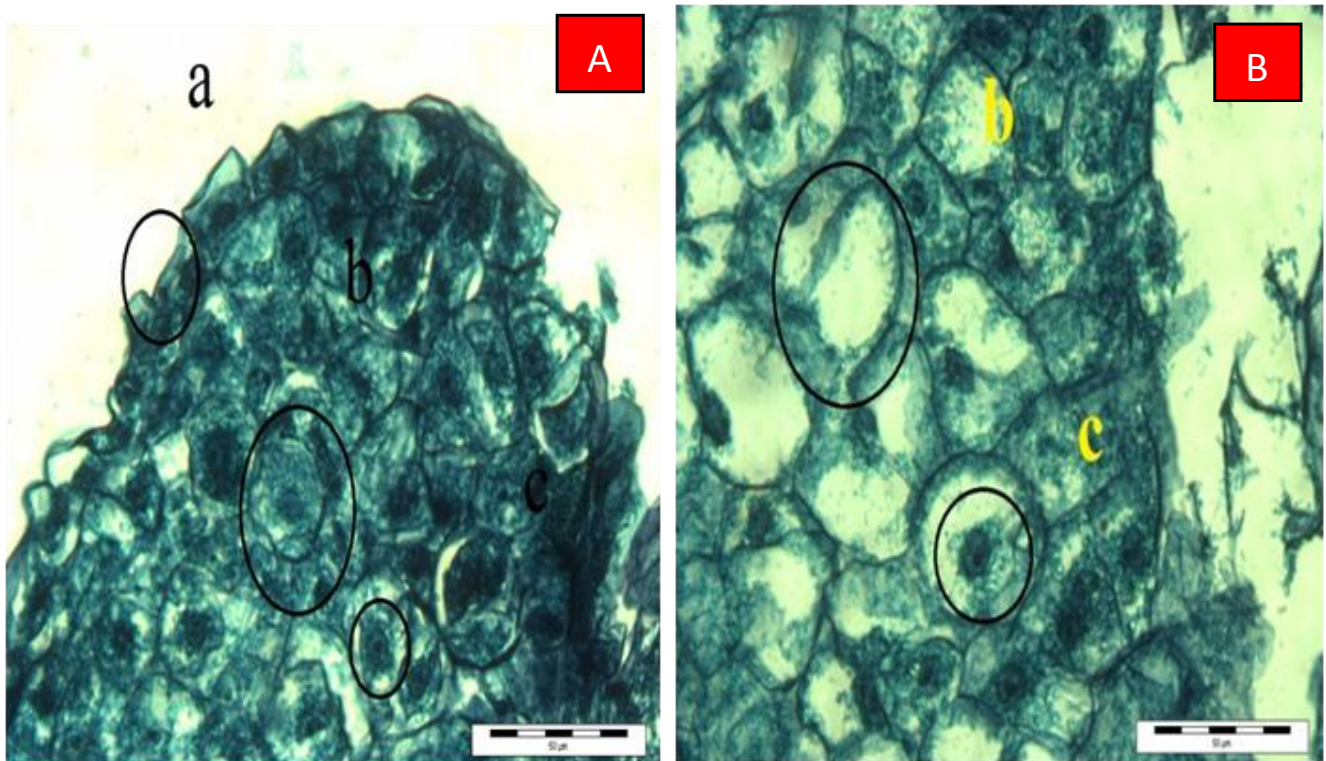


Figure 9. A and B represent cell structure of PLBs after treatment at 25°C. Histological section of *Dendrobium sonia-28* PLBs. (a) Border of cell surface, (b) cell wall, (c) nucleus, and (d) cytoplasm.

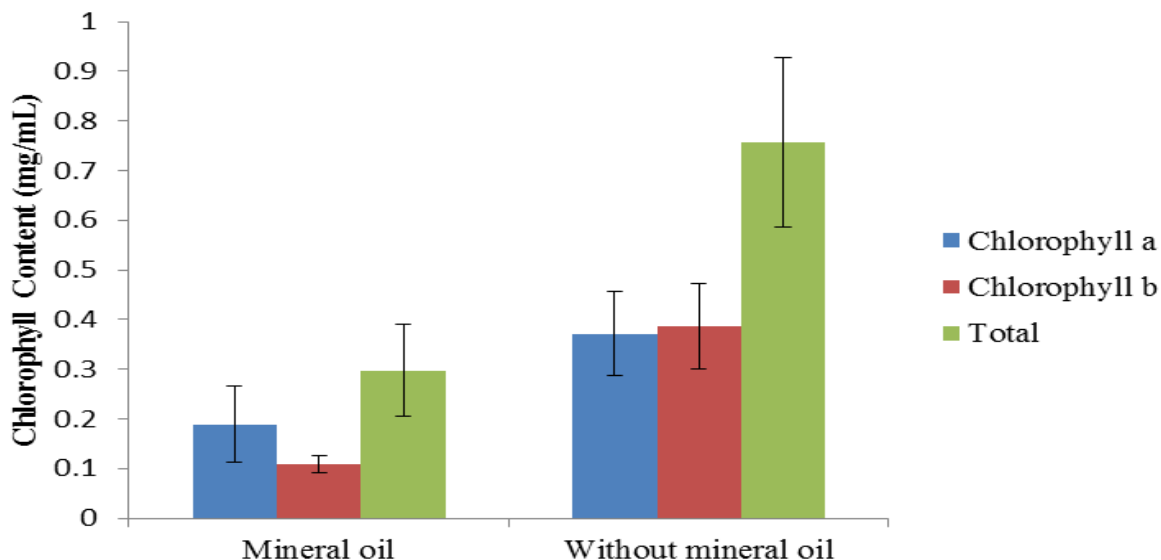


Figure 10. Chlorophyll *a*, chlorophyll *b* and total chlorophyll content (mg/mL) of PLBs cultured under mineral oil and without mineral oil after treatment for 3 weeks. Error bars show mean \pm 0.5 of standard error.

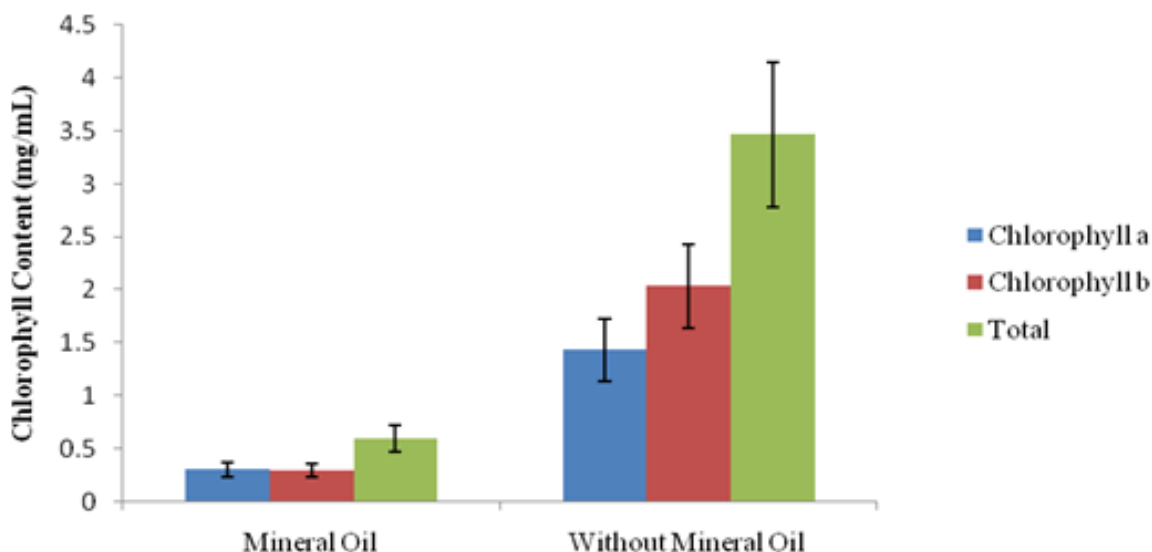


Figure 11. Chlorophyll *a*, chlorophyll *b* and total chlorophyll content (mg/mL) of PLBs cultured under mineral oil and without mineral oil after 12 days of growth recovery period. Error bars show mean \pm 0.5 of standard error.

Plants cultured under mineral oil have greatly reduced their metabolism due to stress and possess peroxidase activity. The protein degradation is associated to oxidative stress promoted by reactive oxygen species such as H_2O_2 (Palma et al., 2002). In plants, peroxidase is specifically presented in the detoxification of H_2O_2 (Asada, 1992). Cellular damage induced by oxidative stress conditions in plants may be alleviated by protective metabolism involving antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase. Both Li et al. (2001) and Chen et al. (2002) reported that the growth inhibition caused by copper treatment was

related to an induction of peroxidase activity. In addition, anionic peroxidases are involved in lignification process and cell wall cross linking which can cause extensibility of the cell is reduced (Fry, 1986) and might produce growth inhibition (Pandalfinii et al., 1992).

The rate of survival and shoot growth of PLBs

PLBs cultured under the mineral oil recorded the lowest survival and shoot growth rates (Figure 15). Hassan (2004) reported that banana shoots restored their growth

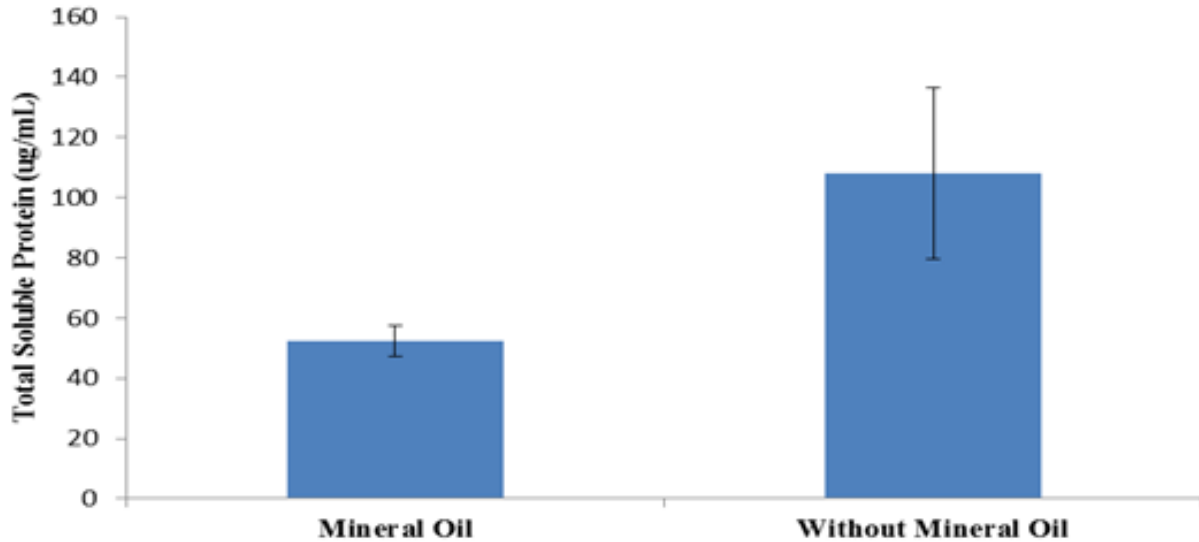


Figure 12. The total soluble protein content ($\mu\text{g/mL}$) of PLBs cultured under mineral oil and without mineral oil after treatment for 3 weeks. Error bars show mean ± 0.5 of standard error.

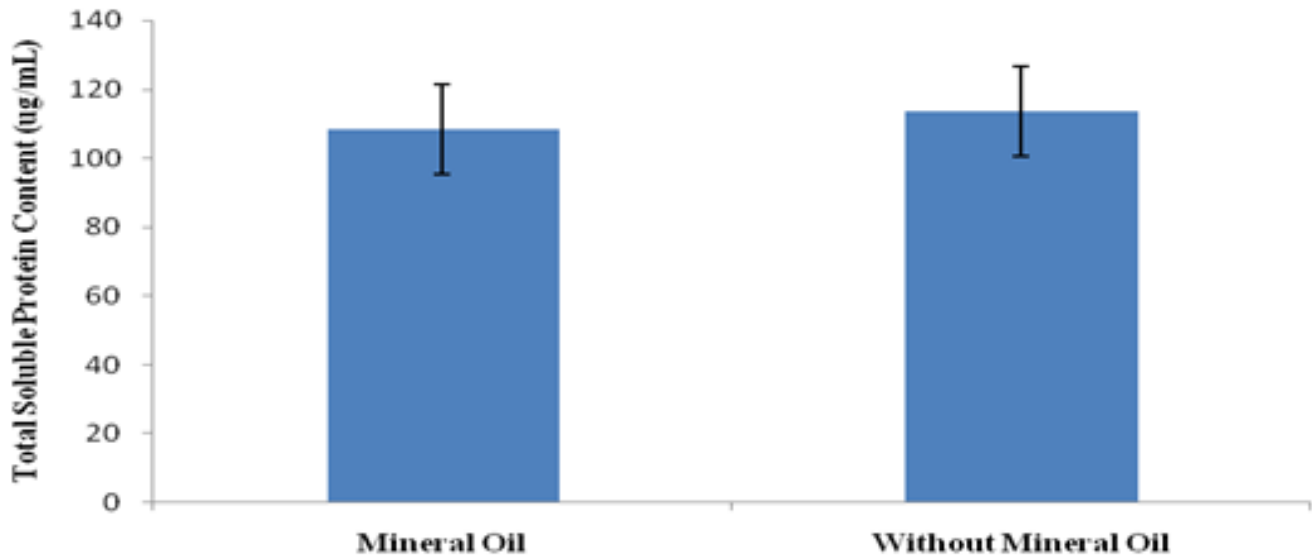


Figure 13. The total soluble protein content ($\mu\text{g/mL}$) of PLBs cultured under mineral oil and without mineral oil after 12 days of growth recovery period. Error bars show mean ± 0.5 of standard error.

capability after being transferred to a standard MS medium composition. According to ANOVA ($P < 0.05$) analysis, the rate of survival data obtained was significant. Thus, PLBs cultured under mineral oil does affect shoot growth of PLBs. Mathur et al. (1991) reported that the rate of growth and morphogenesis in cultures under mineral oil decreased compared to the control. Similarly, Augereau et al. (1986) reported that plant tissues grow at a very slow rate under mineral oil. For example, an increase of 20% of dry weight after six months was observed with 47 *C. roseus* C₄ strain under mineral oil treatment, and finally 70% recovered after they had been

transferred to a standard culture condition.

Histological analysis of PLBs

After 12 days of recovery period from the storage under mineral oil layer, the PLBs were used for histological analysis. The structure of PLBs under mineral oil showed that some of the cell walls and nucleus were damaged (Figure 16A) with some oil droplets surrounding the cells (Figure 16B). However, nucleolus was still clearly visible compared to untreated PLBs since nucleus was still intact

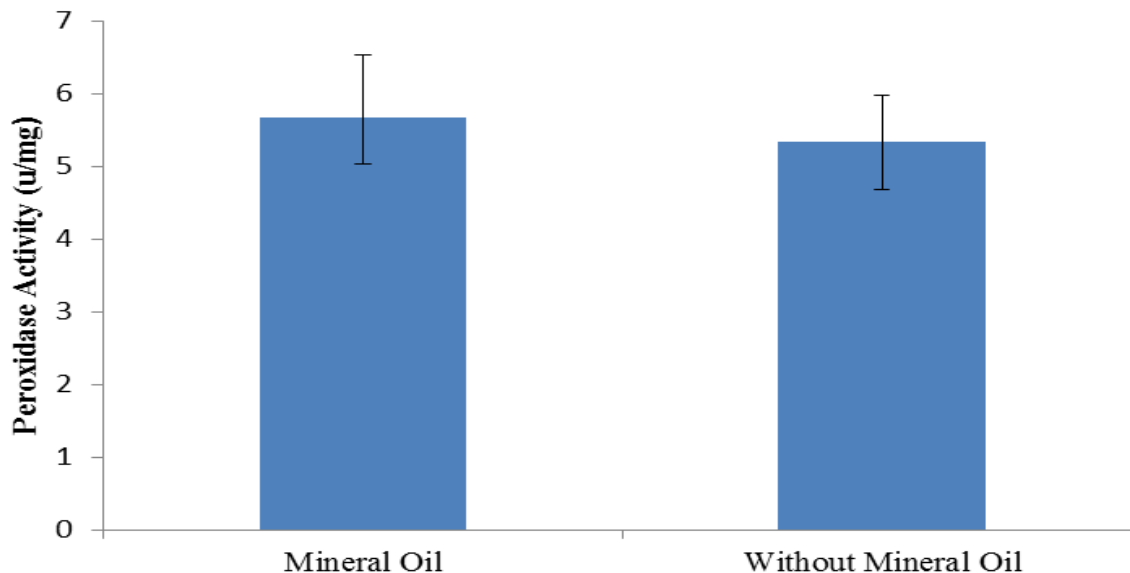


Figure 14. The peroxidase activity of PLBs cultured under mineral oil and without mineral oil. The data was determined after treatment at 12 days of growth recovery period after 3 weeks of storage treatment previously. Error bars show mean \pm 0.5 of standard error.

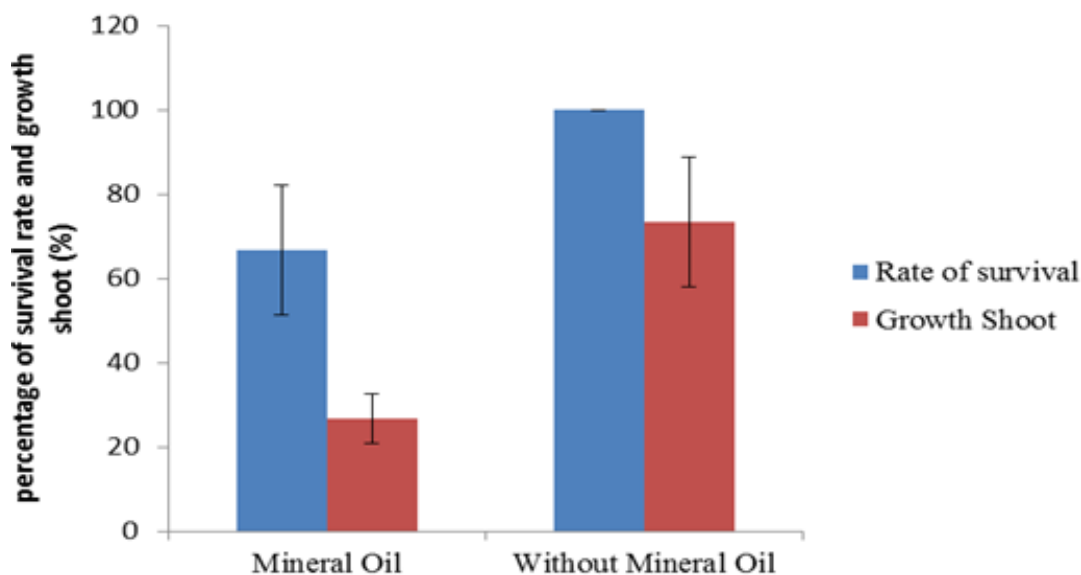


Figure 15. Survival rate and shoot growth of PLBs cultured under mineral oil and without mineral oil treatments after 12 days of growth recovery period. Error bars show mean \pm 0.5 of standard error.

(Figure 16A and B). There were clusters of cells that indicated the cells were still actively dividing. The surface of cell wall was maintained and this will protect the cell from collapsing.

Conclusions

PLBs cultured at 25C and without mineral oil layer have

the highest total chlorophyll contents and total soluble protein as expected of control treatment. PLBs cultured in 5°C and under mineral oil layer have the lowest chlorophyll content and total soluble proteins while exhibiting poor survival rate and shoot growth. This corresponds to the significantly elevated levels of peroxidase activity found at 5°C and under mineral oil layer, indicating that abiotic stress has affected the metabolism and growth of treated PLBs.

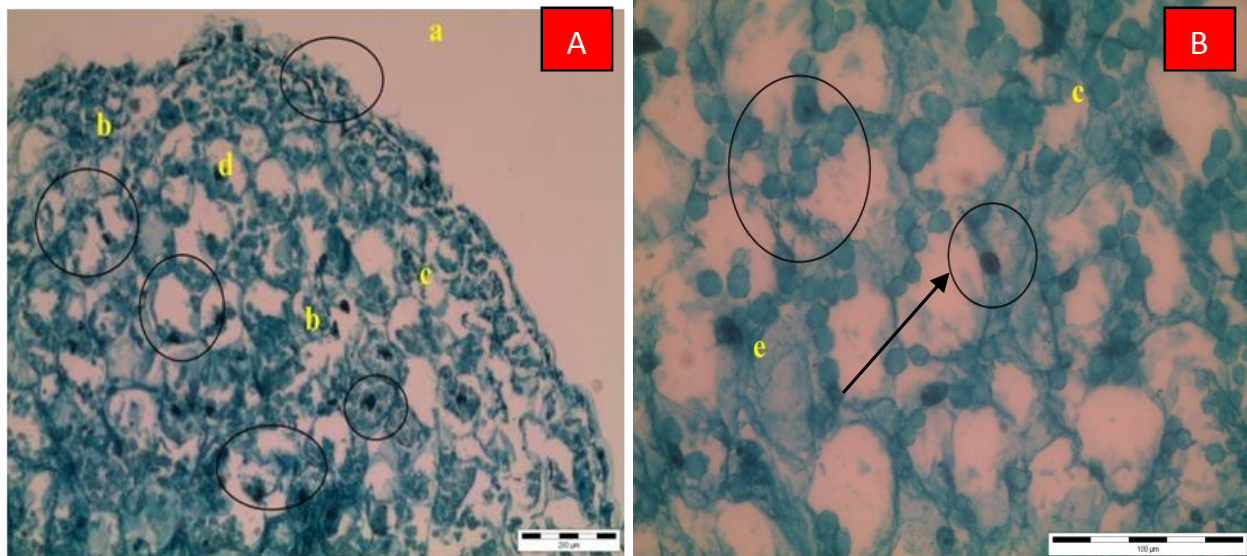


Figure 16. A and B represents cell structure of PLBs after treatment under mineral oil layer. Histological section of *Dendrobium sonia*-28 PLBs. (a) Border of surface, (b) cell wall, (c) nucleus, (d) cytoplasm, and (e) oil droplets.

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