Borneo Journal of Resource Science and Technology (2016) 6(2): 35-42

Nutrient and Physiological Requirements for Biomass Production of *Pestalotiopsis* sp. UMAS P14 and *Pseudopestalotiopsis* sp. UMAS P2005/2592

LATEEF ADEBOLA AZEEZ*[1,2], SEPIAH MUID[1] & MOHAMAD HASNUL BOLHASSAN[1]

[1]Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia; [2]Department of Plant Biology, Faculty of Life Science, University of Ilorin, Nigeria
*Corresponding author: alateef123@yahoo.com

ABSTRACT

Fungi are important decomposers in our ecosystem and are useful in metabolite production, bio-degradation and bio-sorption of different substrates and wastes through their mycelial biomass. Fungal species are known to have different preferences for their growth requirements. Two fungal species *Pestalotiopsis* and *Pseudopestalotiopsis* useful in many biotechnological applications were studied. Nutrient and physiological requirements for mycelial biomass production such as carbon and nitrogen, pH, light and temperature were investigated. Both fungal isolates were grown in liquid basal medium supplemented separately with various carbon and nitrogen source and incubated under different light, pH and temperature conditions for 15 days. In general, *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp. showed significant preferences for monosaccharide and disaccharide carbon source as compared to sugar alcohols and polysaccharides, whereas ammonium tartrate was more preferred as a nitrogen source compared to ammonium nitrate, ammonium sulphate and other ammonium salts. These two fungal species were able to grow and produce good mycelial biomass (223.33 mg for *Pseudopestalotiopsis* and 136.67 mg for *Pestalotiopsis*) at temperature range of 15°C to 30°C and (290.00 mg for *Pseudopestalotiopsis* and 256.67 mg for *Pestalotiopsis*) on media pH of slightly acidic to slightly alkaline. However, they showed no significant preferences between constant light, total darkness and alternate light conditions. The results from this study will be very useful for the mycelial biomass production of *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp. for their biotechnological applications.

Keywords: Carbon source, liquid culture, nitrogen source, temperature, pH

INTRODUCTION

The fungal genera of *Pestalotiopsis* Steyaert and *Pseudopestalotiopsis* Maharachch., K.D. Hyde & Crous have a close phylogenetic relationship, as *Pseudopestalotiopsis* was recently carved out of the genus *Pestalotiopsis* (Maharachchikumbura *et al.*, 2014). *Pestalotiopsis* species are useful in the production of important metabolites including Taxol; an anticancer agent (Heinig *et al*., 2013; Shukla *et al*., 2014; Gu *et al.*, 2015), and also in biodegradation of chemicals and plastics (Russell *et al.*, 2011).

Most microfungal species are easy to grow and can produce high yield of biomass which can be directly used for the removal of waste (Park *et al*., 2005) and also be utilised as inoculum for other applied uses. However, knowledge of the physiology of microfungal species are usually overlooked, in which having physiological data on a microfungus can ease the characterization process of such species prior to their use in various biotechnological processes such as for bioremediation, bio-prospecting, protein production, mycofiltration as well as biological control.

Production of mycelial biomass a microfungus is influenced by many factors which includes the carbon and nitrogen nutrients used in the growth medium, light exposure, temperature, pH and aeration (Madan & Thind, 1998). Earlier studies on physiology of different *Pestalotiopsis* species were conducted using agar media (Mandahar & Narwal, 1970; Sati & Bisht, 2006; Ren *et al*., 2013). However, for biotechnological applications, fungi are mostly grown in liquid media for their biomass.

The purpose of this study was to investigate the ability of both *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp. mycelial biomass production under different growth conditions such as temperature, pH, light, carbon and

nitrogen in liquid media for biotechnological applications. The result from this study will be a contribution to our understanding of the physiology of both *Pestalotiopsis* and *Pseudopestalotiopsis* species, which will aid their further use in biotechnological applications such as bioremediation, biodetoxification and biological control.

MATERIALS AND METHODS

Isolation of the Microfungi and Preparation of Basal Medium

Isolates of *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp. have been discovered from green leaves of *Shorea macrophylla* from Kubah National Park and green leaves of *Baccaurea* sp. from Gunung Gading National Park in Sarawak, Malaysia, respectively. The isolates were purified and the pure cultures were grown on malt extract agar (MEA) (Oxoid by Thermo Scientific) and maintained at room temperature until ready for use.

Evaluation of the physiological requirements for mycelial biomass growth of the isolates were done following the method of Granade *et al*. (1985) and Prasher & Chauhan (2015) in which the biomass was assessed based on their dry weight. The basal liquid medium was prepared following the protocol used by Lateef *et al*. (2015). Briefly, the basal medium was made up of 20.00 g glucose, 2.00 g yeast, 0.30 g KH_2PO_4, 0.30 g $MgSO_4$, 0.20 g $CaCl_2$, 0.20 mg $FeSO_4$, 0.10 mg $CuSO_4$, 0.5 mg pyridoxine, 0.5 mg niacin, 0.3 mg $ZnSO_4$ and then made up to one litres with distilled water.

Mycelial Biomass Production of the Isolates Using Different Carbon Sources

Isolates of *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp. were grown in the basal medium supplemented with different carbon sources, which were malt extract, starch, glycerol, sucrose and myo-inositol, by replacing the glucose in the basal medium at the same quantity (i.e. 20 g/L) and the basal medium liquid without any carbon source served as the control.

30 mL of the basal medium was dispensed into 150 mL conical flasks, pH regulated to 6.5 and then autoclaved at 121°C for 15 min. After cooling, each conical flask was inoculated with one mycelia disc of 5.0 mm diameter from the colony edges of 5-7 days old pure cultures of *Pestalotiopsis* sp. and *Pseudopestalotiopsis* sp., three replicates were prepared for each isolate and then incubated in stationary condition at room temperature for 15 days (Troncozo *et al*., 2015) using a completely randomised design.

Mycelial Biomass Production of the Isolates Using Different Nitrogen Sources

Yeast extract in the basal liquid medium was replaced separately by different nitrogen sources namely ammonium tartrate, ammonium nitrate, ammonium sulphate, ammonium acetate, sodium nitrate and peptone respectively, in the same amount of 2 g/L.

Mycelial Biomass Production of the Isolates at Different Temperature

Temperature requirements for mycelial biomass production was studied using 30 mL of the basal medium with glucose as the carbon source. After sterilization process, the media were allowed to cool before inoculating the flasks with one mycelial disc of the isolates, then incubated at temperatures (± 2°C) of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.

Mycelial Biomass Production of the Isolates at Different Ph

pH requirement for mycelial biomass was done by adjusting the basal medium with glucose as the carbon source to pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 1 M sodium hydroxide and 1 M hydrochloric acid. After which the media were autoclaved, inoculated and incubated as described earlier.

Mycelial Biomass Production of the Isolates Under Different Light Conditions

The inoculated basal medium of both isolates were incubated under three different light conditions which were constant white fluorescent light, constant darkness and alternate light and darkness (each 12 hr).

All the experimental set-up described above were grown for 15 days, the mycelia were harvested, rinsed with distilled water, dried at

60°C on a pre-weighed 9 cm diameter Whatman's No. 1 filter paper and weighed using a Mettler Toledo weighing balance (model PL4002) until constant weight is obtained.

The weight of the mycelial biomass produced were expressed in means and analysed using Analysis of Variance (ANOVA) in SPSS v.22. Means were separated using Duncan multiple range test (DMRT) at significant value of $p \leq 0.05$.

RESULTS AND DISCUSSION

Mycelial Biomass Production of the Isolates Using Different Carbon Sources

Pseudopestalotiopsis sp.

The study showed that *Pseudopestalotiopsis* sp. produced highest mycelial biomass when malt extract was used as the carbon source (Figure 1(a)). However, the biomass produced by *Pseudopestalotiopsis* sp. in malt extract supplemented medium was not significantly different from that of glucose and sucrose. The biomass produced in malt extract medium was significantly higher than that obtained on control medium (with no carbon supplemented), myo-inositol, glycerol and starch supplemented growth media. This result clearly indicates that *Pseudopestalotiopsis* sp. prefers a monosaccharide or dissacharide carbon nutrient for growth and this can be explained as a result of the fungus producing enzymes that easily assimilate monosaccharide and disaccharide carbon nutrient (Madan & Thind, 1998).

This result is supported by the findings of Mandahar and Narwal (1970) that *Pestalotiopsis theae* [now renamed *Pseudopestalotiopsis theae* (Maharachchikumbura *et al*., 2014)] grew best in media incorporated with monosaccharide and disaccharide carbon source.

Monosaccharides have also been reported as the most easily assimilated source of carbon for high mycelial biomass growth for *Pestalotiopsis microspora* (Ren *et al*., 2013), *Colletotrichum gloeosporioides* (Nithya & Muthumary, 2009), *Dactylaria eudermata*, and *Arthrobotrys oligospora* (Anamika, 2015). Practical application of this result would be that this microfungal species can be easily grown or used to assimilate substrates mainly composed of monossacharide or dissacharide carbon compounds.

Pestalotiopsis sp.

For *Pestalotiopsis* sp., the highest mycelial biomass growth (453.33 mg ± 17.64) was obtained when sucrose was supplemented as the carbon source in the basal growth medium (Figure 1(b)), however, this value was not significantly different ($p>0.05$) from those obtained in glucose, starch and malt extract supplemented media, while the growth in sucrose, glucose, starch and malt extract media were significantly different from the other carbon sources such as myo-inositol, and glycerol, as well as the control media. Also, the biomass produced by *Pestalotiopsis* sp. in medium supplemented with myo-inositol was significantly different from that in glycerol and control media, thus, the carbon source preferences of this fungal species as evidenced by this study, therefore occur in the order of: sucrose, glucose, starch, malt extract > myo-inositol > glycerol, control (no carbon).

Interestingly, this result indicates that this fungal species prefers either a monossacharide, dissacharide and a polysaccharide carbon source as compared to sugar alcohols; myo-inositol and glycerol. The high mycelial biomass obtained in sucrose, glucose, starch and malt extract media can be attributed to the easy assimilation and break down of these carbon sources for use in the metabolic pathways of this species (Madan & Thind, 1998). Glucose, sucrose and starch have also been reported to produce the highest mycelial biomass growth for *Pestalotiopsis submersus*, *Flagellospora penicillioides*, and *Tetrachaetum elegans*, respectively (Sati & Bisht, 2006). Myo-inositol was reported to be a poor carbon source for mycelial biomass growth of *Lepiota procera* (Gbolagade, 2006). However, the low biomass growth observed in the sugar alcohols can be attributed to their complex structures (Gbolagade *et al.*, 2006) which requires to be broken down by certain enzymes probably produced in low quantities by this microfungal species. On the other hand, Adejoye *et al.* (2007) reported that sugar alcohols produced the highest mycelial biomass growth for *Schizophyllum commune*.

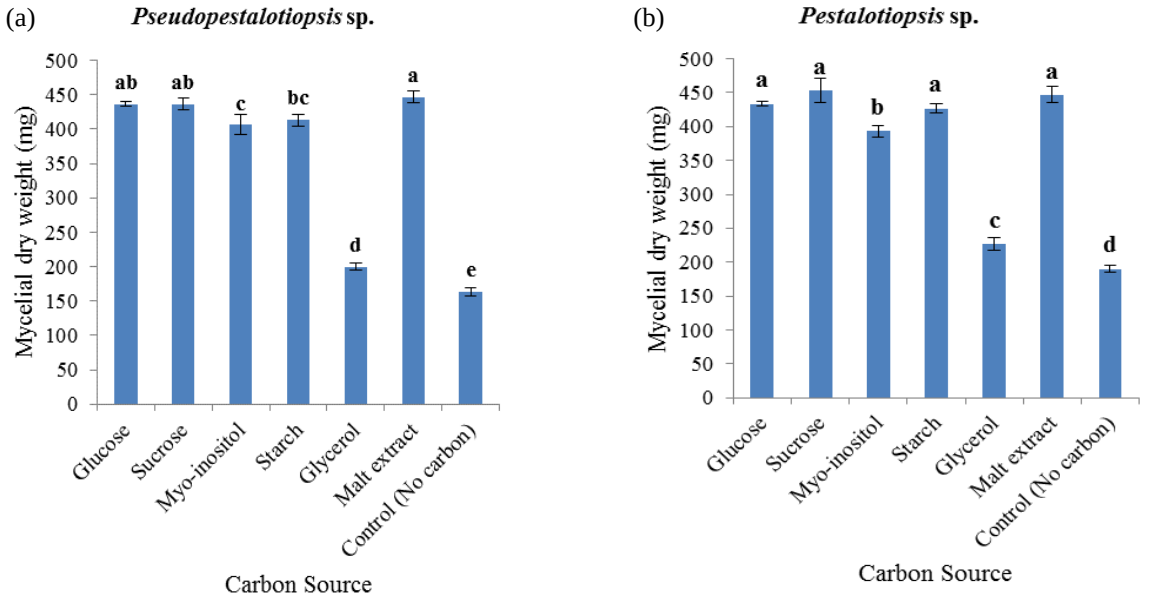

Figure 1. Mean mycelial biomass growth (mg ± S.E.M) of (a) *Pseudopestalotiopsis* sp. and (b) *Pestalotiopsis* sp. using different carbon sources after fifteen days of growth in liquid media. Bar charts with different alphabet superscripts are significantly different from each other ($p \leq 0.05$) using Duncan multiple range test (DMRT).

Mycelial Biomass Production of the Isolates Using Different Nitrogen Sources

Pseudopestalotiopsis sp.

In regard to nitrogen source preference, this fungal species showed no significant difference between all the nitrogen sources tested including the control (without any nitrogen) (Figure 2(a)). *Pseudopestalotiopsis* sp. grew well utilizing both organic and inorganic nitrogen sources for growth.

This result suggests that *Pseudopestalotiopsis* sp. can utilize both organic and inorganic nitrogen sources in the basal medium for its growth and this result is similar to that of Ren *et al*. (2013) on the growth of *Pestalotiopsis* sp. on both organic and inorganic nitrogen sources.

Pestalotiopsis sp.

Ammonium tartrate, as an inorganic nitrogen source, gave the best mycelial biomass growth (236.67 mg) for *Pestalotiopsis* sp. compared to ammonium sulphate (66.67 mg), ammonium nitrate (66.67 mg), peptone (130.00 mg) and yeast extract (80.00 mg) including the control which gave the least biomass growth (30.00mg) (Figure 2(b)).

Preference for ammonium tartrate by *Pestalotiopsis* sp. can be explained by the fact that ammonium tartrate is a salt of a weak acid (Griffin, 1996) which during the process of nitrogen assimilation, the nitrogen compound is broken down into ammonia (NH_3) and hydrogen ions (H^+) and increase in H^+ ions decreases the pH value of the medium, thus increasing the acidity of the medium. The utilization of ammonium tartrate as a nitrogen source in the growth medium thereby releases less H^+ which does not acidify the medium beyond levels tolerable for the fungal's growth (Tong & Rajendra, 1992).

However, results from this study is not in agreement with that of Ren *et al*. (2013) who reported ammonium nitrate and potassium nitrate as the best nitrogen source for mycelial growth of *Pestalotiopsis* sp. The difference in the two results can be attributed to the fact that Ren *et al*. (2013) did not use ammonium tartrate in their study, so its effect on growth was not assessed. The variation in nitrogen source preference can be due to genetic differences in the fungal species used (Madan & Thind, 1998).

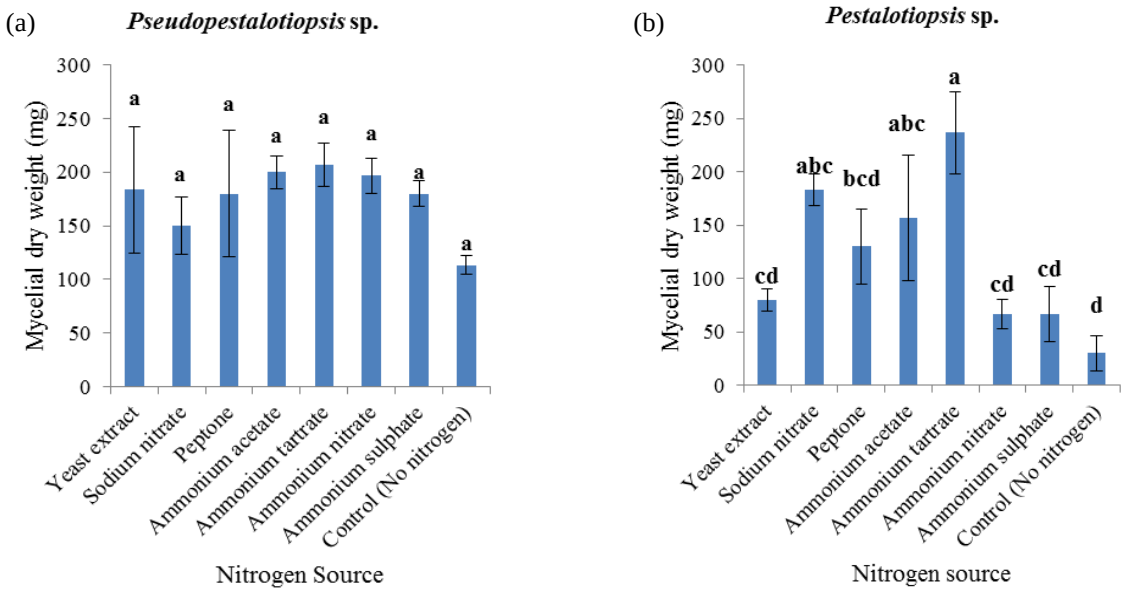

Figure 2. Mean mycelial biomass growth (mg ± S.E.M) of (a) *Pseudopestalotiopsis* sp. and (b) *Pestalotiopsis* sp. using different nitrogen sources after fifteen days of growth in liquid media. Bar charts with different alphabet superscripts are significantly different from each other ($p \leq 0.05$) using Duncan multiple range test (DMRT).

Mycelial Biomass Production of the Isolates at Different Temperature

Pseudopestalotiopsis sp.

High mycelial biomass growth (196.67 – 223.33 mg) of *Pseudopestalotiopsis* sp. was obtained in media incubated at temperature range from 15°C to 30°C in which there was no significant differences between the biomass growths within this temperature range (Figure 3(a)). However, the biomass production of *Pseudopestalotiopsis* sp. from the temperature range of 15 – 30°C was significantly higher than those obtained at 5°C, 10°C and 40°C, respectively. The lowest biomass growth was obtained at 35°C and 40°C which were significantly lower than those obtained at temperatures of 5°C and 10°C. Most microfungi are known to grow well at 15 – 30°C with very few species being able to grow at extreme temperatures of 50°C and above (Madan & Thind, 1998) and the result obtained for *Pseudopestalotiopsis* sp. revealed its optimum biomass production temperature between 15 – 30°C.

Pestalotiopsis sp.

There was high mycelial biomass production of *Pestalotiopsis* sp. (136.67 mg, 170.00 mg and 146.67 mg) in media incubated at 15°C, 20°C and 25°C (Figure 3(b)), which were significantly higher than those obtained in media incubated at other temperatures. The lowest biomass production was observed in media incubated at 40°C (20.00 mg). There was no significant difference between the biomass produced at 5°C, 35°C and at 40°C. The optimum biomass growth temperature was however observed at 20°C with a weight of 170.00 mg ± 5.77. This result is similar to that obtained for *Pseudopestalotiopsis* sp. above and it follows the trend reported for most fungal species (Madan & Thind, 1998).

Mycelial Biomass Production of the Isolates at Different Ph

Pseudopestalotiopsis sp. *and Pestalotiopsis* sp.

Pseudopestalotiopsis sp. and *Pestalotiopsis* sp. displayed a similar trend in their mycelial biomass growth at the various pH values tested in this study. The basal media at pH 5 to pH 10 were favourable for the biomass production of *Pseudopestalotiopsis* sp. with no significant differences in the mycelial biomass weights obtained between this pH range, but significantly different from that of pH 3 and pH 4 (Figure 4(a)).

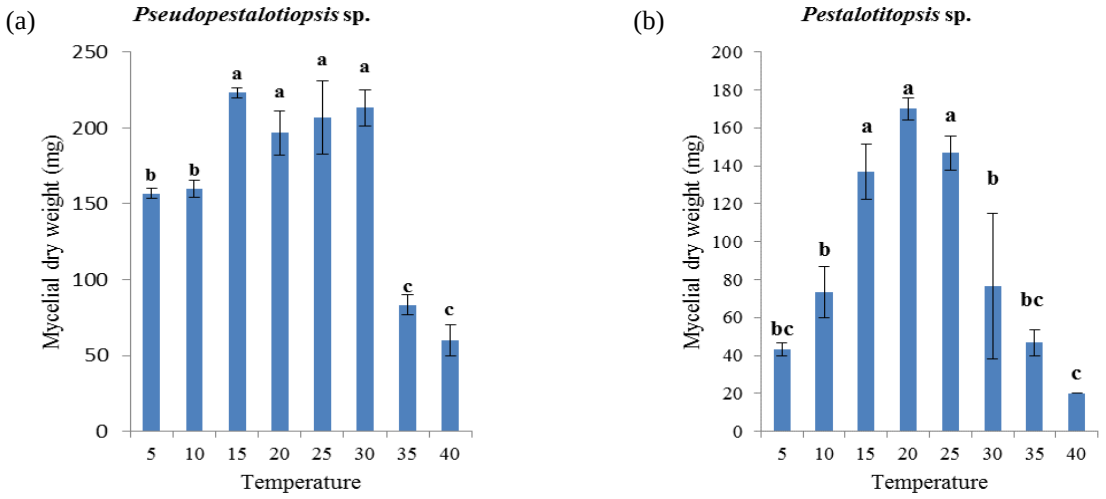

Figure 3. Mean mycelial biomass growth (mg ± S.E.M) of (a) *Pseudopestalotiopsis* sp. and (b) *Pestalotiopsis* sp. at different temperatures after fifteen days of growth in liquid media. Bar charts with different alphabet superscripts are significantly different from each other ($p \leq 0.05$) using Duncan multiple range test (DMRT).

The same growth pattern was observed in *Pestalotiopsis* sp. (Figure 4(b)) but the highest mycelial biomass was obtained from pH 9 basal medium which was significantly different from the other pH. This result suggests that a very acidic medium is not favourable for the mycelial biomass growth of these fungal species with implication that they will not grow well in media or soils with very high acidity but will do well in moderately acidic, neutral or alkaline medium as obtained in this study. This result is supported by that of Ren *et al*. (2013) of a similar trend of good mycelial biomass growth between pH 5-pH 10 from three isolates of *P. microspora*.

Mycelial Biomass Production of the Isolates Under Different Light Regime

Pseudopestalotiopsis sp.

As shown in Table 1, there was no significant difference between the three light regimes on mycelial biomass production of *Pseudopestalotiopsis* sp. with the highest weight of 313.33 mg ± 27.29 in media incubated under constant darkness to 256.67 mg ± 20.28 under constant light. This result conformed to Ren *et al*. (2013) which have earlier reported that light regime did not affect the growth of two strains of *Pestalotiopsis microspora*.

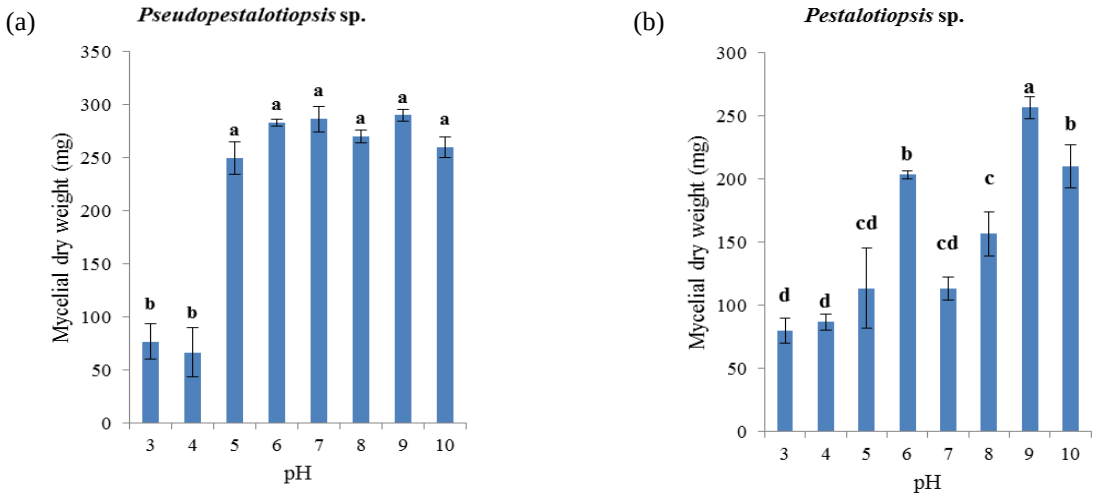

Figure 4. Mean mycelial biomass growth (mg ± S.E.M) of (a) *Pseudopestalotiopsis* sp. and (b) *Pestalotiopsis* sp. at different pH after fifteen days of growth in liquid media. Bar charts with different alphabet superscripts are significantly different from each other ($p \leq 0.05$) using Duncan multiple range test (DMRT).

Pestalotiopsis sp.

Constant darkness and alternate light regimes were significantly better for the mycelial biomass growth of *Pestalotiopsis* sp. than constant light (Table 1). As reported by Yusef and Allam (1967), responses of fungi to light might differ based on species, duration of exposure, intensity and quality of the light. In this study, constant light over 15 days was not favourable for optimum biomass production of *Pestalotiopsis* sp. and this can be explained by the fact that this might be an isolate specific requirement. Whenever the light threshold required by a fungus has been reached and then exceeded, it thereafter becomes over-dosage and thus suppress the fungus' growth (Hill, 1976).

CONCLUSION

In general, it is clear that different fungal species have different preferences for mycelial biomass production with the implication that fungal species have different priorities for their growth conditions. In terms of nutrient, a carbon source that is less preferred by one fungal species could be more preferred by another fungal species and same applies to other growth requirements of fungi. The conditions that can favour the mycelial biomass growth of *Pestalotiopsis* and *Pseudopestalotiopsis* species revealed from this study might aid their use in bioremediation, mycofiltration and other applications that use fungal mycelial biomass.

Table 1. Effect of light on mycelial biomass production (mg) of *Pseudopestalotiopsis* sp. and *Pestalotiopsis* sp., after fifteen days of growth in liquid media.

Light	Mycelial dry weight after 15 days (Average dry weight ± S.E.M)	
	Pseudopestalotiopsis sp.	*Pestalotiopsis* sp.
Constant light	256.67±20.28[a]	173.33±8.82 [b]
Constant darkness	313.33±27.29[a]	266.67±32.83[a]
Alternate light condition	296.67±43.33[a]	286.67±21.72[a]

Means with same alphabet down the columns are not significantly different from each other ($p > 0.05$), Duncan Multiple range test (DMRT).

ACKNOWLEDGEMENTS

The authors wish to thank the Sarawak government for permission to collect samples from the National Parks. The first author is also grateful to Universiti Malaysia Sarawak and University of Ilorin for the scholarship award.

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