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Effects of benzyladenine purine and its interaction with polyamines on growth of *Spathoglottis plicata* PLBs

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Abstract: *Spathoglottis plicata* is highly sought after by florists and orchid growers due to its unique structure and colors. However, this orchid is a slow-growing type and thus the planting materials are acutely limited. Therefore, there is a need to improve its multiplication efficiency and speed up its growth in order to meet the market demand. Benzyladenine purine (BAP) has been widely used to improve plantlet multiplication in plant cultures. Polyamines have also been reported to improve the growth and development of in vitro cultures. The objective of this study was to investigate the effects of BAP and different polyamines on the physical and biochemical changes of protocorm-like bodies (PLBs) of *S. plicata*. Different concentrations of BAP and polyamines (putrescine, spermine, and spermidine) were used. BAP and polyamines were supplemented singly in half-strength MS medium and PLBs were cultured for 2 weeks. It was observed that 5 µM BAP and 25 µM spermidine resulted in the highest fresh weights of 0.38 g and 0.31 g, respectively. The total soluble protein and carbohydrate content for PLBs treated with 25 µM spermidine was 4.85 mg/g FW and 6.36 mg/g FW. A separate experiment was carried out to investigate the interactive effects of 25 µM spermidine with 5 µM BAP. The presence of spermidine in the media reduced the peroxidase and catalase activities by increasing the nitrate reductase activity.

Key words: Benzyladenine purine, micropropagation, orchid, polyamine, *Spathoglottis*

1. Introduction

Spathoglottis plicata is a type of orchid that captures the interest of horticulturists because of its beautiful flowers and good stature. This orchid is free-blooming and it comes in several colors. *Spathoglottis unguiculata* has dark purple flowers while *S. aurea* is popular with its striking yellow flowers, which can be found in the Genting Highlands (Jin et al., 2012). *Spathoglottis* is a slow-growing type of orchid and has low multiplication frequency. However, this problem can be overcome using micropropagation methods with suitable additives. There are some reports suggesting the ability of cytokinin and polyamine in improving the growth of plants. However, the effect of interactions between these compounds on in vitro cultures plants is still unknown. Therefore, studies need to be carried out in order to gain more information about this.

Cytokinin was reported to be used in various types of orchid and improved the growth and development of this plant. Godo et al. (2010) reported that the germination efficiency of orchid seeds was increased when cultured on 0.2 mg/L benzyladenine purine (BAP). Protocorm-like bodies (PLBs) were induced from the shoot tips of *Oncidium* Gower Ramsey by inoculating them on half-strength MS medium fortified with BAP (Mengxi et al., 2010). PLBs were also formed from stem nodes of *Zygopetalum mackayi* after treatment with thidiazuron (Hong et al., 2010). Polyamines are organic compounds having 2 or more primary amino groups and found in plants both in free and bounded form. They appear to be important in cell division, plant growth, and senescence, as well as stress responses (Alcázar et al., 2006). Putrescine, for instance, has been proven to increase the proliferation of *Dendrobium* Sonia PLBs (Saiprasad et al., 2004). Ying et al. (2009) reported that the application of spermidine and putrescine increased the endogenous polyamine levels and resulted in the increased frequency of conversion of PLBs to shoots in *D. huoshanense*. Ganesan and Jayabalan (2006) proved that adding putrescine into media increased the formation of multiple shoots of *Gossypium hirsutum* L. plantlets. The objective of this study is to investigate the effects of BAP and polyamine and the interactions between these 2 compounds on the physical and metabolic changes of *S. plicata* PLBs. Putrescine, spermine, and spermidine were selected as the studied polyamines.

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2. Materials and methods

2.1. Seed germination and induction of PLBs

The PLBs used in this experiment were initiated from seeds derived from calli cultured on half-strength MS B5 vitamin medium with 5 µM 2,4-D. Three-week-old PLBs were subcultured onto basal medium added with 5 µM BAP for further subsequent experiments.

2.2. Medium preparation

PLBs were cultured on half-strength MS medium supplemented with vitamin B5, 2% (w/v) sucrose, and 0.3% (w/v) Gelrite. The pH of the culture medium was adjusted to 5.75. All cultures were maintained at 25 °C in the culture room condition under a 16-h photoperiod of 40 µmol m^{-2} s^{-1} light provided by cool white florescent tubes.

2.3. The effect of different concentrations of BAP on the growth of PLBs

The PLBs (0.15 g) were separately cultured onto half-strength MS medium fortified with BAP (0, 2.5, 5.0, 7.5, and 10.0 µM). The PLBs were cultured for 8 weeks. The fresh and dry weights were recorded every week. The data obtained from this experiment were used to understand the growth conditions of PLBs and to determine the optimum time for subculture.

2.4. Optimization of polyamine concentrations on growth and biochemical changes of *S. plicata* PLBs

Putrescine, spermine, and spermidine (0, 25, 50, 75, and 100 µM) were added as supplements to basal media. Basal media without polyamine were used as a control. The fresh and dry weights were recorded after 2 weeks of culture. The total soluble protein and carbohydrate contents were measured.

2.5. Influence of spermidine interacting with BAP on growth and biochemical changes of *S. plicata* PLBs

In order to compare the effect of spermidine with BAP, 25 µM spermidine with and without 5 µM BAP was used. This concentration was chosen after the optimization of polyamine concentration in the previous experiment. Half-strength MS medium and 5 µM BAP was used for the control. All experiments were carried out by culturing 0.15 g of PLBs on the basal media. The results were collected after 2 weeks of culture. The fresh and dry weights were recorded and total soluble protein and carbohydrate contents were measured. Catalase, peroxidase, and nitrate reductase activities were also measured. These enzymes were assayed to determine the effect of spermidine and BAP on oxidative stress and nitrate assimilation in PLBs.

2.6. Total soluble protein content

The soluble protein content was determined according to the method of Bradford (1976). The PLBs were weighed and protein was extracted by grinding PLBs with 100 mM Tris-HCl buffer containing 1 mM EDTA and 0.1% β-mercaptoethanol at pH 7.8 (with a ratio of 1:10). The homogenate was centrifuged at 12,000 rpm for 30 min. A total of 50 µL of sample extract and 450 µL of Tris-HCl buffer was placed in the vial, and 5.0 mL of Bradford reagent was added to it. The mixture was mixed thoroughly and was allowed to stand for 1 min before the absorbance at A_{595} was recorded. The total protein content was determined using bovine serum albumin as the standard.

2.7. Total soluble carbohydrate content

The total soluble carbohydrate content was measured using a method based on that of Jermyn (1975) with glucose as the standard. The sugar was extracted with 1.5 mL of 75% ethanol and the homogenate was centrifuged at 12,000 rpm for 30 min. Next, 100 µL of the extracted sample was mixed well with 5.0 mL of anthrone reagent (80% v/v sulfuric acid, 20% v/v distilled water, and 0.2% w/v anthrone) in the test tubes. The test tubes were incubated for 1 min at 90 °C and cooled to room temperature. The absorbance was read at A_{625}.

2.8. Catalase Activity Assay

The enzyme was extracted by grinding the sample in 0.1 M phosphate buffer (0.1 M $KHPO_4$, 0.1 M K_2HPO_4, 1 mM EDTA, 1.25 mM PEG 4000, and 0.1% β-mercaptoethanol, pH 7.5). The homogenate was centrifuged at 12,000 rpm for 30 min. The enzyme extracts (50 µL) were mixed with 0.1 M phosphate buffer (pH 7.0, 1.95 mL) and 260 mM H_2O_2 (100 µL), and the kinetics of changes in absorbance at 240 nm for 100 s were measured. The enzyme activity was calculated using the following formula:

$$\text{CAT } (mmol_{H2O2}\, min^{-1}\, mg_{protein}{}^{-1}\, g\ FW^{-1}) = ((\Delta A_{240} / \Delta t)\, V_{mix} / \varepsilon\, d\, P\, V_{extr})\ FW.$$

2.9. Peroxidase activity assay

The enzyme extracts (50 µL) prepared for catalase enzyme assay were mixed with 0.1 M phosphate buffer (pH 6, 1.35 mL), 20 mM H_2O_2 (500 µL), and 18 mM guaiacol (100 µL). The enzyme reaction was measured by reading absorbance of the mixture at A_{470} for 150 s. The calculation for the enzyme reaction was as follows:

$$\text{POX } (mmol_{tetraguaiacol}\, min^{-1}\, mg_{protein}{}^{-1}\, g\ FW^{-1}) = ((\Delta A_{470} / \Delta t) V_{mix} / \varepsilon\, d\, P\, V_{extr})\ FW.$$

2.10. Nitrate reductase activity assay in PLBs and plantlets of *S. plicata*

Nitrate reductase is an enzyme that catalyzes the NAD(P)H reduction of nitrate to nitrite. This enzyme was assayed to estimate nitrate assimilation in the *S. plicata* PLBs. Nitrate reductase was extracted by grinding PLBs with 0.1 M phosphate buffer of pH 7.3 (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, and 1.0 mM EDTA). The homogenate was centrifuged at 14,000 rpm for 10 min. The mixture contained 0.1 M phosphate buffer (500 µL), 0.1 M potassium nitrate (200 µL), 20 µM FAD (100 µL), 10.0 mM NADH (100 µL), and

enzyme extract (500 µL) and was incubated at 30 °C for 15 min. The enzyme reaction was stopped by adding 1% w/v of sulfanilamide (1 mL) and 0.02% w/v N-(1-naphthyl ethylene diamine dihydrochloride) (1 mL). The mixture was allowed to stand until a pink color developed. The absorbances were read at A_{540}. The enzyme activity was expressed as nanomoles of nitrite produced per minute per gram of fresh weight.

2.11. Statistical analysis

The mean values were calculated from a total of 3 replicates and the standard error of the mean was determined. One-way ANOVA and Duncan's multiple range test ($P < 0.05$) were applied to determine the significance of the results between different treatments using SPSS 20 for Windows.

3. Results

The fresh and dry weights were recorded weekly for 8 weeks. The results showed that the fresh weights of PLBs cultured on control media increased every week up to 0.31 g at the fifth week, as shown in Figure 1. However, the fresh weight started to decrease from the sixth week, and at the eighth week of culture, the fresh weight of PLBs was 0.2 g. When the PLBs were cultured in 2.5, 5.0, and 10.0 µM BAP, the fresh weight of the PLBs increased up to the seventh week and decreased at the eighth week.

The fresh weight of the PLBs treated with 7.5 µM BAP increased from 0.15 g to 0.29 g at the seventh week and started to decrease at the eighth week to 0.22 g. The highest fresh weight (0.38 g) of *S. plicata* PLBs was recorded during the seventh week for PLBs cultured on 5.0 µM BAP. A similar pattern was observed in the dry weights of the PLBs, as shown in Figure 2. There was some increment in the dry weight of *S. plicata* when treated with different concentrations of BAP. The dry weight of PLBs cultured in 10.0 µM BAP increased until the sixth week (44.6 mg) while at 5.0 µM and 7.5 µM the dry weights were the highest during the seventh and fifth weeks (30.95 mg and 34.6 mg), respectively. When the PLBs were cultured in 2.5 µM BAP, the dry weights increased until the seventh week (37.2 mg).

Figure 3 showed that the fresh weight of the PLBs cultured on media containing 25 µM putrescine and spermine (0.18 g and 0.17 g) was lower when compared to that of the control (0.27 g), but the fresh weight for the PLBs treated with 25 µM spermidine (0.31 g) was higher by 8% than that of the control. Similar trends could be observed for dry weight of the PLBs, whereby the dry weight of PLBs treated with 25 µM putrescine and spermidine was 27.0 mg and 29.0 mg, respectively. The dry weight of PLBs for the control treatment was 40.0 mg and the dry weight of the PLBs in 25 µM spermine was 25.0 mg.

The total soluble protein and carbohydrate contents of PLBs treated with putrescine, as shown in Figure 4, varied according to the different concentrations of polyamine. The total soluble protein obtained from PLBs cultured in 75 µM putrescine was the highest compared to other concentrations, but for total carbohydrate content, the PLBs cultured in 25 µM putrescine had the highest content as compared to other putrescine concentrations. Unlike putrescine, the soluble protein content of PLBs cultured in 100 µM spermine was the highest, but the total soluble protein in PLBs cultured in 75 µM spermine was decreased by 50% compared to the control. However, the total carbohydrate content increased as the concentration of spermine increased, where the total carbohydrate content in PLBs cultured in 100 µM spermine was up by 5-fold.

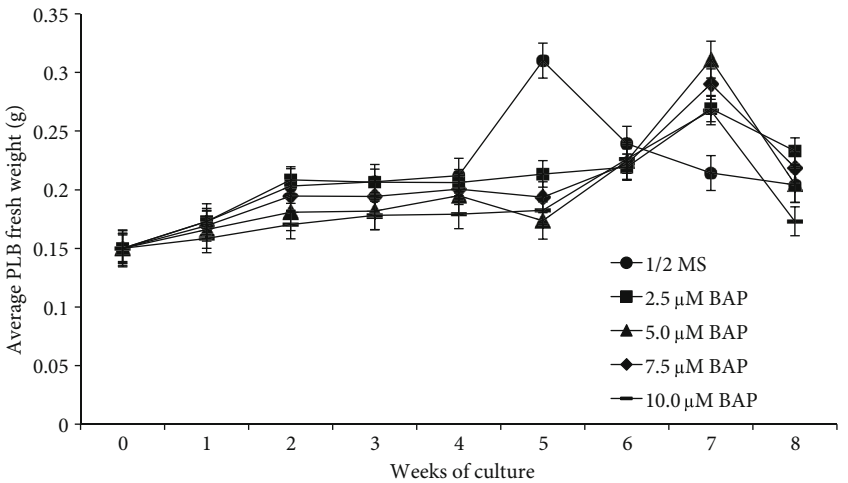

Figure 1. The average fresh weights of *S. plicata* PLBs after 8 weeks of culture on half-strength MS media supplemented with 2.5, 5.0, 7.5, and 10.0 µM BAP. Error bar indicates standard error.

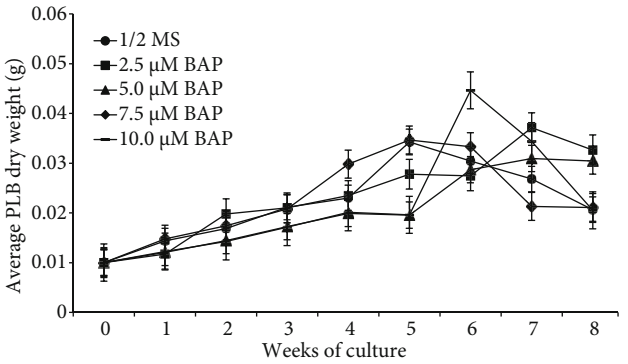

Figure 2. The average dry weights of *S. plicata* PLBs after 8 weeks of culture on half-strength MS media supplemented with 2.5, 5.0, 7.5, and 10.0 µM BAP. Error bar indicates standard error.

The total soluble protein and carbohydrate for PLBs treated with spermidine increased as the concentration increased from 25 µM spermidine to 50 µM spermidine. However, the soluble protein and carbohydrate contents decreased when the concentration was 75 or 100 µM spermidine.

The interaction effect of polyamine and cytokinin on the growth of *S. plicata* PLBs was investigated. The polyamine-treated PLBs produced 0.68 g fresh weight and 31.5 mg dry weight, as shown in Figure 5. The fresh and dry weights obtained from PLBs cultured in 25 µM spermidine were 0.56 g and 0.038 g, respectively, and those in 5 µM BAP were 0.450 g and 38.5 g. The fresh weight of PLBs cultured in 25 µM spermidine with 5 µM BAP was 0.53 g and the dry weight was 39.4 mg. Figure 6 shows the highest total soluble protein content obtained from the PLBs cultured on media supplemented 25 µM spermidine with 5 µM BAP, at 60.4% higher compared to the total soluble protein obtained from PLBs cultured in control media. Additionally, PLBs cultured in 5 µM BAP produced higher total soluble protein compared to 25 µM spermidine. PLBs cultured in 25 µM spermidine give the highest carbohydrate content (17.33 mg/g FW). However, the carbohydrate content obtained from PLBs cultured in 5 µM BAP was reduced by 59.5% compared to the PLBs cultured in control media.

Enzyme assays in which peroxidase, catalase, and nitrate reductase were recorded are shown in Figures 7 and 8. The peroxidase and catalase activities in the PLBs were decreased in the presence of 25 µM spermidine in the media. The peroxidase and catalase activities in PLBs cultured in 25 µM spermidine were reduced to 24% and 12% compared to the control. Meanwhile, the peroxidase and catalase activities in the PLBs cultured in 25 µM spermidine with 5 µM BAP were reduced to 35% and 16%. However, these treatments had different effects on nitrate reductase activity. The presence of 25 µM spermidine increased the nitrate reductase activity in PLBs. Nitrate reductase activity in PLBs cultured in 25 µM spermidine increased by up to 2-fold and for 25 µM spermidine and 5 µM BAP treatment the nitrate reductase activity increased by up to 3-fold.

4. Discussion

The application of cytokinin, and especially BAP, resulted in variable patterns in relation to formation and proliferation of PLBs. According to Nahar et al. (2012), application of 1.0 mg/L BAP increased formation

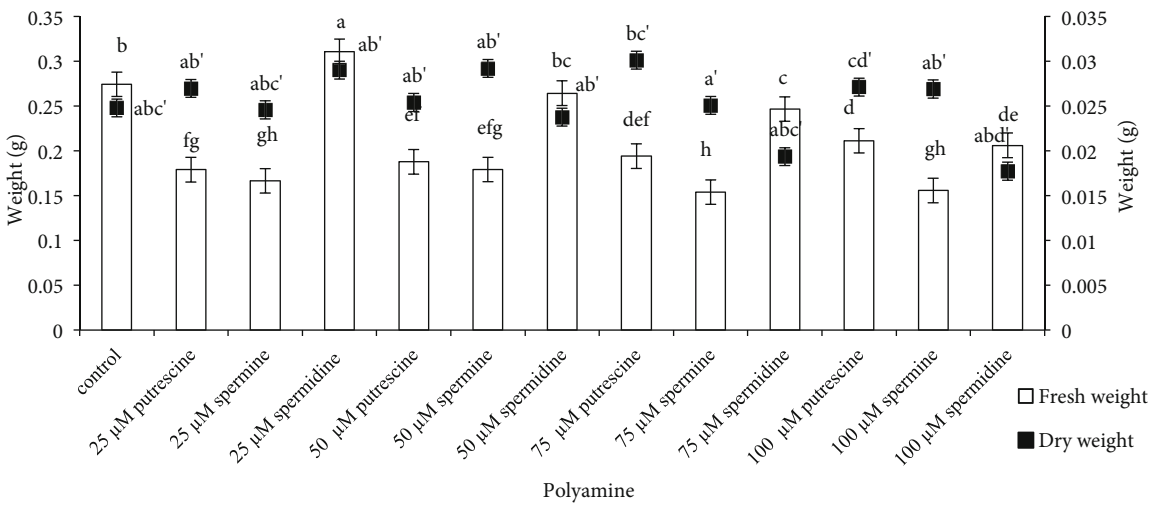

Figure 3. The average fresh weights and dry weights (g) of *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with different types of polyamines at different concentrations (0, 25, 50, 75, and 100 µM). Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

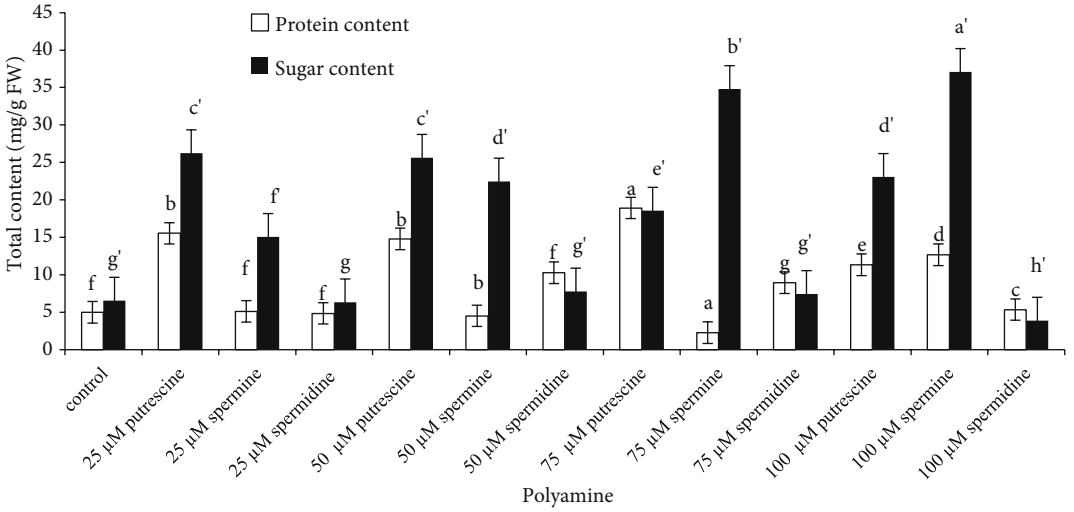

Figure 4. The total soluble protein and carbohydrate contents in *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with different polyamines at different concentrations (0, 25, 50, 75, and 100 µM). Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

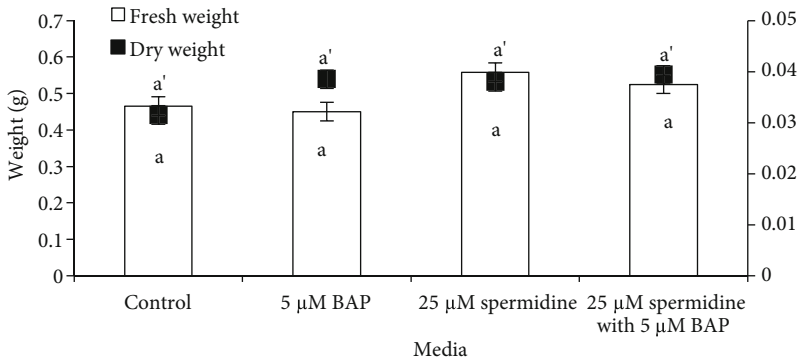

Figure 5. The fresh and dry weights of *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with 5 µM BAP, 25 µM spermidine, and 25 µM spermidine and 5 µM BAP. Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

Cymbidium insigne PLBs by up to 73%. The proliferations of *Renanthera* Tom Thumb 'Qilin' PLBs were affected by the concentration of BAP as reported by Wu et al. (2013). However, Prasertsirivatna and Koolpluksee (2011) reported that the exogenous application of cytokinins did not affect the growth of *D. friedericksianum* since the growth of PLBs was slower than that in cytokinin-added media. Alternatively, the high concentration of cytokinin inhibited PLB proliferation from leaf segments of *Aranda* Wan Chark Kuan 'Blue' × *V. coerulea* Grifft. ex. Lindl (Gantait and Sinniah, 2012). According to Irvani et al. (2010), the effect of certain plant growth regulators depended on the ability of the plant cell to metabolize the plant growth regulator or the capability of that plant growth regulator to induce endogenous hormone in the plant. Based on this study, the best concentration of BAP for maintenance of PLBs was 5 µM BAP, and the PLBs have to be transferred to fresh media every 4 weeks.

PLBs were treated with polyamines at different concentrations (0, 25, 50, 75, and 100 µM) and the effects of these treatments were investigated. Spermidine at different concentrations is effective in increasing fresh and dry weight of the PLBs compared to other polyamines even though the carbohydrate content and soluble proteins were lower. Several studies were carried out on the effect of polyamines on plant growth and development. According to Kumar and Palni (2013), the accumulation of spermine and spermidine at high concentrations in the

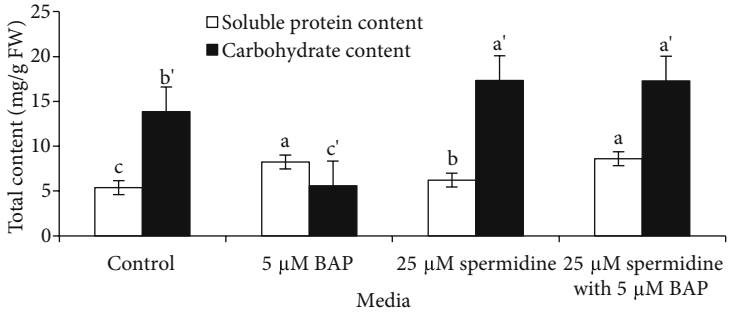

Figure 6. The soluble protein and carbohydrate contents of *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with 5 μM BAP, 25 μM spermidine, and 25 μM spermidine and 5 μM BAP. Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

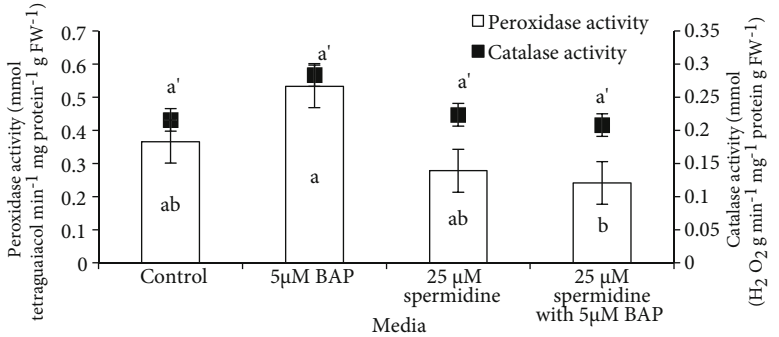

Figure 7. The peroxidase and catalase activities of *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with 5 μM BAP, 25 μM spermidine, and 25 μM spermidine and 5 μM BAP. Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

culture of *Gladiolus hybridus* inoculated on media without any plant growth regulator might had led to the formation of cormlets. Furthermore, Ovono et al. (2010) reported that endogenous putrescine and auxin could produce better growth with the presence of exogenous putrescine in the *Dioscorea cayanensis–Dioscorea rotundata* complex. Additionally, polyamines were also involved in plant stress resistance mechanisms. Accumulation of polyamine in plants during stress will help in plant tolerance mechanisms. For example, production of polyamine and sugar in spring wheat might have been induced by stress when the cultures were treated with PEG (Grzesiak et al., 2013). Putrescine is a diamine, while spermine is tetramine and spermidine is triamine. The methylene group of polyamines will give a hydrophobic interaction for this compound. Therefore, exogenous putrescine might not be absorbed by the PLBs as much so as the other polyamines and putrescine might accumulate in the media; this will induce stress since it is toxic at certain concentrations (Takao et al., 2006). However, this result is in contrast with a report on *Momordica dioica*, wherein 1.0 μM putrescine was the best polyamine in increasing the fresh weight of the embryogenic callus (Thiruvengadam et al., 2013).

According to Dutra et al. (2013), the application of spermidine and spermine increased the changes of cellular structure in proembryogenic masses by producing more embryogenic and suspensor cells in *Araucaria angustifolia*. Exogenous spermidine and spermine also can increase the stress tolerance in *Allium fistulosum* toward waterlogging, by increasing the free proline accumulation, and help in maintaining high relative water content (Yiu et al., 2009). Spermine and spermidine were synthesized from putrescine by adding an aminopropyl group obtained from decarboxylated S-adenosylmethionine (Duan et al., 2008). It appears, therefore, that when spermine and spermidine were added into the media the plant cells were able to absorb and use these polyamines without any further processing because these polyamines were already

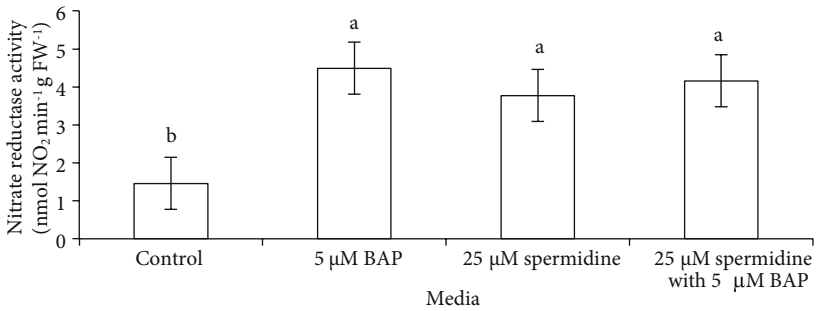

Figure 8. The nitrate reductase activity of *S. plicata* PLBs after 2 weeks of culture on half-strength MS media supplemented with 5 µM BAP, 25 µM spermidine, and 25 µM spermidine and 5 µM BAP. Similar letters show insignificant differences according to the Duncan multiple range test ($P < 0.05$). Error bar indicates standard error.

synthesized in spermidine and spermine form. Thus, 25 µM spermidine was chosen as the best polyamine for growth of *S. plicata* PLBs.

Polyamine was also reported to mediate the hormone effects in plant cells. This compound also acts as a second messenger and works by interacting with plant growth regulators in cell division. According to De-la-Peña et al. (2008), endogenous spermidine and spermine levels in *Coffea canephora* plantlets were decreased to 50% and 30% when the plantlets were culture without BAP. Similarly, Steiner et al. (2007) also reported that polyamines were able to increase the growth of *Araucaria angustifolia* embryogenic cell cultures on media without any plant growth regulators by increasing the ABA level. Therefore, in the last experiment, the PLBs were treated with 25 µM spermidine with and without 5 µM BAP. Based on the results, not all treatments increased the growth as compared to the control. However, PLBs cultured on medium fortified with 25 µM spermidine alone did have lower total soluble protein but high carbohydrate content. Thus, a few enzyme assays were carried out for peroxidase, catalase, and nitrate reductase. All of these enzymes had low activity in the PLBs treated with 25 µM spermidine as compared to other treatments.

The oxidative stress was overcome by exogenous application of spermine and spermidine when these compounds prevented the Cu-induced enhancement of O^{-2} formations and H_2O_2 content (Wang et al., 2007). In addition, polyamine was able to protect the cell when the oxidative damage was reduced via controlling the enzymatic antioxidant system and ROS levels when seedlings of *Cucumis sativus* were under dehydration conditions (Kubis, 2008). Furthermore, according to Rosales et al. (2011), the nitrate reductase activities in *Triticum aestivum* leaves were reduced after treatment with spermine and spermidine. This might be due to the displacing of nitrate reductase with polyamine receptor, which has low affinity for its ligand (Tun et al., 2006).

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