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Effects of micronutrients (Cu, Zn, Mn, and Fe) on the growth of *Spathoglottis plicata* plantlets

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Abstract

The micropropagation of valuable orchid species such as *Spathoglottis plicata* could help in their conservation and increase their propagation rate. The objective of this study was to investigate the effects of micronutrients ($CuSO_4$, $ZnSO_4$, $MnSO_4$ and Fe-EDTA) on the growth of *S. plicata* plantlets. Plantlets of uniform height (1.5 cm) were transferred to a half-strength MS media supplemented with vitamin B5 and different concentrations of selected micronutrients (copper, zinc, manganese and iron). The highest production of soluble protein content (38.98 mg/g of fresh weight, FW) was recorded when plantlets were treated with 25 μM $MnSO_4$. *Spathoglottis plicata* plantlets formed the highest amount of chlorophyll (22.32 mg/g FW) when the growth media were supplemented with 75 μM Fe-EDTA. A total of 25 μM Fe-EDTA induced the production of up to 19.78 mg/g FW of carbohydrates in *S. plicata* plantlets. Furthermore, we demonstrate that different concentrations of micronutrients had different effects on the activities of several enzymes, such as peroxidase, catalase, polyphenol oxidase and nitrate reductase.

Key words: antioxidant enzymes, micronutrients, micropropagation, orchid, *Spathoglottis plicata*

Abbreviations

A – absorbance
T – time of reaction
V_{mix} – total volume of the sample
ε – molar extinction coefficient at 240 nm for H_2O_2 and 470 nm for oxidized guaiacol
d – optical distance
P – protein content in the extract
V_{ext} – volume of extract used in the reaction

Introduction

There are 3000 orchid species in Malaysia. *Spathoglottis plicata* is a tropical species mostly found throughout southern Asia, China, the Malay Archipelago, New Guinea, Thailand, Philippines, Australia and the islands of the southwest Pacific Ocean (Hossain and Dey, 2013). This garden orchid has a high resistance toward both high and cold temperatures and can be cultivated in any environment (Chikmawati, 2013). The plicate leaves that grow from pseudobulbs and its beautiful purple color flower make it an attractive ornamental crop. Besides its evergreen palm-like leaves and stunning purple flowers, this orchid also has medicinal value. According to Rao (2004), the decoction resultant from boiling its leaves is used for the treatment of rheumatism. The ornamental and medicinal characteristics of *S. plicata* contribute to the high demand for this plant material; large scale plantlet production for this orchid should therefore be established.

Different plants have different demands and responses to nutrients that are present in culture media. The establishment of optimum concentrations of nutrients is important to produce healthy plantlets and thus healthy plants. There are several reports on enhancing culture

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conditions through higher levels of certain micronutrients. Kalpana and coworkers (2010) reported that the number of *Stevia rebaudiana* shoot buds increased in the presence of high concentrations of copper. According to Kowalska and coworkers (2012), elevated concentrations of copper in culture media of *Daucus carota* improved the formation of plantlets. In addition, the rate of root proliferation for sorghum plantlets cultured in the MS media supplemented with 1 mg/l of a different type of auxins and of 1 μM $CuSO_4$ increased the production of roots to 56.7 per explant (Liu et al., 2013). Bardar et al. (2015) reported that the number of *Eclipta alba* shoots produced from nodal segments cultured in growth media with 0.55 μM $CoCl_2$ increased to 13 shoots compared to the control plants that produced only 9 shoots, on average. Meanwhile, Schwalbert and coworkers (2016) reported that changing the concentration of micronutrient and macronutrient in MS media to 1/4 and 1/2, respectively, can improve the shoot growth of *Desmodium incanum* plantlets. Many studies have shown that the manipulation of micronutrients in growth media can produce significant effects on the quality of plant cultures. However, to date there have been no reports on the optimization of micronutrients in orchid tissue cultures. Therefore, in this study the effects of copper ($CuSO_4$), zinc ($ZnSO_4$), manganese ($MnSO_4$), and iron (Fe-EDTA) on the growth of *S. plicata* plantlets were investigated.

Materials and methods

Plant material

Spathoglottis plicata flowering plants were bought from a local nursery and were grown and maintained in a glasshouse under normal temperature (32°C) and normal daylight for 12 hours. The plants were watered every day and fertilized with a commercial fertilizer. Mature green seed pods, with the dry petal attached at the tips, were selected for experiments.

Basic culture media preparation and culture incubation conditions

The basic medium used in this study was half-strength MS (Murashige and Skoog, 1962). The media were supplemented with 20.0 g/l sucrose, 2.75 g/l Gelrite and vitamins B5 (containing 100 mg/l myo-inositol, 0.5 mg/l niacin, 0.5 mg/l pyridoxine HCl, 0.1 mg/l thiamine HCl). The medium pH was adjusted to 5.75 and autoclaved for 15 min at 121°C. These media were used as the basal media throughout the study and are referred to as 1/2 MS media. The seeds were germinated and the seedlings were maintained in 25 ml 1/2 MS media to be used in other experiments. Four-week-old seedling cultures were kept in cool white fluorescent tubes at 25°C under 16 h photoperiod of 40 μmol m^{-s} s^{-1}.

Effects of micronutrients (Zn, Cu, Mn and Fe) on the growth of S. plicata plantlets

Four-week-old (1.5 cm) plantlets were cultured on half-strength MS media supplemented with the following concentrations – 0, 25, 50, 75, 100 μM, of either $CuSO_4$, $ZnSO_4$, $MnSO_4$ or Fe-EDTA. Basal 1/2 MS medium was used as the plantlet growth control. The numbers of leaves, roots, plant height, root length, fresh and dry weights were recorded.

Total soluble protein content in S. plicata plantlets

The protein content was determined using the Bradford method (1976). The plantlets were weighed and the proteins were extracted by grinding plantlets with 100 mM Tris – HCl buffer pH 7.8 (100 mM Tris – HCl, 1 mM EDTA, 0.1% β-mercaptoethanol, sample : buffer ratio was 1 : 10). The homogenate was centrifuged at 15294 g for 30 minutes. A total of 50 μl of sample extracts and 450 μl 100 mM Tris – HCl buffer were placed in vials, and 5 ml of Bradford reagent was added. The mixtures were mixed thoroughly and allowed to stand for one minute before absorbance at 595 nm was recorded. Total protein content was determined using bovine serum albumin (BSA) as the standard.

Total carbohydrate content in S. plicata plantlets

The carbohydrate content was measured using the Anthrone method (Jermyn, 1975) with glucose as the standard. The carbohydrates were extracted by grinding the plantlets with 1.5 ml 75% ethanol and the homogenates were centrifuged at 15294 g for 30 minutes. 100 μl of extracts were mixed well with 5 ml of Anthrone reagent (80% v/v sulfuric acid, 20% v/v distilled water and 0.2% w/v Anthrone) in test tubes. The test tubes were incubated for 1 min at 90°C. The tubes were cooled at room temperature and the absorbance was read at 625 nm. A glucose equivalent was used to express the total carbohydrate content in the plantlets.

Total chlorophyll content in S. plicata plantlets

Total chlorophyll content was measured to determine the activity of plant photosystem in the plantlets. The method used was based on the protocol by Arnon (1949), with modifications. Fresh leaves (0.5 g) were ground with 10 ml of 80% acetone. The homogenates were centrifuged at 863 g for 5 min. The supernatants were transferred to clean vials and absorbances were recorded at 645, 652 and 663 nm. The chlorophyll content was determined according to the formula below.

$$20.2\ (A645) + 8.02\ (A663)$$
$$\times \text{ total volume}/1000 \times \text{fresh weight}$$

Influence of micronutrient (Zn, Cu, Mn and Fe) on selected enzymatic activities in S. plicata plantlets

Three plantlets were prepared for each treatment and the total protein was collected from each of the plantlets to be used for the enzyme assays. The mean values were calculated from the total of three replicates and the standard error of the mean was determined. Catalase, peroxidase, polyphenol oxidase, and nitrate reductase activity assays were carried out on plantlets grown on media containing selected micronutrients (25 μM $CuSO_4$, 50 μM $CuSO_4$, 25 μM $ZnSO_4$, 50 μM $ZnSO_4$, 25 μM $MnSO_4$, 50 μM $MnSO_4$, 50 μM FeEDTA and 100 μM Fe-EDTA) and without the addition of micronutrients (as a control) according to the following procedures.

Catalase and peroxidase activity assays in S. plicata plantlets

Catalase and peroxidase activities were assayed according to the Omran (1980) method, with modifications. Enzymes were extracted by grinding the samples in a phosphate buffer (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 1 mM EDTA, 1.25 mM PEG 4000 and 0.1% β-mercaptoethanol, pH 7.5). The homogenates were centrifuged at 15 294 g for 30 minutes.

Catalase activity assay in S. plicata plantlets

The enzyme extracts (50 μl) were mixed with a phosphate buffer, pH 7.0 (1.95 ml), 260 mM H_2O_2 (100 μl) and absorbance was recorded at 240 nm for 100 seconds. The enzymatic activities were calculated using the following formula:

$$\mathrm{CAT}\,(\mathrm{mmol}_{H_2O_2}\,\mathrm{minute}^{-1}\,\mathrm{mg}_{\mathrm{protein}}{}^{-1}\,\mathrm{g\,FW}^{-1}) =$$
$$= ((A_{240}/t)\,V_{mix}/\varepsilon\,d\,P\,V_{extract})\,FW$$

Peroxidase activity assay in S. plicata plantlets

The enzyme extracts (50 μl) obtained from the catalase assay were mixed with a phosphate buffer, pH 6 (1.35 ml), 20 mM H_2O_2 (500 μl), and 18 mM guaiacol (100 μl). The enzymatic activities were measured by reading the absorbances of the mixtures at 470 nm for 150 seconds. The calculation for the reaction was as follows:

$$\mathrm{POX}\,(\mathrm{mmol}_{\mathrm{tetraguaiacol}}\,\mathrm{minute}^{-1}\,\mathrm{mg}_{\mathrm{protein}}{}^{-1}\,\mathrm{g\,FW}^{-1}) =$$
$$= ((A_{470}/t)\,V_{mix}/\varepsilon\,dP\,V_{extract})\,FW$$

Polyphenol oxidase activity assay in S. plicata plantlets

The enzymatic fractions extracted for catalase and peroxidase assays were also used to assay the polyphenol oxidase activity in *S. plicata* plantlets and this enzyme assay was conducted using the method proposed by Arnon (1949), with the following modifications. The enzyme mixtures were prepared by adding a phosphate buffer (phosphate buffer pH 7 (1.95 ml)), 0.1 M catechol (1.0 ml) and enzyme extracts (50.0 μl). The enzymatic activities were determined by reading the absorbances at 410 nm for 5 min.

Nitrate reductase activity assay in S. plicata plantlets

The method used was based on the Hageman and Flesher (1960) procedure, with the following modifications. The nitrate reductase was extracted by grinding plantlets with a phosphate buffer, pH 7.3 (0.1 M KH_2PO_4, 0.1 M K_2HPO_4 and 1.0 mM EDTA). The homogenates were centrifuged at 15294 g for 10 min. Supernatants containing enzymes were used in the assay. A total of 500 μl of enzyme extracts were mixed with the phosphate buffer (500 μl, pH 7.3), 0.1 M potassium nitrate (200 μl), 20 uM FAD (100 μl) and 10.0 mM NADH (100 μl) and incubated at 30°C for 15 min. The enzymatic reactions were stopped by adding 1% w/v of sulfanilamide (1 ml) and 0.02 % w/v N-(1-naphthyl) ethylene diamine dihydrochloride (1 ml). The mixtures were allowed to stand until a pink color developed. The absorbances were read at 540 nm. The enzymatic activities were expressed as nanomoles of the nitrite produced per min per fresh weight of the plantlet.

Statistical analysis

In all experiments, a minimum of three flasks of cultures containing three plantlets were prepared for each treatment. The mean values were calculated from the

total of three 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 version 20 for Windows.

Results

Effects of micronutrients (Zn, Cu, Mn and Fe) on the growth of S. plicata plantlets

Fresh and dry weights (FW and DW, respectively) of the plantlets treated with 25 μM $CuSO_4$ were lowered to 16% and 24% compared to the control, respectively. Similarly, the fresh weight of the plantlets cultured in the medium with 100 μM $MnSO_4$ was 23% lower than that for control plantlets. However, FW of the plantlets cultured in a medium supplemented with 50 μM $ZnSO_4$ was 29% higher and one-fold higher in DW. The plantlets treated with 100 μM Fe-EDTA had a 20% increase in the fresh weight and 50% in the dry weight.

As shown in Table 1, the heights of the plantlets treated with 75 μM $CuSO_4$ increased (by 42%). The height of the plantlets treated with 50 μM $ZnSO_4$ increased (by 50%) and those treated with 100 μM $ZnSO_4$ also increased but to a lesser extent (by 24%). The manganese treatment did not have any significant influence on plantlet height, only for 50 μM $MnSO_4$ was it reduced by 6% compared to the control. Plantlet height in growth media supplemented with 75 μM Fe-EDTA was reduced one-fold.

Those plantlets cultured in media supplemented with 100 μM $CuSO_4$ experienced a two-fold reduction in root length. At the concentration of 75 μM $ZnSO_4$, plantlets had 36% shorter roots compared to the control. Plantlets treated with 50 μM $MnSO_4$ had root length reduced by 22% and with 100 μM $MnSO_4$ by 64%. The root length of plantlets cultured in a medium supplemented with 25 μM Fe-EDTA had a 27% reduction and with 100 μM Fe-EDTA the reduction of root length was 95%.

The supplementation of growth media with $CuSO_4$ and Fe-EDTA did not produce significant effects on the number of leaves grown on the plantlets (Table 1). However, for plantlets treated with 50 μM $ZnSO_4$, a 17% increase in the number of leaves, compared to the control, was observed. Those plantlets treated with 25 μM $MnSO_4$ had a 20% higher number of leaves, whereas for 50 μM $MnSO_4$ 20% the number of leaves was lower. Supplementation with $CuSO_4$, $ZnSO_4$ and Fe-EDTA did not have any significant effect on the number of roots formed (Table 1). Plantlets treated with 50 μM $ZnSO_4$ had a 20% higher number of roots, while the treatment with 100 μM $ZnSO_4$ gave a two-fold lower number of roots from the plantlets.

Plantlets cultured in a medium supplemented with 75 μM $CuSO_4$ changed significantly, showing a 27% increase in the total soluble protein fraction, compared to the control (Fig. 1). Interestingly, the 25 μM $MnSO_4$ treatment gave a 60% higher total soluble protein fraction from the plantlets. Meanwhile for the plantlets cultured in medium fortified with 100 μM FeEDTA, the amount of total soluble protein was 17% higher when compared to plantlets cultured in the control medium. After treatment with $ZnSO_4$ (in the case of all tested concentrations), the plantlets showed no significant change in terms of the total soluble protein fractions.

Figure 1 summarizes the total carbohydrate content in the plantlets after the treatments with selected micronutrients. The total carbohydrate content in the plantlets cultured in medium fortified with 100 μM $CuSO_4$ was three-fold lower, but for 25 μM $CuSO_4$ it was 5% higher, compared to the control. Meanwhile, the total carbohydrate fraction obtained from plantlets treated with 50 μM $ZnSO_4$ was 33% lower. Those plantlets treated with 50 μM $MnSO_4$ had a 50% increase in the carbohydrate content, compared to the plantlets cultured in the control medium. However, from plantlets cultured on medium fortified with 25 μM Fe-EDTA the obtained total carbohydrate fraction was 11% higher, but for 100 μM Fe-EDTA it was one-fold lower.

The chlorophyll content in plantlets (Fig. 1) treated with 25 μM $CuSO_4$ was one-fold lower compared to the control. Plants treated with 75 μM $ZnSO_4$ had a 43% reduction in chlorophyll content. The chlorophyll content obtained from plantlets cultured with 50 μM $MnSO_4$ was 25% lower, while in media supplemented with 75 μM $MnSO_4$ the chlorophyll content increased in plantlets by 31%. Meanwhile, the chlorophyll content obtained from plantlets cultured with 75 μM Fe-EDTA was 35% higher; however, in the case of 100 μM Fe-EDTA concentration the chlorophyll content was reduced by 18%.

Influences of micronutrients on selected enzymatic activities

Peroxidase activity in plantlets cultured with 50 μM Fe-EDTA was one-fold higher than the control; however,

Table 1. The effect of different concentrations of micronutrients on *S. plicata* fresh, and dry weights, plant height, root length, and number of roots and leaves. The same letters with symbols show no significant difference according to the Duncan Multiple Range Test ($P < 0.05$)

Treatments	Concentrations (μM)	Fresh weight (g)	Dry weight (g)	Plant height (cm)	Root length (cm)	Number of roots	Number of leaves
Control		0.18^{ab}	0.02^{b}	9.33^{b}	3.17^{bc}	2.00^{a}	5.00^{a}
$CuSO_4$	25	0.15^{b}	0.01^{b}	8.33^{b}	3.50^{bc}	1.67^{a}	5.00^{a}
	50	0.23^{a}	0.02^{b}	12.50^{a}	5.00^{ab}	2.00^{a}	5.00^{a}
	75	0.21^{ab}	0.02^{a}	13.33^{a}	6.33^{a}	2.00^{a}	4.67^{a}
	100	0.20^{ab}	0.02^{a}	9.50^{c}	1.67^{c}	2.67^{a}	4.67^{a}
Control		$0.18^{ab'}$	$0.01^{b'}$	$7.43^{b'}$	$4.73^{a'}$	$3.00^{ab'}$	$5.67^{ab'}$
$ZnSO_4$	25	$0.13^{b'}$	$0.02^{ab'}$	$8.33^{b'}$	$3.00^{a'}$	$2.67^{ab'}$	$5.00^{b'}$
	50	$0.22^{ab'}$	$0.02^{a'}$	$10.67^{a'}$	$3.00^{a'}$	$4.00^{a'}$	$6.67^{a'}$
	75	$0.23^{a'}$	$0.02^{ab'}$	$10.50^{a'}$	$2.67^{a'}$	$3.00^{ab'}$	$5.67^{ab'}$
	100	$0.21^{ab'}$	$0.02^{ab'}$	$9.17^{ab'}$	$3.67^{a'}$	$1.67^{b'}$	$5.00^{b'}$
Control		0.27^{ab*}	0.03^{ab*}	14.50^{a*}	5.50^{a*}	2.67^{a*}	5.00^{ab*}
$MnSO_4$	25	0.29^{a*}	0.02^{bc*}	12.33^{a*}	4.17^{ab*}	2.00^{a*}	6.00^{a*}
	50	0.31^{a*}	0.03^{a*}	13.50^{a*}	4.33^{ab*}	2.33^{a*}	4.00^{b*}
	75	0.16^{b*}	0.01^{c*}	11.00^{a*}	3.17^{ab*}	2.33^{a*}	4.67^{ab*}
	100	0.21^{ab*}	0.02^{abc*}	11.00^{a*}	2.00^{ab*}	2.33^{a*}	4.67^{ab*}
Control		0.23^{a+}	0.02^{b+}	13.67^{a+}	5.50^{a+}	2.00^{a+}	4.67^{a+}
Fe-EDTA	25	0.11^{b+}	0.02^{b+}	11.67^{ab+}	4.00^{ab+}	2.00^{a+}	4.00^{a+}
	50	0.21^{a+}	0.02^{b+}	12.17^{ab+}	2.83^{bc+}	2.00^{a+}	4.67^{a+}
	75	0.23^{a+}	0.02^{b+}	7.67^{b+}	0.90^{cd+}	1.33^{a+}	4.33^{a+}
	100	0.30^{a+}	0.03^{a+}	10.00^{ab+}	0.23^{d+}	2.00^{a+}	4.00^{a+}

when the plantlets were treated with 50 μM $ZnSO_4$, 50 μM FeEDTA or 100 μM FeEDTA there was no significant change compared to the control (Fig. 2). The catalase activity in plantlets increased only after treatment with 50 μM $CuSO_4$, 50 μM $MnSO_4$ and 50 μM Fe-EDTA. However, for 25 μM $ZnSO_4$ the catalase activity was three-fold lower, compared to the control. The micronutrient treatments reduced the polyphenol oxidase activity in plantlets, except for the 50 μM $CuSO_4$ treatment, which was recorded as showing a three-fold increase (Fig. 2). Nitrate reductase activity in plantlets was also reduced after being treated with micronutrients. Those plantlets treated with 50 μM Fe-EDTA showed a 96% lower nitrate reductase activity and the plantlets cultured with 25 μM $CuSO_4$ had a 32% reduction in this activity (Fig. 2).

Discussion

Many studies have reported that alteration in the concentrations of certain nutrients can help to improve the conditions and growth of the plant cultures. According to Chauhan and Kothari (2004), the shoot bud induction and plant regeneration of *Hordeum vulgare* increased when the nutrient level in culturing media was increased by adding 0.30 mM ammonium nitrate, 1.25 mM potassium dihydrogen phosphate, 1.03 mM sodium molybdate and 0.11 mM cobalt chloride. The shoot bud induction of *Jatropha curcas* was improved when a $CuSO_4$ concentration 10 times higher than normal was applied on MS media (Khurana-Kaul et al., 2010). Based on the results of this study, it has been shown that altering $CuSO_4$, $ZnSO_4$, $MnSO_4$ and Fe-EDTA concentrations (0, 25, 50, 75 and 100 μM) in the culturing media may

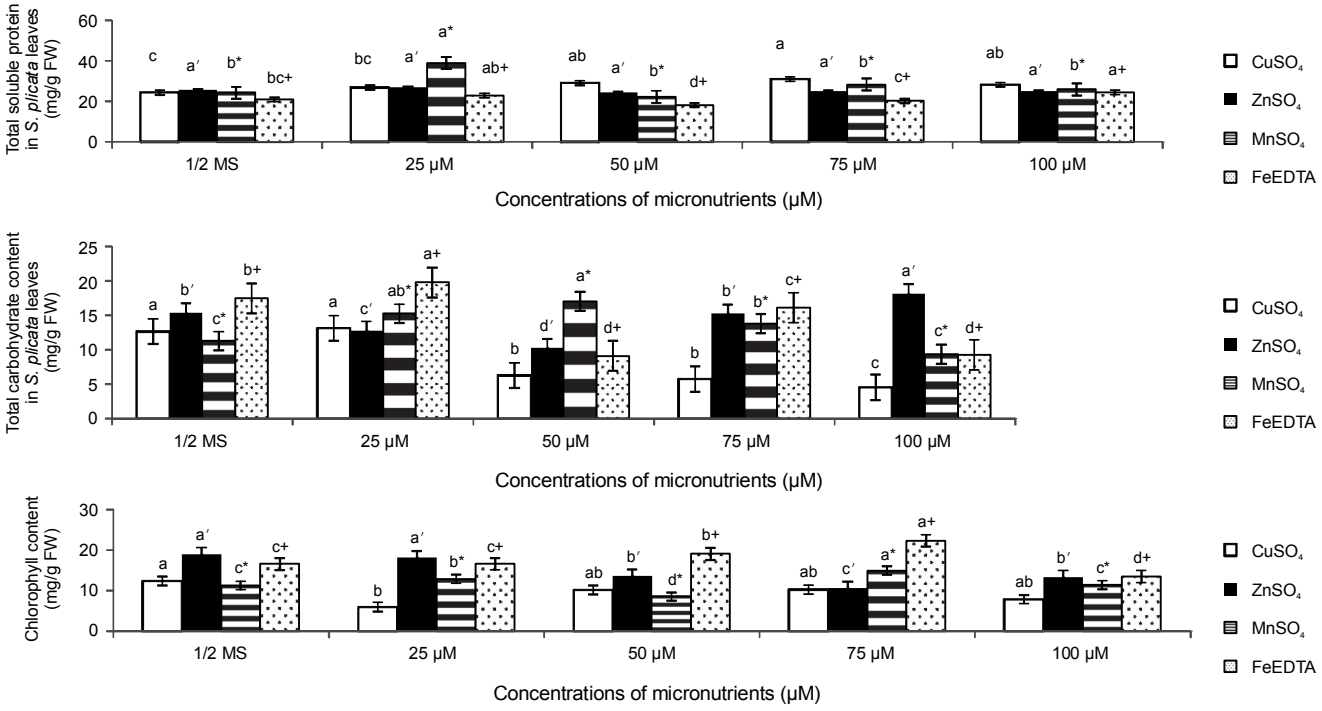

Fig. 1. The effect of different concentrations of micronutrients on *S. plicata* total soluble protein, carbohydrate and chlorophyll contents. The same letters with symbols show no significant difference according to the Duncan Multiple Range Test ($P < 0.05$)

influence the growth of plantlets. Fresh, dry weights and plant heights were affected by different concentrations of these four micronutrients. However, it has been shown that for the root length and the number of root-only plantlets treated with copper there was a limited increase, while in other treatments the result was worse than in the control (Khurana-Kaul et al., 2010). Joshi and Kothari (2007) reported that higher concentrations of copper in the growth media improved the induction and elongation of shoot buds in *Capsicum annuum*. Additionally, Jain and coworkers (2012) reported improved shoot bud induction in *Stevia rebaudiana* when the medium concentrations of $MnSO_4$, KI and $CoCl_2$ were increased. This might be due to the function of micronutrients in the growth and metabolism of plants. These compounds act as cofactors for various enzymes and play roles as secondary messengers in the plant growth pathways (Niedz and Evens, 2007).

Protein, chlorophyll and carbohydrate contents in plant tissues can be used to indicate plant performance in certain growth stages or conditions. The total chlorophyll content can be used as a parameter for stress response in plants, because this compound is bound to membranes. If the membrane of the plant cell is damaged due to stress, the chlorophyll content will be reduced and the photosynthesis process will not be as efficient as it would have been under normal growth conditions. Many studies have shown that a high chlorophyll content can be used as an indicator for healthy plants. Han and coworkers (2009) reported that plants with a higher total chlorophyll content in salt-tolerant *Triticum aestivium* could produce higher yields. According to Jaleel and coworkers (2008), total chlorophyll content was reduced when the plantlets germinated from seeds in *Catharanthus roseus* (L.) G. Don when exposed to salinity. On the other hand, Guo and coworkers (2007) reported a decrease in the amount of a soluble protein fraction in roots and leaves of *Hordeum vulgare* when plantlets were treated with high concentrations of aluminum, copper and cadmium. Moreover, Bernstein and coworkers (2010), used total protein content to investigate plant antioxidative response in *Zea mays* toward salinity and established that the protein content in leaves was higher when the plants were submitted to salinity. Yet, in our experiments, the amount of total soluble proteins did not show any significant differences when the plantlets were treated with different concentrations of micronutrients, except for supplementation with

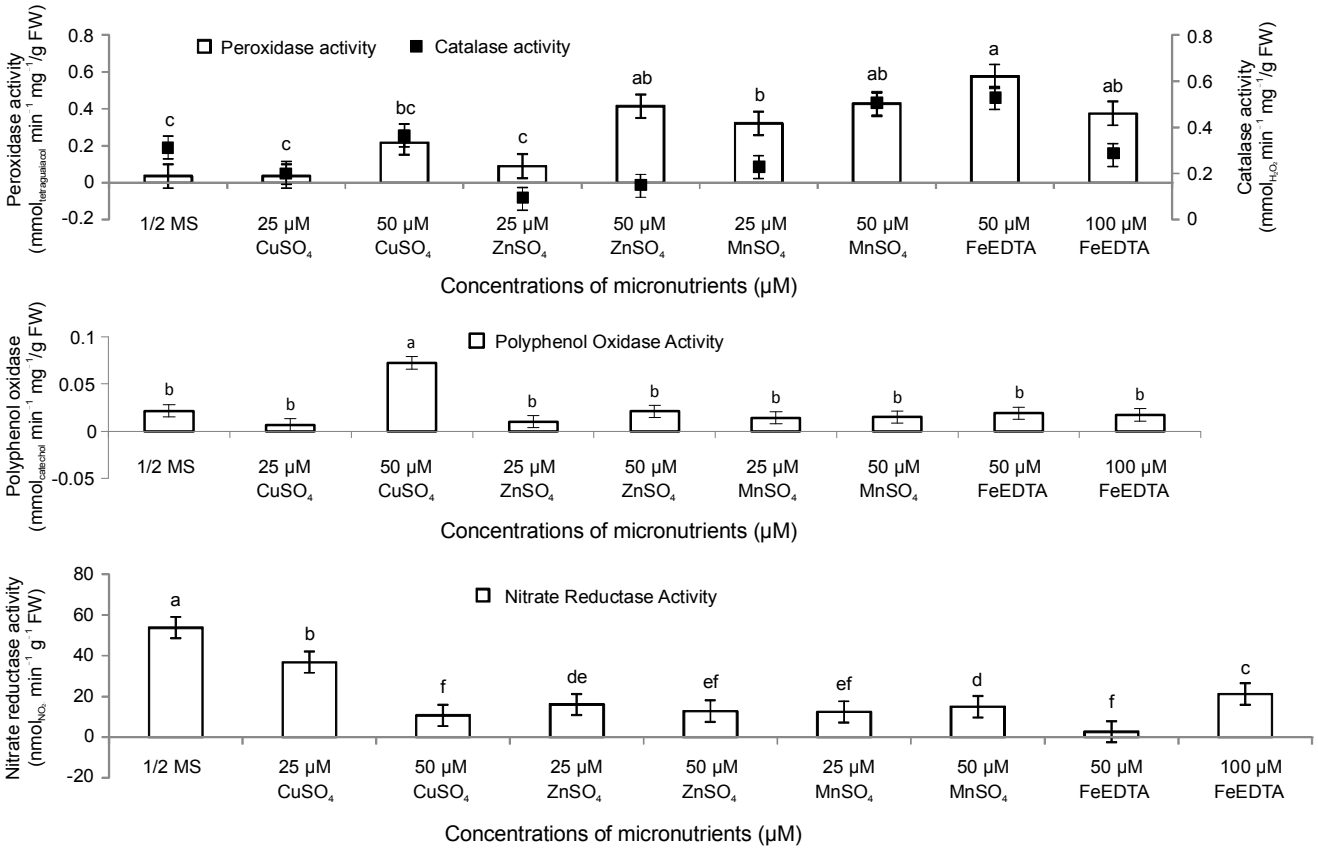

Fig. 2. Enzymatic activities in *S. plicata* plantlets after six weeks of culturing on 1/2 MS media supplemented with different concentrations of micronutrients. The same letters with symbols show no significant difference according to the Duncan Multiple Range Test ($P < 0.05$)

25 µM MnSO4 where we recorded a 60% increase in the total soluble protein fraction.

The total sugar (carbohydrates) content in plants has also been used in many studies to estimate the conditions of growing plants. For example, Sales and coworkers (2013) reported that high levels of total soluble sugar in the stalk of *Arachis pintoi* indicated that this organ had a photoassimilate transportation function. The carbohydrate content in leaves of *Pisum sativum* treated with mycorrhiza, before being exposed to water stress, decrease compared to the control (Shinde and Thakur, 2015). Similar to the results of our experiments, the chlorophyll content was also increased in platelets grown in a medium fortified with $ZnSO_4$. $MnSO_4$ and Fe-EDTA. However, the total carbohydrate content in the plantlets was affected by different concentrations of micronutrients. It has been shown that increasing the concentrations of $CuSO_4$ and $ZnSO_4$ in growth medium affects the production of chlorophyll and carotenoids in *Withania somnifera* (Fatima et al., 2011). Borowiak and coworkers (2015) reported that the carbohydrate content in the hybrid *Salix purpurea* x *triandra* x *viminalis* increased, as the concentrations of zinc in the culture media increased. Similarly, the chlorophyll content in the *Rauvolfia serpentina* plantlets increased, when 25 µM $ZnSO_4$ and 20 µM $CuSO_4$ concentrations were applied in the growth medium (Ahmad et al., 2015).

The activities of peroxidase and catalase in plantlets grown in media supplemented with micronutrients increased under most of the conditions analyzed in our study. These enzymes possess antioxidant properties; therefore, an increase in their activity probably reflects the need to protect the plant against oxidative stress which is generated by the increment of micronutrients in the growth media. Similarly, as reported by Rahimizadeh and coworkers (2007), superoxide dismutase, catalase and glutathione peroxidase activities in leaves of *Helianthus annuus* were elevated after the plant had been fertilized with micronutrients. The polyphenol oxidase activity in the plantlets was assayed, because per-

oxidase plays a role in the accumulation of phenolic compounds in plants. However, based on the results of our study, it may be stated that polyphenol oxidase activity in plantlets supplemented with micronutrients is lower compared to the plantlets cultured in the control media. Peroxidase usually catalyzes certain inorganic or organic compounds, and hydrogen peroxide in particular does so (Khatun et al., 2008). Therefore, the activities of these enzymes depend on the amount of these compounds present in plants.

As seen in our experiments, the elevated oxidative enzyme activities do not have any harmful effects on the growth of plantlets; on the contrary, they probably protect the cells from oxidative damage such as lipid peroxidation and protein carboxylation. Furthermore, copper, which is a redox active metal, can help to block the formation of hydroxyl, peroxyl and alkoxyl compounds (Gao et al., 2008). These compounds are toxic to plants; and thus, by preventing the formation of these compounds, copper is a part of the antioxidant defense system against any oxidative damage occurring in plant cells.

Nitrogen is an essential mineral for plant growth and development. Therefore, we also assayed the nitrate reductase activity in plantlets fortified with different concentrations of micronutrients. Interestingly, nitrate reductase in plantlets cultured in different concentrations of micronutrients was lower in all tested conditions compared to the control. According to Xiong and coworkers (2006), this decrease in the nitrate reductase activity might be caused by the interruption of the nitrate transporter system in plants by copper, or due to zinc, manganese or iron. The information acquired in our experiments could be useful in the production of an appropriate and efficient media culture for orchids, especially *S. plicata*.

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