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Full Length Article

Effects of Copper on Total Phenolics, Flavonoids and Mitochondrial Properties of *Orthosiphon stamineus* Callus Culture

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Abstract

Plants need an appropriate amount of copper to sustain their normal growth. However, excess of copper may interrupt plant development and cause stress. In this study, the responses of *Orthosiphon stamineus* callus culture under various copper concentrations were investigated. Callus cells at 24-days old were treated with 100, 150, 200 and 250 µM of copper solution and harvested after one week. Mitochondria from the calluses were isolated and the total mitochondrial proteins were determined. We used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to compare the mitochondrial protein profiles in normal medium and copper-containing medium. The activities of cytochrome c oxidase, as well as the total phenolic and flavonoid contents were determined. The results of atomic absorption spectroscopy (AAS) analysis have shown significant amount of copper accumulated in *O. stamineus* callus cells, depending on the amount of copper supplied. With the BCF values of more than 10, this species can be categorized as copper hyperaccumulator. When the amount of copper supplied increased, the cells exhibit higher cytochrome c oxidase activities and produce more phenolic compounds, in spite of decreasing amount of total mitochondrial proteins and a lesser number of visible bands visualized from SDS-PAGE analysis. Overall, our findings highlight the dynamic responses of *O. stamineus* cells when exposed to various levels of copper, notably by alteration of biochemical and mitochondrial properties as their protection mechanism under stress.

Keywords: Heavy metals; Plant mitochondria; Cultured cells; *Orthosiphon stamineus*

Introduction

Copper is one of the most important micronutrients in plants. In general, it is required as a cofactor for many enzymes involve in respiration, photosynthesis and protein metabolisms (Yruela, 2005). However, high level of copper will lead to toxicity and disturb the normal processes in plant tissues, cells, and organelles including mitochondria (Keunen *et al*., 2011; Rahman *et al.*, 2013).

Mitochondria in plants have various roles, especially in the generation of energy (Hanson and Day, 2013). Mitochondrial proteins are mostly encoded by plant nuclear genome, while the mitochondrial genome encodes a few components of the ETC (electron transport chain) (Cooper, 2000). During respiration, proteins involved in the primary metabolic reactions in tricarboxylic acid cycle are catalyzed by copper and iron; thus, facilitate the electron transfer in ETC (Xu *et al*., 2013; Garcia *et al*., 2014). Hence, appropriate amount of copper is essential for proper mitochondrial functions and whole plant development.

Nowadays, environmental pollution due to excess of heavy metals has become a serious issue. Copper contamination in soil and water bodies may come from natural and anthropogenic activities such as agriculture, mining, and metallurgical industries (He *et al*., 2005; Nagajyoti *et al*., 2010). As adaptive measures towards copper stress, plants may change their physiological and biochemical properties to continue survival. If plants are tolerant to the metal, they can be used for phytoremediation purposes. However, this will depend on plant individual capacity and the amount of metals present in their surroundings.

To date, studies related to mitochondria from the callus culture have not been widely reported. Previously, the streptomycin resistance of tobacco cultured cells and the response of mitochondria from calluses-forming potato

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tuber disks towards ethanol and growth temperature were reported (Plas and Wagner, 1980; Hemrika-Wagner *et al*., 1982; Zamski and Umiel, 1982). Quite recently, the effects of nitric oxidase to mitochondrial respiration of *Arabidopsis thaliana* calluses were investigated (Wang *et al*., 2010). Hence, the information on mitochondrial response of the cultured cells under stress is limited.

This study attempted to better understand the biochemical and mitochondrial properties of *Orthosiphon stamineus* calluses when exposed to copper stress. *O. stamineus* is a type of medicinal herb widely found in Malaysia. The leaves extract of this plant is known to possess strong antioxidant capacity (Akowuah *et al*., 2004). In specific, the objectives of this study were to explore the responses of *O. stamineus* cultured cells in terms of copper accumulation level, and the production of phenolics and flavonoid compounds, besides profiling the mitochondrial properties subsequent to its mitochondrial isolation. Overall, this study will enable us to assess the capacity of this species to be used in copper remediation.

Materials and Methods

Plant Materials

Misai Kucing (*O. stamineus*) aged 3 weeks old were purchased from a local supplier in Johor, Malaysia. Plants were placed in the glasshouse, watered twice per day with tap water early in the morning and late in the evening. The day length is 12-h, similar to the night length. The range of daily temperature is 26ºC to 33ºC and the relative humidity is above 80%.

Callus Development and Surface Sterilization

Matured leaf explants were selected from *O. stamineus* plants and smeared in commercial liquid soap prior to the washing step under running tap water for 30 min. Whole leaves were placed in sterilized jars and ethanol 70% (v/v) were applied for one minute. 20% of bleach solution was used for 30 min with an addition of a few drops of Tween 20, followed by three times rinse with sterile distilled water. Plant leaves were cut with sterilized cutter blade to establish 1 cm × 1 cm square shape each. Five pieces of cut leaves were cultured into each Petri dish containing agar MS medium plus NAA 1 mg/L and 2,4-D 1 mg/L for callus induction. Callus of *O. stamineus* were established until maturation by day-24.

Treatment of Callus Culture

The callus culture placed in jars, each containing MS medium containing 1 mg/L NAA and 1 mg/L 2, 4-D with different concentration of $CuSO_4.5H_2O$. The treatments were indicated as T1=control, T2=100 µM, T3=150 µM, T4=200 µM, T5= 250 µM. Calluses were harvested after one week of $CuSO_4.5H_2O$ treatments.

Determination of Heavy Metal Content

Prior to heavy metal analysis, callus samples were dried in the oven at 80ºC for 72 h. Dried samples were pulverized into powder and approximately 0.1 g samples were subjected to acid digestion process. Nitric acids followed by perchloric acid (2:1) were added to the powdered samples. Digested sample was further diluted and aliquots were used for estimation of copper concentration. The measurements of these metal elements were conducted using Atomic Absorption Spectrophotometer (AAS) Model AAnalyst 400 (Perkin Elmer, Massachusetts, USA). Sample preparation and heavy metal analysis were conducted based on the standard method by the American Public Health Association (APHA-AWWA-WPCF, 1980).

Heavy Metal Analysis

The accumulations of copper in *O. stamineus* was calculated using bio-concentration factor (BCF) as follows: BCF = metal concentration in callus/metal concentration in medium.

Mitochondrial Isolation

Mitochondria were isolated using a method from (Wilson and Chourey, 1984) with some modifications. Callus tissues were grounded in a pre-chilled mortar and pestle which consisted of 10 mM n-tris (hydroxymethyl)methyl-2-aminoethane-sulphonic acid (TES), pH 7.2, 0.5 M mannitol, 1 mM ethylene glycol-bis-(2-aminoethylether)-n, n'tetraacetic acid (EGTA), 0.2% w/v BSA, and 0.05% w/v cysteine. After that, the suspension was filtered through 4 layers of cheesecloth into pre-chilled tubes. Using a fresh buffer, the tissues were re-grounded two more times and the pooled filtrate was centrifuged at 1000 x g for 10 min at 4°C. Next, mitochondria were collected from the supernatant by centrifugation at 16,000 × g for 10 min at 4°C. A small paint brush was used to resuspend the mitochondrial pellet in a 10 mL pre-chilled buffer that consisted of 0.3 M sucrose and 0.05 M Tris, pH 7. The mitochondria were pelleted by centrifugation at 12,000 x g for 20 min at 4 °C. The mitochondrial pellet was resuspend in a 10 mL buffer and re-pelleted at 14,000 x g for 10 min at 4°C. Next, the pellet was resuspend in a 10 mL sucrose wash buffer and stored in -80° C freezer prior to usage.

Mitochondrial Protein Quantification

The protein concentrations of isolated mitochondria were determined using Optiblot Bradford Reagent (Abcam, USA). A standard for protein was prepared from BSA (1 mg/mL). Sample (20 µL) and 300 µL reagent were mixed and absorbance at 595 nm was recorded (Bradford, 1976).

Mitochondrial Cytochrome C Oxidase Test

The activity of cytochrome c oxidase in isolated mitochondria was determined using Cytochrome c Oxidase Assay Kit (Sigma-Aldrich, USA) based on the instruction recommended by the manufacturer. This colorimetric assay is based on an observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis SDS-PAGE

SDS-PAGE was carried out based on (Bollag *et al*., 1996). The stacking gel solution (125 mM Trishydrochloride (Tris-HCl) pH 6.8, 0.1% (w/v) SDS, 5% (w/v) acrylamide (acrylamide: bis-acrylamide ratio of 37.5: 1), 0.07% (w/v) ammonium persulphate and 0.03% (v/v) N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) was overlaid on the separating gel solution (373 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 12% (w/v) acrylamide, 0.04% (w/v) ammonium persulphate and 0.05% (v/v) TEMED). Samples were mixed with 5 X sample buffer (60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 14.4mM 2-mercaptoethanol) and heated for 5 min at 90ºC, followed by centrifugation at 10,000 x g for 5 min at 25ºC. The same amount of protein samples was loaded on the gel and separated at 150 V until the bromophenol blue reached the bottom of the gel. The gel was stained with Coomassie Blue: The gel was incubated in Coomassie blue R-250 (0.1% (w/v) coomassie blue R-250, 10% (v/v) acetic acid, 45% (v/v) methanol) for 16 h and transferred to destaining solution (10% (v/v) methanol, 10% (v/v) acetic acid). The gel was destained until the protein bands are visible and the image was then captured.

Aqueous Extracts Preparation

Dried samples were pulverized using mortar and pestle and extracted with autoclaved deionized water at the ratio of 1:40 (dry weight: volume), at 90ºC for 1 h. The heat incubated homogenate was vacuum-filtered and the filtrate was centrifuged at 9000 rpm at 4ºC for 15 min. The supernatant obtained was immediately aliquoted (500 µL each) and stored at -20ºC prior to analysis.

Determination of Total Phenolic Content

Determination of total phenolic contents in the extracts was conducted using a Folin-Ciocalteu colorimetric assay (Waterhouse, 2001). A mixture containing extract (0.2 mL), deionized water (0.8 mL), and Folin Ciocalteu reagent (0.1 mL) was incubated at room temperature for 3 min. Next, 0.3 mL of Na_2CO_3 (20% w/v) was added and the mixture was incubated at room temperature for 120 min. Absorbance of the mixture was recorded at 765 nm. A standard curve was prepared from 0 to 100 mg/L gallic acid. Total phenolic content was expressed in mg gallic acid equivalents/g dry matter.

Determination of Total Flavonoid Content

The content of flavonoids in the extracts was determined as previously described (Zou *et al*., 2004). Plant extract (0.2 mL) was added to 0.15 mL of $NaNO_2$ (5% w/v) and the mixture was incubated at room temperature for 6 min. Then, 0.15 mL of $AlCl_3.6H_2O$ (10% w/v) was added to the mixture and incubated for 6 min at room temperature, followed by an addition of 0.8 mL of NaOH (10% w/v). The mixture was incubated at room temperature for 15 min. and absorbance of the mixture was recorded at 510 nm. A standard curve was prepared from 0 to 500 µg/mL quercetin dissolved in 80% ethanol and total flavonoid content was expressed in mg quercetin equivalents/g dry matter.

Data Analysis

Experiments were carried out in triplicates. Data were analyzed using Microsoft Excel 2007 and reported as mean±standard error. Tukey's test was used at the 0.05 level of probability for comparison of the set of means.

Results

***Orthosiphon stamineus* Calluses Have the Capacity to Accumulate Copper**

The concentrations of copper in *O. stamineus* callus cells were increased as the concentrations of copper supplied increased. Although the magnitude of the BCF varied with the concentration of copper supplied in media, the BCF values of more than 10 indicate good copper-hyperaccumulation properties in *O. stamineus* (Table 1). The highest BCF of 12, was determined from calluses in mediums containing 200 µM and 250 µM of copper.

Copper Stress Reduced the Total Mitochondrial Protein Content in *O. stamineus* Calluses

Callus cells contain mitochondria although the density was lower than the differentiated cells. The results from our analysis showed a significantly lower amount of mitochondrial proteins in the callus exposed to copper treatments compared to control (Table 2). The concentrations of mitochondrial protein were 1.1, 1.3, 1.4, and 1.3-fold lesser than the control for 100, 150, 200 and 250 µM copper treatments, respectively. A similar trend for the yield of protein in isolated mitochondria was observed (Table 2). Comparisons between different copper treatments also exhibit significant differences in mitochondrial proteins contents and yield, except between the treatments using 150

Table 1: Concentrations of copper in *O. stamineus* callus and the bioconcentration factor

Level of Cu supplied in medium (µM)	Level of Cu in callus (µM)	Bioconcentration factor (BCF)
Control	0[a]	0
100	1115.83 ± 5.40[b]	11.2
150	1522.04 ± 29.11[c]	10.1
200	2445.79 ± 10.10[d]	12.2
250	3017.73 ± 13.53[e]	12.1

Data are means ± standard errors (n =3). Different lowercase letters indicate significant differences among treatments within a column according to Tukey's Test ($p<0.05$)

Table 2: Mitochondrial protein concentrations and yield from *O. stamineus* callus grown in different copper treatments

Copper treatment (µM)	Protein concentrations (mg/ml)	Yield (mg)
Control	16.86 ± 0.18[a]	168.60 ± 1.78[a]
100	15.70 ± 0.12[b]	157.04 ± 1.17[b]
150	12.58 ± 0.33[c]	125.78 ± 3.26[c]
200	12.33 ± 0.03[dc]	123.26 ± 0.32[dc]
250	13.26 ± 0.06[e]	132.58 ± 0.59[e]

Data are means ± standard errors (n =3). Different lowercase letters indicate significant differences among treatments within a column according to Tukey's Test ($p<0.05$)

Table 3: Total phenolic and flavonoid contents in the callus of *O. stamineus*

Parameter	Total phenolics (mg GAE/g)	Total flavonoids (mg Quercetin/g)
Control	167.30 ± 0.25[a]	9.18 ± 0.17[a]
100µM	169.88 ± 2.02[ab]	9.64 ± 0.11[a]
150µM	171.62 ± 0.16[ab]	9.38 ± 0.18[a]
200µM	175.94 ± 2.98[b]	9.47 ± 0.07[a]
250µM	172.25 ± 0.61[ab]	9.44 ± 0.06[a]

Data are means ± standard errors (n =3). Different lowercase letters indicate significant differences among treatments within a column according to Tukey's Test ($p<0.05$)

and 200 µM of copper solution.

Copper Treatment Increased the Activity of Mitochondrial Cyt C Oxidase

Our results revealed that the activity of cytochrome c oxidase has been increased with increasing amount of copper, in spite of the reduction of total mitochondrial protein contents. The detection of cytochrome c oxidase activity signified the success of our mitochondrial isolation. We demonstrated that the activity of cytochrome c oxidase increased significantly at 5.3-fold when treated with 200 µM copper, while at the highest copper treatment (250 µM), the cytochrome c oxidase activity was 11.3-fold higher compared to control (Fig. 1).

The Profile of Mitochondrial Protein from *O. stamineus* Calluses

The protein profiles of mitochondria from *O. stamineus* calluses were obtained from SDS-PAGE. The results showed that the protein bands were more visible in the control, compared to samples treated with copper. There were three clear protein bands in the control treatments with molecular weight slightly less than 75 kDa, approximately 50 kDa, and 25 kDa. The former band intensity increased with copper treatment. However, the intensity of ~50 kDa and ~25 kDa protein bands decreased with copper treatments (Fig. 2).

Total Phenolic Contents Increased in Copper Treated *O. stamineus* Calluses

Analysis of ethanol extracts of *O. stamineus* with different amount of copper showed significantly 5.2% higher amount of total phenolic content in the callus treated with 200 µM copper, compared to control. Although there was a slight reduction in total phenolics content when cells were exposed to 250 µM copper, this change was not significantly different when compared to other treatments. The total flavonoid however, did not differ significantly between all treatments (Table 3).

Discussion

Understanding the response of plants under stress is important to evaluate plant capacity to be used in bioremediation. Generally, the BCF value is one of the indicators for plant capacity to accumulate or tolerate heavy metals. The BCF values of 1 to 10 indicate the plant ability as hyperaccumulator (Audet and Charest, 2007). High BCF values for *O. stamineus* calluses showed good copper-hyperaccumulation properties. Our result was in agreement with a previous report on the ability of *O. stamineus* whole plants to absorb heavy metals (Abdu *et al*., 2011), and further proved that plants in Lamiacea family are potential metal accumulators as reported by (Saxena and Misra 2010). Earlier, heavy metal tolerance in the cultured cells has been demonstrated in their respective intact plants, for instance in *Anthoxantum odoratum* (Qureshi *et al*., 1981). However, unlike the intact plants, the mechanisms of heavy metal tolerance in callus cells are not fully elucidated, especially in relation to plant mitochondrial properties. Nevertheless, it is known that metal stress triggers the production of antioxidant, which acts a defense mechanism in callus cells (Gallego *et al*., 2002; Xu *et al*., 2011; Lukatkin *et al*., 2014).

Different amount of protein content in plants during stress is an indicator for alteration in plant metabolisms. Our study revealed the decreased level of mitochondrial protein concentrations and yield with increasing amount of copper treatment. Previously, reductions of total protein content in plants due to copper stress have been reported, although they were highly depending on the duration of exposure (Zengin and Kirbag, 2007; Vinod *et al*., 2012; Brahim and Mohamed, 2013). While those studies focused on total proteins, our research is focusing on mitochondrial

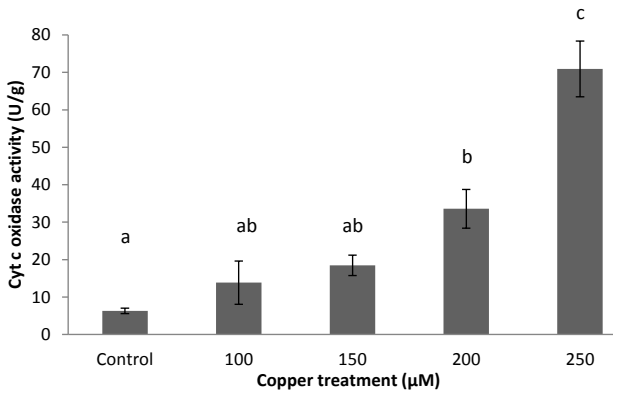

Fig. 1: Cytochrome c oxidase activity in *Orthosiphon stamineus* callus grown in different copper concentrations. Data are means ± standard errors (n =3). Different lowercase letters indicate significant differences among treatments within a column according to Tukey's Test ($p<0.05$)

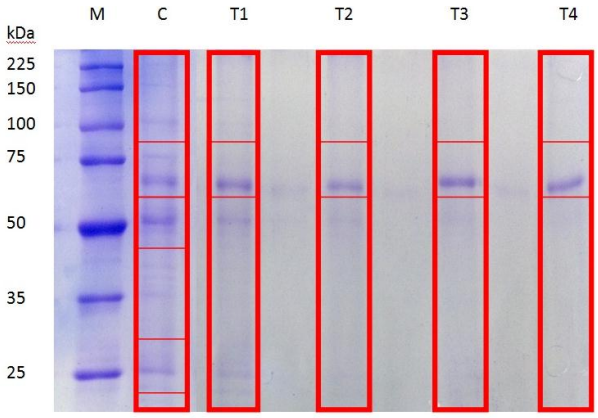

Fig. 2: Protein expression profiles of mitochondrial proteins from *Orthosiphon stamineus* treated with different concentrations of Cu. M= Molecular weight protein marker kDa. C= Control. T1= 100 µM Cu. T2= 150 µM Cu. T3= 200 µM Cu. T4= 250 µM Cu

proteins. Proteins in plant mitochondria are complex with up to 3000 gene products (Millar *et al*., 2007). These proteins are involved in various functions such as respiration transport, stress response, and many more (Zeng *et al*., 2007). It was previously reported that high level of copper inhibits plant respiration and induces the production of alternative oxidases in cultured cells (Padua *et al*., 1999). In this study, we detected a much lesser amount of total mitochondrial proteins under copper stress, indicating that certain processes in mitochondria may be compromised.

The activity of cytochrome c oxidase, an important enzyme in mitochondrial ETC, increased when higher amount of copper is applied. Generally, copper toxicity causes oxidative stress and possibly leads to dysfunction of cytochrome c (Srinivasan and Avadhani, 2012). However, in our case, excess of copper may promote the activity of cytochrome c oxidase that naturally requires copper as a cofactor and functions as its terminal electron acceptor (Pilon *et al*., 2006).

Then, we further analyzed the protein profiles from SDS-PAGE and observed differential expressions of polypeptides during metal stress. We predict that some mitochondrial proteins might have small molecular weight, thus, the bands are invisible on the gel. Based on molecular weight, the protein band with decreased intensity is most probably the heat shock protein-60 (HSP-60) or known as chaperonin 60. This is a mitochondrial chaperonin accountable in transportation and protein refolding from the cytoplasm into the mitochondrial matrix (Deocaris *et al*., 2006). Earlier works on human mitochondria also revealed the expression of HSP 60, as well as several other proteins when exposed to copper ions (Choi *et al*., 2012).

Since mitochondria are very synonym with reactive oxygen species, we determined the total phenolic and flavonoid content in our samples. The phenolic and flavonoid are secondary antioxidant compounds normally produced by plants to defend themselves under stress condition by chelating the metals that interfere normal plant processes (Michalak, 2006), as well as inhibit ROS generation (Deng *et al.*, 1997; Grael *et al.*, 2010). Although there was a significant increase in total phenolic content with increasing copper concentrations, the percentage of increment seems low, probably due to low level of oxidative stress in cells.

Conclusion

The findings described in this paper show that *O. stamineus* calluses react dynamically toward copper stress. The callus absorbed a significant amount of copper from the medium, hence, categorized as hyperaccumulator. The active response of callus to stress has been shown by the increasing amount of total phenolic compounds and higher activities of cytochrome c oxidase. In addition, changes in the total mitochondrial protein content and different intensity of several bands with respect to copper amount may reflect the reaction of these cells under stress. It also proved that mitochondria are important organelles in plants that function synergistically with other components in the cells for normal plant growth and development. Taken together, since *O. stamineus* species has high potential in surviving copper stress, we suggest that this species might potentially be used as phytoremediation agent in the future.

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