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ORIGINAL ARTICLE

Effect of copper–induced oxidative stress on sclerotial differentiation, endogenous antioxidant contents, and antioxidative enzyme activities of *Penicillium thomii* PT95

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Abstract *Penicillium thomii* PT95 strain is able to form abundant orange, sand-shaped sclerotia in which carotenoids accumulate. We have studied the effects of copper (Cu)-induced oxidative stress on sclerotial differentiation, biosynthesis of some endogenous antioxidants, and activities of a number of the antioxidative enzymes of strain PT95. The association between sclerotial biomass, carotenoid, ascorbate and glutathione contents, and the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductases (GR) were also analyzed in this fungal strain. We found that the oxidative stress induced by Cu was directly dependent on the concentrations of $CuSO_4$ in the media, with higher $CuSO_4$ concentrations resulting in higher oxidative stress. Cu-induced oxidative stress in strain PT95 was characterized by the initiation of lipid peroxidation. Under Cu-induced oxidative stress growth conditions, the initiation of exudates and sclerotia in strain PT95, as well as sclerotial maturation, were advanced by 1−2 days. Cu-induced oxidative stress favored sclerotial differentiation and the biosynthesis of endogenous antioxidants, i.e., carotenoids, ascorbate, and glutathione. Comparison of SOD, CAT, and GR activities at 0 and 100 μg/ml Cu revealed a 1.1-, 1.8-, and 1.2-fold increase, respectively, at the higher Cu concentration; comparison of their activities at 100 and 300 μg/ml Cu revealed a 1.4-, 3.1-, and 2.2-fold decrease, respectively, at the higher Cu concentration. APX activity decreased linearly with increasing $CuSO_4$ concentration. Our results suggest that the ability of the *P. thomii* PT95 strain to cope with metal stress is related to its ability to trigger an efficient defense against oxidative stress. These findings may contribute to a better understanding of the response mechanisms of sclerotia production in *Penicillium* strains to metal stress and to better insights into metal–fungi interactions in natural environments.

Keywords Copper · Oxidative stress · *Penicillium thomii* · Endogenous antioxidants · Antioxidative enzymes

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Introduction

The toxicity of heavy metals to microorganisms has attracted considerable research attention in recent years (Mukherjee et al. 2010). Heavy metals can be introduced into ecosystems through industrial effluents and wastes, agricultural fungicides, domestic garbage dumps, and mining activities (Merian 1991). Copper (Cu), a heavy metal, is an essential micronutrient that plays key roles in different physiological processes and metabolic pathways and is a structural and catalytic component of many proteins (Elleuch et al. 2013). As an essential trace element it acts as a cofactor of multiple enzymes, including superoxide dismutases (SOD; for protection against free radicals), cytochrome *c* oxidase (mitochondrial electron transport chain), tyrosinase (pigmentation), peptidylglycine α-amidating monooxygenase (neuropeptide and peptide hormone processing), and lysyl oxidase (collagen maturation) (Uauy et al. 1998; Peña et al. 1999).

Fungi can accumulate, store, and tolerate high levels of heavy metals (Brady et al. 1994; Joho et al. 1995). To this end, fungi, similar to all living organisms, have evolved a set of mechanisms that control and respond to the uptake and accumulation of heavy metals. Biochemical responses include precipitation of heavy metals in cell walls or associated compounds (Rizzo et al. 1992; Suresh and Subramanyam 1996), production of organic acids, polysaccharides, melanins, and/

or proteins to bind metal ions (Gadd 1993; Martino et al. 2002; Baldrian 2003), chelation in the cytosol and transport into vacuoles (Hayashi and Mutoh 1994a, b), chemical transformation of metals (Gadd 1993), and/or transport of metal cations (Blaudez et al. 2000a). Another response process involves the synthesis of compounds containing thiol functional groups (Gadd 1993), which are known to have a high affinity for metal ions (Romero–Isart and Vašák 2002).

The toxicity of metals can be due to the generation of reactive oxygen species (ROS) that may cause wide-ranging damage to proteins, nucleic acids, and lipids, eventually leading to cell death (Bai et al. 2003). The toxic effects of ROS may involve inhibition of growth (Guillén and Machuca 2008; Krumova et al. 2009), substitution of essential ions and blocking of functional groups on proteins (Borkow and Gabbay 2005; Dupont et al. 2011), inactivation of enzymes (Gokhale and Cowan 2005; Wang et al. 2010), disturbances of metabolic pathways (Chillappagari et al. 2010), alterations of membrane integrity and production of ROS by membrane-bound Cu (Krumova et al. 2009, 2012; Sharma and Dietz 2009; Dávila Costa et al. 2011). Consequently, the tolerance of fungi to different metals has been associated with its ability to remove ROS (Fujs et al. 2005).

Fungi display several antioxidant enzymes against ROS, including SOD, catalases (CAT), ascorbate peroxidase (APX), and glutathione reductases (GR), all capable of removing oxygen radicals and their products and/or repairing oxidative damage (Jamieson 1998; Bai et al. 2003). In addition, molecules such as glutathione and ascorbate not only play an important role in cellular protection during oxidative stress, but they may form a complex with metals in cells (Pócsi et al. 2004). Carotenoids are antioxidants and reduce oxidative stress by acting as scavengers of ROS (mainly singlet oxygen) (Stratton and Liebler 1997). β-Carotene and other carotenoids have been associated with fungal photomorphogenesis and development (Mohr and Schopfer 1995; Georgiou and Petropoulou 2001a, b, c; Georgiou et al. 2001a, b; Zervoudakis et al. 2003; Han et al. 2005). In most organisms, ascorbic acid has important functions in biochemical reactions, such as in cell growth and differentiation. It has been shown to play an important antioxidant role in sclerotial differentiation of phytopathogenic fungi (Georgiou and Petropoulou 2001a, b, c; Georgiou et al. 2003; Li et al. 2006). Glutathione is considered to be a very important signaling molecule which acts as a link between environmental stress and adaptive responses; it is regenerated from glutathione-*S*-transferases by GR activity (Navari–Izzo et al. 1997).

Penicillium thomii PT95 strain studied here was isolated from a soil sample and is able to form abundant orange, sand-shaped sclerotia in which carotenoids accumulate (Han 1998). In earlier studies, we examined the effect of iron-induced oxidative stress on sclerotial differentiation in strain PT95 (Han et al. 2005; Li et al. 2006). The aim of the study reported here was to investigate the effects of Cu-induced oxidative stress on sclerotial differentiation, sclerotia biomass, the level of lipid peroxidation [based on malondialdehyde (MDA level)], activities of enzymatic antioxidants (SOD, CAT, APX, GR), and the contents of endogenous antioxidants (such as carotenoids, glutathione, ascorbate). The association between sclerotial biomass, endogenous antioxidant contents, and antioxidative enzyme activities of this fungus was also analyzed. The results should provide data which will help explain the role and correlation between enzymatic and non-enzymatic antioxidants in the prevention or limitation of metal stress injury.

Materials and methods

Preparation of inoculum

Strain PT95 was isolated from soil samples collected close to Fenyang in Shanxi Province. It was identified as *Penicillium thomii* (GenBank accession nr. KC966728) based on rDNA–ITS sequence analysis. Additional details on its isolation and a complete bibliography are presented in Han et al. (1998). Strain PT95 was cultured on Czapek's agar plates in an incubator maintained in the dark condition and at 25 °C. The sclerotia to be used as inoculum were obtained from 14-day-old Czapek's agar plate cultures. To purify sclerotia, we first centrifuged the plate cultures and then rinsed the sclerotia aseptically five times with sterilized water to remove the spores.

Oxidative stress growth conditions

The different oxidative stress growth conditions to be assessed were designed using potato dextrose agar (PDA) medium supplemented with different concentrations of $CuSO_4$ (100, 200, or 300 μg/ml, respectively). PDA medium without $CuSO_4$ supplementation served as the control. Using three point inoculations (Pitt 2000), three grains of sclerotia of the PT95 strain were inoculated onto 25 ml of medium in a 9-cm Petri dish. The plates were incubated in the dark at 25 °C for 20 days.

Sclerotial biomass, carotenoid extraction, and determination

The sclerotia which formed on the agar surface in the petri plates were separated and washed thoroughly with distilled water and dried at 50 °C to a constant weight to determine sclerotial biomass. The sclerotia were then observed with an anatomical lens (model SMZ–168; Motic China Group Co., China).

The extraction and determination of pigments were performed as described by Li et al. (2006) with minor modifications. Briefly, 1 g of dried sclerotia was manually ground with a glass homogenizer and extracted three times with 10-ml

aliquots of acetone. The acetone extracts were combined in a separatory funnel, and 10 ml of chloroform and a few milliliters of a saturated NaCl solution were added to help break the emulsions. The chloroform extract was collected after removal of the acetone layer, which was then re-extracted. Absorbance of the chloroform extract was measured at 475 nm. The content of carotenoids was calculated by using a 1 % extinction coefficientof2,500 by the formula:

$$\text{Content of carotenoids}(\mu g/\text{g dry sclerotia}) = (\text{ml of chloroform} \times A_{475})/\text{sclerotia dry weight} \times 2500)$$

Ascorbate assay

The ascorbate assay was performed according to Georgiou and Petropoulou (2001a). Ascorbate concentration was expressed in units of μg ascorbate/g sclerotia dry weight (DW).

Glutathione assay

The sclerotia (0.5 g DW) were ground in 2 ml of ice-cold 5 % (w/v) sulfosalicylic acid. The extract was then centrifuged at 4 °C for 20 min at 12,000*g* in a cooled centrifuge and the supernatant used for the assay of glutathione. Glutathione and its oxidized product glutathione disulfide (GSSG) were measured together spectrophotometrically according to Griffith (1980) with some modifications. In brief, total glutathione was measured in a 5-ml reaction mixture containing 4.29 ml phosphate buffer (47.5 mM $Na_2HPO_4 \cdot 12H_2O$, 2.5 mM $KH_2PO_4 \cdot 5H_2O$, 2.5 mM EDTA–Na_2), 0.5 ml of 1.25 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 60 μl 15 mM NADPH, and 100 μl acid extract. The acid extract was composed immediately prior to starting the reaction by the addition of 50 μl of 5 U of GR activity (bakers' yeast, type III; Sigma Chemical Co., St. Louis, MO). Change in the absorbance of the reaction mixture was measured at 412 nm.

The reduced form of glutathione (GSH) was measured by similar method, but with ddH_2O instead of the coenzyme II reduction system. The amounts of oxidized glutathione (GSSG) were calculated by subtracting the GSH content from total glutathione content (GSH+GSSG). All values are reported as glutathione equivalents.

Evaluation of lipid peroxidation

Lipid peroxidation in the sclerotia of strain PT95 was evaluated by determining the levels of the peroxidation products. Thiobarbituric acid reactive substances (TBARS), such as aldehydes, malondialdehyde (MDA), and endoperoxides were determined according to the methods of Hodges et al. (1999). MDA, which isroutinely used as an indicator of lipid peroxidation, was extracted with 5 % (w/v) trichloric acid. The reaction with TBA was conducted at 95 °C for 30 min. After the samples were chilled, absorbance was measured at 532 and 432 nm and the results expressed as μmol MDA/g DW sclerotia.

Measurement of antioxidant enzyme activity

Sclerotia (1 g DW) were homogenized in 50 mM ice-cold phosphate buffer, pH 7.8, containing 0.2 mM EDTA−Na_2 and 4 % insoluble polyvinylpyrrolidone (1 ml buffer/100 mg DW). The extract was then centrifuged at 4 °C for 20 min at 12,000*g* in a cooled centrifuge. The supernatant was used for the assays of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), and GR (EC 1.6.4.2).

SOD activity was measured according to Giannopolitis and Ries (1977). The reaction mixture contained 1.3 mM riboflavin, 13 mM methionine, 63 mM nitroblue tetrozolium (NBT), 0.1 mM EDTA in 50 mM phosphate buffer (pH 7.8), and 50 μl of the enzyme extract in a final volume of 10 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were immersed in a bath at 25 °C and illuminated with a fluorescent lamp (4,000 lx). Identical tubes which were not illuminated served as blanks. After illumination for 20 min, absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity which inhibited the photoreduction of NBT to blue formazan by 50 %, and SOD activity of the extracts was expressed as SOD units/g sclerotia.

CAT activity was measured according to Li et al. (2005) using absorbance of a complex compound (peroxomolybdates) at 405 nm due to H_2O_2 decomposition based on results in a previous experiment. One unit of CAT activity was defined as 1 μmol decomposed H_2O_2 per one milligram sclerotia per second.

Total GR activity was assayed by the DTNB method, with minor modifications (Sun et al. 2007). The reaction mixture (300 μl) consisted of 100 mM phosphate buffer (pH7.8), 0.1 mM NADPH, 1.0 mM GSSG, and 20 μl enzyme extract. The reaction was started by the addition of GSSG, and glutathione reduction rate was monitored at 420 nm for 5 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol GSH per minute .

The assay for APX was based on monitoring the rate of oxidation of ascorbate according to the method of Nakano et al. (1981). Absorbance readings were taken at 290 nm using the spectrophotometer blanked against an aliquot of buffer (50 mM phosphate buffer, pH7.0, 0.3 mM ascorbate). A 3.85-ml aliquot of this buffer was placed in a glass cuvette, 50 μl extract was added, followed by 1 ml of 0.015 % chilled H_2O_2. The cuvette was inverted three

times as quickly as possible and placed in the spectrophotometer. The reaction was monitored observing the decrease in extinction at 290 nm for 1 min.

Statistics

All experiments were replicated in three plates, and the data are presented as the arithmetic mean±standard error (SE). Duncan's multiple range test (Ray 1985) was used on the isolation means to test for significant differences at the 5 % level of confidence.

Results

Effect of Cu-induced oxidative stress on sclerotial differentiation, sclerotial biomass, and carotenoid contents in sclerotia of strain PT95

Observation of the macroscopic colony characters of strain PT95 on each plate culture suggested that the colonies of strain PT95 growing under different oxidative stress conditions differed from each other (Fig. 1). Table 1 shows that with increased oxidative stress increased, the time of exudate initiation, sclerotial initiation, and sclerotial maturation were advanced by 1 −2 days.

There were apparent differences in sclerotial biomass and carotenoid contents in sclerotia of strain PT95 under different oxidative growth conditions (Table 2), with higher sclerotial biomass and carotenoid contents in sclerotia positively correlated with the higher $CuSO_4$ concentration. When the fungus was grown under the high oxidative stress condition induced by 300 μg/ml $CuSO_4$, its sclerotial biomass (i.e., dry sclerotia weight) increased by 1.56-fold with respect to the control. Carotenoid contents in sclerotia under the high oxidative stress condition increased by 1.19-fold with respect to the control. Statistical analysis revealed that sclerotial biomass values were positively correlated ($p<0.05$) with the carotenoid content values in sclerotia ($R=0.9581$).

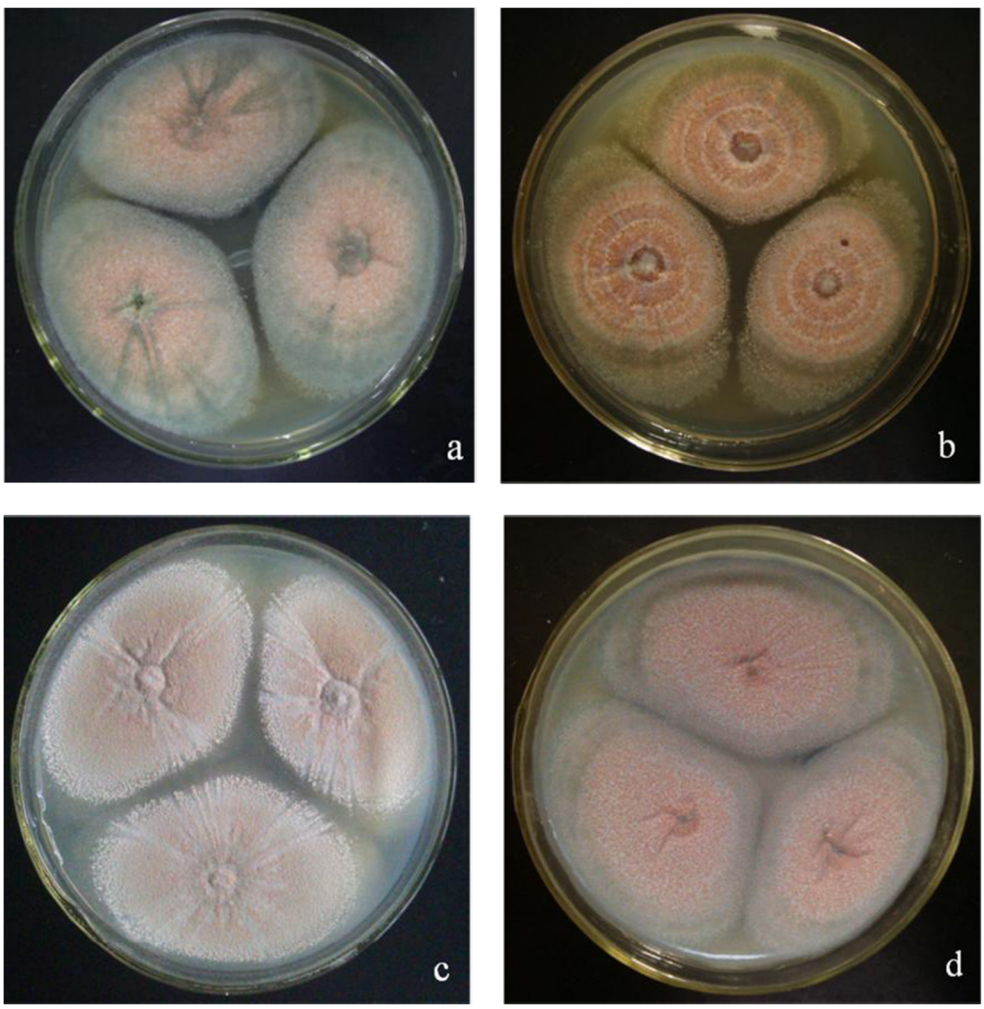

Fig. 1 Colonies of *Penicillium thomii* PT95 strain growing for 14 days on (**a**) potato dextrose agar (PDA) without added $CuSO_4$, (**b**) PDA+100 μg/ml $CuSO_4$, (**c**) PDA+200 μg/ml $CuSO_4$, (**d**) PDA+ 300 μg/ml $CuSO_4$, at 25 °C. Petri dish size: 9 cm diameter

Table 1 Effect of copper-induced oxidative stress on sclerotial differentiation of *Penicillium thomii* PT95 strain

Oxidative growth condition	Time of exudate initiation (days)	Time of sclerotial initiation (days)	Time of sclerotial maturation (days)
Control (no $CuSO_4$)	5	7	13
$CuSO_4$ (100 μg/ml)	4	5	12
$CuSO_4$ (200 μg/ml)	4	5	12
$CuSO_4$ (300 μg/ml)	4	5	12

One conclusion that can be drawn from these results is that copper-induced oxidative stress favored sclerotial differentiation and endogenous carotenogenesis of the PT95 strain.

Effect of $CuSO_4$ concentrations in media on lipid peroxidation in sclerotia of strain PT95

Lipid peroxidation is an important indicator of oxidative stress and is accompanied by the formation of aldehydic lipid hydroperoxide decomposition products, such as MDA (Esterbauer et al. 1991). The formation of MDA is considered to be evidence of the occurrence of free radical-mediated reactions; therefore, the concentration of MDA is used as a measure of the degree of this stress (Yazdanpanah et al. 1997). We therefore tested the effect of $CuSO_4$ concentrations in media on lipid peroxidation in sclerotia of strain PT95 and found that the MDA content in sclerotia was significantly correlated ($p<0.05$) with the $CuSO_4$ concentrations in the media ($R=0.9771$) (Fig. 2). This result indicates that the oxidative stress induced by Cu was clearly dependent on the concentration of $CuSO_4$ in the medium.

Effect of Cu-induced oxidative stress on ascorbate content in sclerotia of strain PT95

We determined the contents of reduced and oxidized ascorbate in the sclerotia of strain PT95 under different oxidative stress growth conditions (Table 3). The contents of non-enzymic antioxidants, i.e., ascorbate content, in sclerotia showed a trend to increase with increased $CuSO_4$ concentration in media. Under the oxidative stress growth condition induced by 300 μg/ml $CuSO_4$, the levels of both oxidized and reduced ascorbate reached their maximum values (10.3 and 8.87 μg/g, respectively), which represents a 3.1- and 4.5-fold increase, respectively, relative to the control. The reduced ascorbate/oxidized ascorbate ratio increased gradually.

One conclusion to be drawn from these results is that Cu-induced oxidative stress favored the accumulation of endogenous ascorbate in sclerotia of strain PT95.

Table 2 Effect of copper-induced oxidative stress on sclerotial biomass and carotenoid contents in sclerotia of *P. thomii* PT95 strain

Oxidative growth condition	Sclerotial biomass (mg/plate)	Carotenoid content (μg/g sclerotia DW)
Control (no $CuSO_4$)	315.7±11.6 a	10.7±0.9 a
$CuSO_4$ (100 μg/ml)	352.3±12.7 b	12.4±0.9 b
$CuSO_4$ (200 μg/ml)	422.3±19.8 c	12.7±0.8 b
$CuSO_4$ (300 μg/ml)	493.7±10.4 d	11.7±1.3 a

DW, Dry weight

Values are presented as the mean ± standard error (SE). Values in the same column followed by different lowercase letters are significantly different at $p<0.05$ according to Duncan's multiplerange test

Effect of Cu-induced oxidative stress on glutathione content in sclerotia of strain PT95

Similarly, the contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) in sclerotia were strongly dependent on the oxidative stress growth condition (Table 3). Both GSH and GSSG contents in sclerotia showed a trend to increase with an increase of $CuSO_4$ concentrations in the medium. The GSH/GSSG ratio was maintained at a high level in all

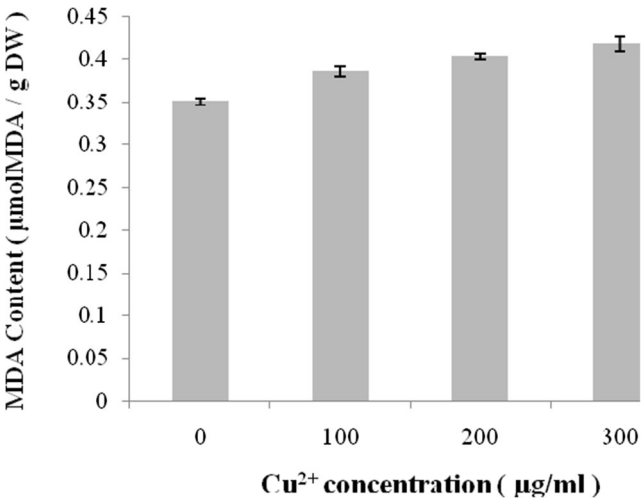

Fig. 2 Effect of $CuSO_4$ concentrations in media on lipid peroxidation of sclerotia of *Penicillium thomii* PT95 strain based on malondialdehyde (*MDA*) content. Results represent mean values of 3 independent experiments± standard deviation (SD)

Table 3 Oxidized glutathione (GSSG) and reduced glutathione (GSH) content, GSH/GSSG ratio, reduced ascorbate and oxidized ascorbate content, and reduced ascorbate/oxidized ascorbate ratio in sclerotia of *P. thomii* PT95 strain

Oxidative growth condition	GSH content (μmol/g DW)	GSSG content (μmol/g DW)	GSH/ GSSG	Reduced ascorbate content (μg/g DW)	Oxidized ascorbate content (μg/g DW)	Reduced ascorbate/ oxidized ascorbate
Control (no $CuSO_4$)	1.51±0.02 a	1.10±0.06 a	1.36	1.97±0.31 a	3.3±0.3 a	0.59
$CuSO_4$ (100 μg/ml)	1.75±0.01 b	1.21±0.03 b	1.45	3.53±0.5 b	4.87±0.25 b	0.72
$CuSO_4$ (200 μg/ml)	1.78±0.04 bc	1.22±0.01 b	1.46	7.47±0.5 c	9.03±0.45 c	0.83
$CuSO_4$ (300 μg/ml)	1.81±0.03 c	1.24±0.02 b	1.46	8.87±0.42 d	10.3±0.26 d	0.87

Values are presented as the mean ± standard error (SE). Values in the same column followed by different lowercase letters are significantly different at $p<0.05$ according to Duncan's multiplerange test

treatments and showed a 1.08-fold increase relative to the control, possible due to nonprotein thiol (NP−SH) synthesis. Under the high oxidative stress condition (200–300 μg/ml $CuSO_4$), the GSH/GSSG ratio gradually increased, reaching a maximum at 300 μg/ml (1.46-fold increase).

Effect of Cu-induced oxidative stress on SOD activity in sclerotia of strain PT95

Data on SOD activity of sclerotia of strain PT95 is shown in Fig. 3. Based on our results, increasing the $CuSO_4$ concentration in the medium from 0 to 100 μg/ml correlated with a significant increase in SOD activity, while increasing the $CuSO_4$ concentration in the medium from 100 to 300 μg/ml did not cause a significant increase SOD activity. This observation suggests that SOD activity in sclerotia subjected to the oxidative stress condition induced by $CuSO_4$ at a concentration of 100 μg/ml had reached its maximum value of 135.93 U/g DW. The values of SOD activities under different oxidative stress conditions showed a moderately negative correlation with the contents of reduced and oxidized ascorbate, respectively (R_{red}= −0.45, R_{oxi}= −0.4783), with the carotenoid contents in sclerotia (R= −0.5411), and with the contents of GSH and GSSG, respectively (R_{GSH}=−0.172, R_{GSSG}= −0.661). These results suggest that under higher oxidative stress, strain PT95 may fail to significantly enhance SOD activity in sclerotia to scavenge ROS generated by the oxidative stress. However, the strain may further differentiate to form more sclerotia for long-term survival or produce more endogenous antioxidants to counter ROS formation.

Effect of Cu-induced oxidative stress on CAT activity in sclerotia of strain PT95

Information on the CAT activity of sclerotia is presented in Fig. 4. CAT activity significantly increased when the $CuSO_4$ concentration in the medium increased from 0 to 100 μg/ml, but it significantly decreased with an increase of $CuSO_4$ concentration in the medium from 100 to 300 μg/ml. Under

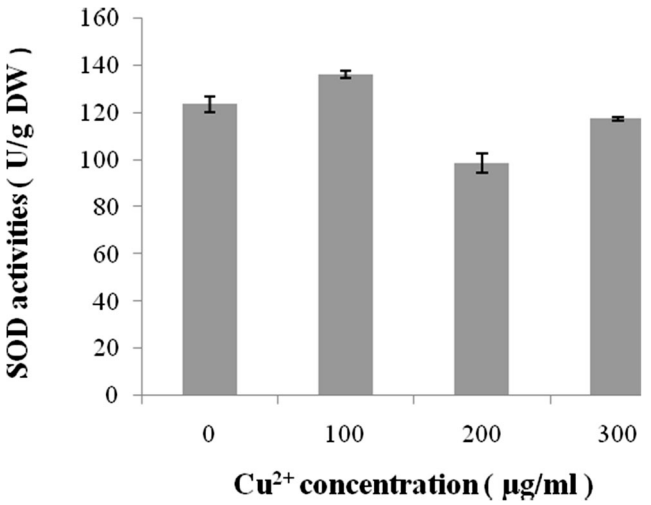

Fig. 3 Effect of Cu-induced oxidative stress on sclerotia's superoxide dismutase (*SOD*) activities of *P. thomii* PT95 strain. Results represent mean values of 3 independent experiments± SD

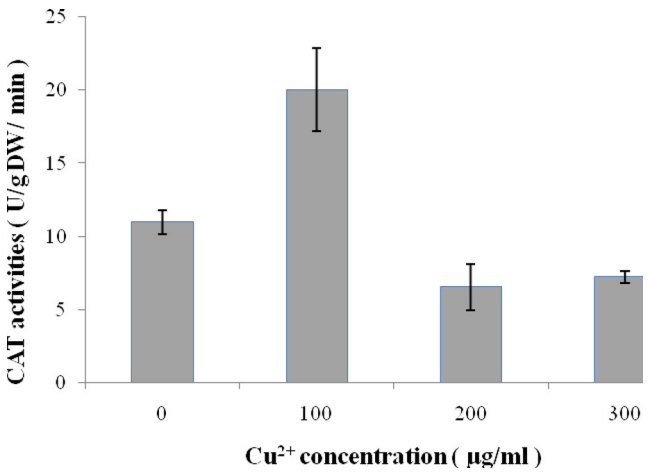

Fig. 4 Effect of Cu-induced oxidative stress on catalase (*CAT*) activity of the sclerotia of *P. thomii* PT95 strain. Results represent mean values of 3 independent experiments± SD

the oxidative stress condition induced by 100 μg/ml $CuSO_4$, CAT activity of sclerotia reached its maximum value of 20.02 U/g DW/min. The values of CAT activity had a moderately positive correlation with those of SOD activity (R=0.8615). In contrast, they had a moderately negative correlation with the contents of reduced and oxidized ascorbate (R_{red}= −0.6290, R_{oxi}= −0.6435), respectively, with carotenoid contents in sclerotia (R= −0.4990), and with GSH content (R_{GSH}=−0.392), and they had a significantly negative correlation (p<0.05) with GSSG content (R_{GSSG}=−0.950). These results suggest that under the relatively lower oxidative stress, strain PT95 may significantly enhance CAT activity in sclerotia to scavenge ROS generated by oxidative stress.

Effect of Cu-induced oxidative stress on GR activity in sclerotia of strain PT95

As shown in Fig. 5, GR activity increased by almost 1.19-fold with the addition of 100 μg/ml $CuSO_4$ to the medium relative to the control, indicating higher enzyme activity either in response to the superoxide anions formed due to Cu toxicity or for the increased synthesis of glutathione to trap intracellular Cu. A gradual decrease in enzyme activity was observed with an increase in $CuSO_4$ concentration, with GR activity decreasing by almost 1.88-fold at 300 μg/ml $CuSO_4$ relative to the control. This decrease in enzyme activity is indicative of the severity of the oxidative stress imposed by the higher $CuSO_4$ concentration on the test strain. The values of GR activity showed a moderate positive correlation with those of the SOD and CAT activities (R_{SOD}=0.442, R_{CAT}=0.419, respectively). In contrast, the values of GR activity had a moderately negative correlation with the contents of reduced and oxidized ascorbate (R_{red}=−0.924, R_{oxi}=−0.920), with carotenoid contents in sclerotia (R= −0.701), and with GSH and GSSG contents (R_{GSH}=−0.845, R_{GSSG}=−0.872), respectively.

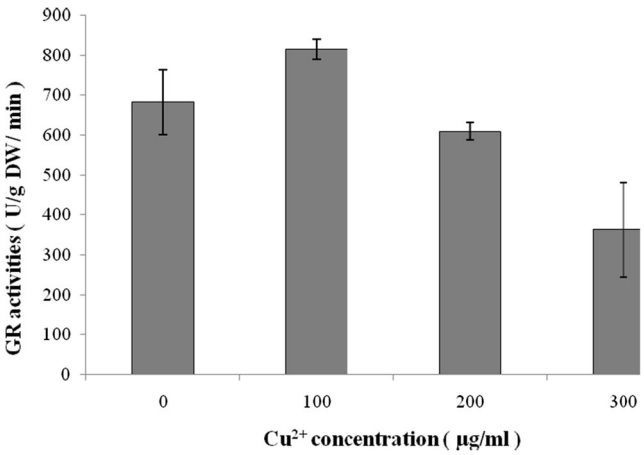

Fig. 5 Effect of Cu-induced oxidative stress on sclerotia's glutathione reductase (*GR*) activities of *P. thomii* PT95 strain. Results represent mean values of 3 independent experiments± SD

Effect of Cu-induced oxidative stress on APX activity in sclerotia of strain PT95

Peroxidases [APX and glutathione peroxidase (GPX)] are distributed throughout the cell and catalyze the reduction of hydrogen peroxide (H_2O_2) to H_2O. APX uses ascorbate as electron donor in the first step of the ascorbate–glutathione cycle and is considered to be the most important plant peroxidase involved in H_2O_2 detoxification (Noctor and Foyer 1998). As shown in Fig. 6, under Cu-induced oxidative stress, APX activity decreased linearly with increasing $CuSO_4$ concentration (R= −0.988) (p<0.05). The highest concentration of Cu (300 μg/ml) proved to be extremely toxic, resulting in a decline in APX activity. Compared with the control, APX activity decreased by 3.3-fold. The values of APX activity had a moderately positive correlation with the values of SOD, CAT, and GR activities (R_{SOD}=0.475, R_{CAT}=0.786, R_{GR}= 0.688). The values of GR activity had a moderately negative correlation with the contents of reduced and oxidized ascorbate (R_{red}=−0.924, R_{oxi}=−0.920), significantly (p<0.01) negative correlation with carotenoid contents in sclerotia (R = −0.994), and a moderately negative correlation with GSH and GSSG contents (R_{GSH}=−0.881, R_{GSSG}=−0.350).

Discussion

Georgiou (1997) advanced a theory which proposed that sclerotial differentiation in fungi was triggered by oxidative stress. Our previous experiments (Han et al. 2005; Li et al. 2006) showed that the sclerotial biomass produced by strain PT95 grown under a high oxidative stress condition was 1.23-fold greater than that produced when grown under a low oxidative stress condition. The data of this study provide additional evidence in support of Georgiou's theory and are also in accordance with the general theory of microbial

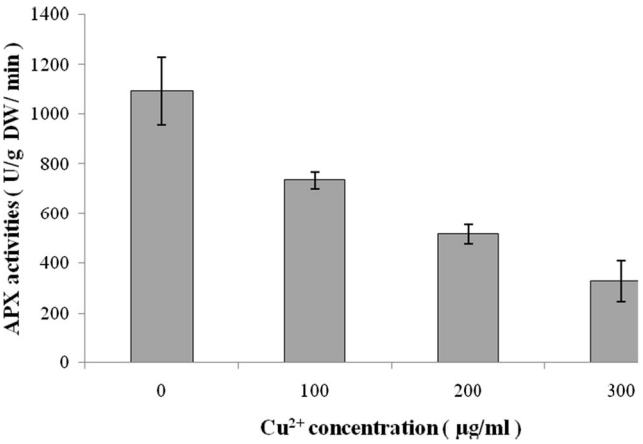

Fig. 6 Effect of Cu-induced oxidative stress on sclerotia's ascorbate peroxidase (*APX*) activity of *P. thomii* PT95 strain. Results represent mean values of 3 independent experiments± SD

differentiation, which also postulates that the latter is induced by oxidative stress (Burton and Ingold 1984).

Metal toxicity is often driven by the generation of ROS either directly via the catalytic production of superoxide (O_2^-) by the Haber–Weiss and Fenton reactions or indirectly by other mechanisms (Yamamoto et al. 2002; Boscolo et al. 2003) and participates in the initiation and propagation of lipid peroxidation. These last findings provide indirect evidence that excessive ROS were produced after the Cu treatment, resulting in membrane lipid deterioration measured as MDA. Our results indicate that excess Cu increases oxidative stress, as was evident from the increased lipid peroxidation, which is in accordance with previous findings showing that MDA accumulated greatly after Cu exposure and that the cell membrane was the primary site affected by Cu toxicity (Thounaojam et al. 2012), possibly due to the overproduction of ROS under Cu stress which is highly destructive to the cell membrane. This toxic effect resulting from the cellular oxidative stress may be allayed by several antioxidant systems. The increased activity of antioxidative enzymes in the sclerotia indicates the formation of ROS. In other words, the sclerotia analyzed in this study contained sufficiently high Cu concentrations to activate ROS production and subsequently oxidative stress.

The protection provided by the antioxidant system against these ROS is complex and highly organized. In this system, the SOD constitutes the primary line of defense as it dismutates superoxide radicals to H_2O_2 (Fatima and Ahmad 2005). H_2O_2 degradation to water and oxygen is carried out by the CAT localized in the peroxisomes and, as a constituent of ascorbate–glutathione cycle, by the APX, GR, ascorbate and glutathione (Gratão et al. 2005).

However, we noted that the activities of the various antioxidant enzymes were affected differently by high Cu concentrations (=high oxidative stress). SOD is the first enzyme of the detoxifying process. A disproportionate amount of the superoxide anion (O_2^-) was catalyzed by SOD, which catalyzes the formation of H_2O_2 (Asada 2006). Under the low oxidative stress condition (100 μg/ml), SOD activity was significantly increased compared to control; in contrast, under the higher oxidation stress condition (100–300 μg/ml Cu), SOD activity decreased, possibly due to its sensitivity to high H_2O_2 content in the cell. This decreased SOD activity may be related to lower levels of $O_2\cdot$ because of the Haber–Weiss reaction. Roughly paralleling the changes in SOD, CAT, and GR activities increased with increasing Cu concentrations from 0 to 100 μg/ml, but decreased with an increase in Cu concentrations from to 300 μg/ml. According to Willekens et al. (1997), it is likely that an excess production of ROS caused by heavy metals can inactivate CAT, probably by inactivating the enzyme-bound heme group. CAT is only present in peroxisomes, but it is indispensable for ROS detoxification during stress when high levels of ROS are produced. APX has a higher affinity for H_2O_2 than CAT and can therefore scavenge small amounts of H_2O_2 in more specific locations (Asada 1992; Radic et al. 2010). In our experiments, Cu treatments induced APX activities in the sclerotia of strain PT95 strain, suggesting that APX activity decreased linearly with increasing $CuSO_4$ concentration. The highest concentration of Cu (300 μg/ml) proved to be extremely toxic, resulting in a decline of APX activity. The hyperactivity of peroxidase under Cu stress indicates its role in the constant detoxification of H_2O_2. Glutathione is considered to be a very important signal molecule which acts as a link between environmental stress and adaptive responses, and it is regenerated from GST by GR activity (Navari–Izzo et al. 1997).

Carotenoids can be antioxidant since they are known to inhibit oxidative stress by acting as quenchers of singlet oxygen and scavengers of hydroxyl, alkoxyl, and alkoperoxyl radicals (Burton and Ingold 1984; Simic 1992; Stratton and Liebler 1997; Georgiou and Petropoulou 2001a). Ascorbate can directly scavenge ROS (including hydroxyl and superoxide radicals and H_2O_2) either nonenzymatically or enzymatically (McKersie and Leshem 1994). In the latter case, it is used as a substrate for the H_2O_2-splitting enzyme APX (Nakano and Asada 1981). It can also indirectly act as an antioxidant by regenerating the membrane-bound α-tocopherol which is involved in the scavenging of peroxyl radicals and singlet oxygen (Schraudner et al. 1997). In our experiment, carotenoids, ascorbate, and glutathione were accumulated in the sclerotia of strain PT95. However, the effect of Cu-induced oxidative stress on carotenoid content in the sclerotia was different from that on ascorbate content and glutathione content. We found that the carotenoid content in sclerotia had a weak, positive correlation with the $CuSO_4$ concentration in the medium (R=0.480) and that the total ascorbate content in sclerotia had a significantly positive correlation (p<0.05) with the $CuSO_4$ concentrations in the medium (R=0.915). The GSH content in sclerotia had a significantly positive correlation (p<0.05) with the $CuSO_4$ concentrations in the medium (R_{GSH}= 0.924). The highest carotenoid content was obtained from PDA plates supplemented with 200 μg/ml $CuSO_4$. The total ascorbate content was the sum of the reduced and oxidized ascorbate content, and the highest total ascorbate content was obtained from PDA plates supplemented with 300 μg/ml $CuSO_4$ (19.17 μg/g dry sclerotia). The highest GSH and GSSG contents were obtained from PDA plates supplemented with 300 μg/ml $CuSO_4$ (Table 3). These results indicate that the oxidative stress induced by a lower amount of $CuSO_4$ (about 200 μg/ml) favored endogenous carotenogenesis by strain PT95, whereas the oxidative stress induced by a higher amount of $CuSO_4$ (about 300 μg/ml) favored the accumulation of ascorbate and glutathione in sclerotia.

Conclusions

In light of these results, we suggest that higher Cu levels cause oxidative stress in *P. thomii* PT95 cells and may cause membrane damage through the production of ROS. Under the Cu-induced oxidative stress growth conditions in this study, the time of exudate initiation, sclerotial initiation, and sclerotial maturation of strain PT95 advanced by 1−2 days. Therefore, the data shown here can be used to illustrate how *P. thomii* PT95 strain responds to its stressful environment. Cu-induced oxidative stress favored sclerotial differentiation in strain PT95 as well as the biosynthesis of endogenous antioxidants, i.e., carotenoids, ascorbate, and glutathione. Among the antioxidative enzymes, SOD, CAT, APX, and GR appear to play key roles in this fungus' antioxidative defense mechanisms under conditions of Cu toxicity. Our results suggest that the ability of *P. thomii* PT95 strain to cope with metal-induced stress is related to its ability to incite an efficient defense against oxidative stress. These findings may contribute to a better understanding of the response mechanism of producing sclerotia by this *Penicillium* strain following exposure to metal stress and further insights into metal–fungi interactions in natural environments.

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