Environmental & Analytical
Toxicology

Zhao and Han, J Environ Anal Toxicol 2016, 6:1
http://dx.doi.org/10.4172/2161-0525.1000338

Research Article Open Access

Effect of H_2O_2-Induced Oxidative Stress on Sclerotial Differentiation and Asa-GSH Cycle of *Penicillium thomii* Q1 Strain

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Abstract

Penicillium thomii Q1 strain was able to form abundant orange, sand-shaped sclerotia in which carotenoids were accumulated. The aim of this work was to determine the effects of H_2O_2-induced oxidative stress on the sclerotial differentiation and ascorbate-glutathione (AsA-GSH) cycle of Q1 strain. Results showed that the oxidative stress induced by H_2O_2 was clearly dependent on the H_2O_2 concentrations in media. The higher the concentration of H_2O_2 was, the higher the sclerotial biomass and carotenoids content in sclerotia ($R_{biomass}$=0.972, $R_{content}$=0.941) (P<0.05). H_2O_2-induced oxidative stress favored the sclerotial differentiation. Results indicated that H_2O_2-induced oxidative stress can improve the contents of reduced form of ascorbate (ASC) and dehydroascorbate (DHA) and the ratio of ASC/DHA in sclerotia of *Penicillium thomii* Q1 strain (R_{ASC}=0.727, R_{DHA}=0.640, $R_{ASC/DHA}$=0.929). The contents of DHA and ASC were increased by 1.9-fold and 2.4-fold with respect to control. At the same oxidative stress condition, DHA content was higher than ASC content. Oxidative stress may increase the contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) in sclerotia of *Penicillium thomii* Q1 strain (R_{GSH}=0.888, R_{GSSG}=0.997). The value of the ratio of GSH/GSSG had a negative correlation with the oxidative stress (R=-0.933). At the same oxidative stress condition, GSH content was higher than GSSG content. The values of Ascorbate peroxidase (APX) and Glutathione reductase (GR) activities had negative correlation with the oxidative stress (R_{APX}=-0.555) (R_{GR}=-0.420). The values of Monodehydroascorbate reductase (MDHAR) and Dehydroascorbate reductase (DHAR) activities had positive correlation with the oxidative stress (R_{MDHAR}=0.448) (R_{DHAR}=0.603).

Keywords: AsA-GSH cycle; H_2O_2; Oxidative stress; *Penicillium*; Sclerotia

Abbreviations: ROS: Reactive oxygen species; H_2O_2: Hydrogen peroxide; CAT: Catalase; POD: peroxidase; APX: Ascorbic Acid Peroxidase; GR: Glutathione Reductase; AsA-GSH: Ascorbate-Glutathione; ASC: Reduced form of Ascorbate; DHA: Dehydroascorbate; MDHAR: Monodehydroascorbate Reductase; DHAR: Dehydroascorbate reductase; GSH: Reduced Glutathione; GSSG: Oxidized Glutathione; PDA: Potato Dextrose Agar; MDA: Malondialdehyde; TBA: Thiobarbituric acid

Introduction

Reactive oxygen species (ROS) and their subsequent reaction with macromolecules like proteins, lipids, polysaccharides and nucleic acids leading to altered membrane fluidity, loss of enzyme function and genomic damage. ROS cause an imbalance in cellular redox systems in favour of oxidized forms. Of the ROS generated, hydrogen peroxide (H_2O_2) is the most stable and is a source of the most damaging and reactive hydroxyl radical. The oxidative damage to different cellular components by H_2O_2 could be minimized either by catalase (CAT) and peroxidase (POD) activities or by a reaction sequence known as ascorbate-glutathione (AsA-GSH) cycle that involves the redox pairs of ascorbate-dehydroascorbate and glutathione-glutathione disulphide [1,2]. In this system, APX can reduce H_2O_2 by the oxidation of reduced form of ascorbate (ASC) to form the monodehydroascorbate radical, which is removed by photosynthetic electron flow through ferredoxin [3] or further reduced into ASC by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR). The resulting radical may be spontaneously disproportionate to ASC and dehydroascorbate (DHA) [4]. DHA is then reduced to generate ASC by dehydroascorbate reductase (DHAR) using glutathione as an electron donor [5]. In the meantime, this oxidized glutathione can be reduced to glutathione by GR, which utilizes the reducing equivalents from NAD(P)H. These low molecular weight antioxidants and enzymatic antioxidants have been associated with the overall tolerance and ultimate survival of a plant during periods of adverse environmental stresses [6].

GSH is associated with stress resistance [7]. It is an important antioxidant in the cellular milieu and is responsible for maintenance of the antioxidative machinery of the cells intact under stress.

Glutathione is present in cells in its reduced form (GSH) and most of its proposed functions are related to the thiol group and its use as a reductant. It is oxidized during the antioxidative process to GSSG. A high GSH:GSSG ratio is necessary to achieve optimal protein synthesis in cells. GSSG inhibits protein synthesis by converting an initiation factor into its inactive form. It is re-converted into GSH by glutathione reductase using photosynthetically generated NADH or NADPH [8]. GSH is the main component of the free, low molecular weight thiol pool in cells and is the precursor for enzymatic synthesis of metal binding peptides with the general structure (g-glutamylcysteinyl) *n* glycine [9]. Consumption of GSH for peptide synthesis and in its direct antioxidant role under metal stress leads to activation of its biosynthetic system.

Carotenoids are antioxidants as they are known to reduce oxidative stress by acting as scavengers of ROS (mainly singlet oxygen) [10]. The β-carotene and other carotenoids have been associated with fungal

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Received November 12, 2015; **Accepted** November 30, 2015; **Published** December 27, 2015

Citation: Zhao W, Han J (2016) Effect of H_2O_2-Induced Oxidative Stress on Sclerotial Differentiation and Asa-GSH Cycle of *Penicillium thomii* Q1 Strain. J Environ Anal Toxicol 5: 338. doi:10.4172/2161-0525.1000338

photomorphogenesis and development [11-17]. Ascorbic acid plays important biochemical roles, such as in cell growth and differentiation, in most organisms. It has been shown to play an important antioxidant role in sclerotial differentiation of phytopathogenic fungi [12-14,16,18].

Penicillium thomii Q1 strain was isolated from a soil sample, and able to form abundant orange, sand-shaped sclerotia in which carotenoids accumulated. We had studied the effect of Cu-induced oxidative stress on sclerotial differentiation and antioxidants contents of Q1 strain [19]. The AsA-GSH cycle was very widespread in the study of adverse resistant plants, but in the microbial study reported little. Therefore, the present study was designed to investigate the effects of H_2O_2-induced oxidative stress on the sclerotial differentiation and AsA-GSH cycle of *Penicillium thomii* Q1 strain. It should help to explain Q1 strain by AsA-GSH cycle in ASC and DHA, GSH and GSSG of non-enzymatic antioxidants and antioxidant enzymes MDHAR, DHAR to help it through adverse environment.

Materials and Methods

Preparation of inocula

Strain Q1 was isolated from roots soil of pine forest, collected close to Wutai Mount, Shanxi Province. By rDNA-ITS sequence analysis, strain Q1 was identified as *Penicillium thomii* (its GenBank accession number was KC966729). Strain Q1 was cultured on potato dextrose agar (PDA) plate in a dark incubator at 25°C. The sclerotia as inoculum were obtained from 14-day-old PDA plate cultures of this strain. To purify sclerotia, the plate cultures were centrifuged, rinsed aseptically five times with sterilized water to remove mass spores.

Oxidative stress growth conditions

Potato dextrose agar (PDA) media supplemented respectively with H_2O_2 of 3 μmol/L, 6 μmol/L and 10 μmol/L were as the different oxidative stress growth conditions. The PDA medium without supplementation of H_2O_2 served as the control. Using three point inoculations [20], three grains of sclerotia of Q1 strain were inoculated at a 9-cm Petri dish containing 25 mL of medium. Plates were incubated in the dark at 25°C for 20 days.

Sclerotial biomass, carotenoids extraction and determination

The sclerotia on the agar surfaces in Petri plates were separated and washed thoroughly with distilled water and dried at 50°C to constant weight to determine sclerotial biomass. Then, the sclerotia were observed with an anatomical lens (model SMZ-168, Motic China Group Co., China).

The extraction and determination of pigments were performed as a modified procedure described by ref. [18]. One gram of the dry sclerotia was manually ground with a glass homogenizer and extracted three times with 10 mL aliquots of acetone. The combined acetone extracts were combined in a separatory funnel, and 10 mL of chloroform and few milliliters of a solution of saturated NaCl to help break emulsions. The chloroform extract was collected after removal of the acetone layer, which was re-extracted. Absorbance of the chloroform extract was measured at 475 nm. The content of carotenoids was calculated by using the 1% extinction coefficient=2500 by the formula:

$$\text{Content of carotenoids}\left(\frac{\mu g}{g}\text{dry sclerotia}\right)=\frac{\text{ml of chloroform}\times A475}{\text{sclerotia dry weight}}\times 2500 \quad (1)$$

Ascorbate assay

The ascorbate assay was performed by a procedure described by Georgiou et al. [12]. Ascorbate concentration was expressed in units of μg ascorbate/g sclerotia dry weight.

Glutathione assay

The sclertia (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 centrifuged. The supernatant was used for the assay of glutathione. GSH and its oxidized product GSSG were measured together spectrophotometrically [21] with some modifications. 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 DTNB, 60 μL 15 mM NADPH and 100 μL acid extract. The acid extract was done immediately prior to starting the reaction by the addition of 50 μL of 5 units GR activity (bakers'yeast, type III, Sigma Chemical Company). Change in absorbance of the reaction mixture was measured at 412 nm.

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

Evaluation of lipid peroxidation

The levels of lipid peroxidation in the sclerotia of Q1 strain were evaluated by their peroxidation product determination. Thiobarbituric acid reactive substances (TBARS): aldehydes, mainly malondialdehyde (MDA) and endoperoxides were determined according to the methods described by Hodges *et al.* [22]. MDA-routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloric acid. The reaction with thiobarbituric acid (TBA) was conducted at 95°C for 30 min. After the samples were chilled absorbance was measured at 532 nm and 432 nm. The results were expressed as MDA μmol per gram of dry sclerotia.

Measurement of antioxidant enzyme activity

The sclertia (1 g DW) were homogenized in ice-cold 50 mM pH 7.8 phosphate buffer, 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 12000 *g* in a cooled centrifuge. The supernatant was used for the assay of APX (EC 1.11.1.11), GR (EC 1.6.4.2), DHAR (EC 1.8.5.1) and MDHAR (EC 1.6.5.4).

Total GR activity was assayed by the DTNB method, with minor modifications [23]. The reaction mixture (300 μL) consisted of 100 mM phosphate buffer (pH 7.8), 0.1 mM NADPH, 1.0 mM GSSG and 20 μL enzyme extract. The reaction was started by the addition of GSSG and the GSH reduction rate was monitored at 420 nm for 5 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 μmol GSH per minute.

The assay for APX was done by monitoring the rate of oxidation of ascorbate according to the method of Nakano and Asada [24]. Absorbance readings were taken at 290 nm using the spectrophotometer blanked against an aliquot of buffer (50 mM phosphate buffer, pH 7.0, 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 3 times as quickly as possible, and placed in the spectrophotometer. The reaction was monitored by decrease in extinction at 290 nm for 1 min.

MDHAR activity was measured according to Krivosheeva *et al.*

[25]. The reaction mixture (3 mL) consisted of 2.49 mL 2 mM AsA (pH 5.6 PBS), 0.06 mL 2 mM ascorbate oxidase (pH 5.6 PBS), 0.15 mL 2 mM NADPH (pH 7.6 PBS) and 0.3 mL enzyme extract. The reaction was monitored by increase in extinction at 340 nm for 1 min. The extinction coefficient was 6.2 mmol/L/cm.

DHAR activity was measured according to Dalton *et al.* [26]. The reaction mixture (1 mL) consisted of 100 mM Hepes-KOH (pH 7.0), 1 mM EDTA, 2.5 mM GSH and 0.2 mM DHA. The reaction was started by the addition of DHA. The reaction was monitored by increase in extinction at 265 nm for 1 min. The extinction coefficient was 14 mmol/L/cm.

Statistics

All experiments were replicated in three plates and the data are presented as the arithmetic mean ± standard error. Duncan's multiple range test [27] was used on the isolation means to test for significant differences at the 5% level of confidence.

Results

Effect of H_2O_2-induced oxidative stress on sclerotial differentiation, sclerotial biomass and carotenoids content in sclerotia of Q1 strain

The macroscopic colony characters of Q1 strain was observed for each plate culture. The results (Figure 1) showed that on the media with different oxidative stress growth conditions, the colonies of Q1 strain were apparent different. Different colonies could be observed from the colonies' composition and sclerotia's color and sclerotia's biomass. Table 1 showed that when the level of oxidative stress increased, the time of exudates initiation, sclerotial initiation and sclerotial maturation were advanced in 2 days.

There were apparent differences in the sclerotial biomass and carotenoids content in sclerotia of Q1 strain under different oxidative growth conditions (Table 2). The higher the concentration of H_2O_2 was, the higher the sclerotial biomass and carotenoids content in sclerotia ($R_{biomass}$=0.972, $R_{content}$=0.941) (P<0.05). When the fungus was grown at high oxidative stress condition induced by H_2O_2 of 10 μmol/L, its sclerotial biomass (i.e., dry sclerotia weight) increased by 1.26-fold with respect to the control. Carotenoids content in sclerotia under high oxidative stress condition increased by 1.58-fold with respect to the control. It could be found that the values in sclerotial biomass had a moderate positive correlation with the values in the carotenoids content in sclerotia (R=0.899).

One conclusion from these results was that H_2O_2–induced oxidative stress favored the sclerotial differentiation and endogenous carotenogenesis of Q1 strain.

Effect of H_2O_2 concentrations in media on lipid peroxidation in sclerotia of Q1 strain

Lipid peroxidation is an important indicator of oxidative stress and is accompanied by the formation of aldehydic lipid hydroperoxide decomposition products such as MDA [28]. The MDA formation is considered evidence that free radical-mediated reactions have taken place and its concentration is used as a measure of the degree of this stress [29]. Therefore, we tested the effect of H_2O_2 concentrations in media on lipid peroxidation in sclerotia of Q1 strain (Figure 2). It was found that the MDA content in sclerotia had a moderate positive correlation with the H_2O_2 concentrations in media (R=0.824). This result indicated that the oxidative stress induced by H_2O_2 was clearly dependent on the H_2O_2 concentrations in media.

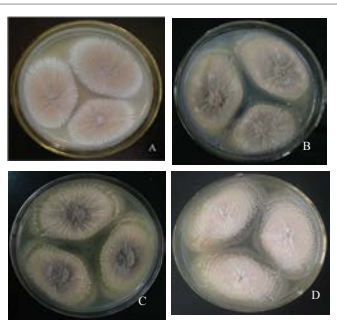

Figure 1: Colonies of *Penicillium thomii* Q1 strain on PDA (A), PDA+H_2O_2 of 3 μM/L (B), PDA+H_2O_2 of 6 μM/L (C) and PDA+H_2O_2 of 10 μM/L (D) at 25°C, after 14 days. Petri dish size=9 cm diam.

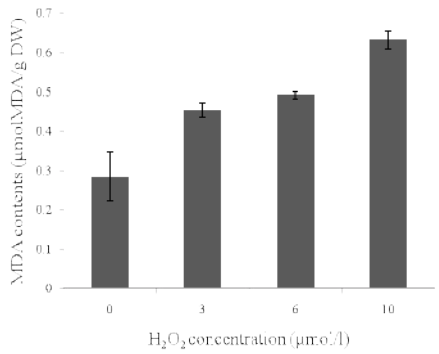

Figure 2: Effect of H_2O_2 concentrations in media on lipid peroxidation of sclerotia of *Penicillium thomii* Q1 strain. Results represent mean values of 3 independent experiments ± standard deviation.

Oxidative growth conditions	Time of exudates initiation (days)	Time of sclerotial initiation (days)	Time of sclerotial maturation (days)
Control (H_2O_2 of 0 μM/L)	5	7	13
H_2O_2 of 3 μM/L	4	5	12
H_2O_2 of 6 μM/L	4	5	12
H_2O_2 of 10 μM/L	4	5	12

Table 1: Effect of H_2O_2–induced oxidative stress on sclerotial differentiation of *Penicillium thomii* Q1 strain.

Oxidative growth conditions	Sclerotial biomass (mg/plate)	Carotenoid content (μg/g dry sclerotia)
Control (H_2O_2 of 0 μM/L)	322.7 ± 24.8a*	10.9 ± 0.8a
H_2O_2 of 3 μM/L	365.0 ± 4.6b	13.8 ± 0.3b
H_2O_2 of 6 μM/L	392.7 ± 7.5c	13.7 ± 1.1b
H_2O_2 of 10 μM/L	405.3 ± 8.7d	17.2 ± 1.2c

Table 2: Effect of H_2O_2–induced oxidative stress on sclerotial biomass and carotenoids content in sclerotia of *Penicillium thomii* Q1 strain.

*Mean values in the same column followed by different letters are significantly different at the P<0.05 level according to Duncan's multiple−range test.

Effect of H_2O_2-induced oxidative stress on ascorbate content in sclerotia of Q1 strain

We determined the contents of reduced and oxidized ascorbate in sclerotia of Q1 strain under different oxidative stress growth conditions (Table 3). The non-enzymic antioxidants, viz., ascorbate

Oxidative growth conditions	GSH content (μmol/g dw)	GSSG content (μmol/g dw)	GSH/GSSG	ASC content (μg/g dw)	DHA content (μg/g dw)	ASC/DHA
Control (H_2O_2 of 0 μM/L)	1.65 ± 0.04a*	0.37 ± 0.02a	4.46	2.07 ± 0.12b	3.57 ± 0.35b	0.58
H_2O_2 of 3 μM/L	1.69 ± 0.03b	0.67 ± 0.03b	2.52	1.80 ± 0.40a	2.70 ± 0.26a	0.67
H_2O_2 of 6 μM/L	1.94 ± 0.23c	1.05 ± 0.02c	1.85	4.97 ± 0.15d	6.80 ± 0.20d	0.73
H_2O_2 of 10 μM/L	2.85 ± 0.06d	1.29 ± 0.01d	1.5	3.83 ± 0.38c	5.23 ± 0.35c	0.73

*Mean values in the same column followed by different letters are significantly different at the P<0.05 level according to Duncan's multiple−range test.

Table 3: Oxidized glutathione (GSSG) and reduced glutathione (GSH) content, GSH/GSSG ratio, reduced ascorbate (ASC) and oxidized ascorbate (DHA) content, and ASC/DHA ratio in sclerotia of *Penicillium thomii* Q1.

content in sclrotia showed increasing trends with the increase of H_2O_2 concentrations in media. Under the oxidative stress growth condition induced by H_2O_2 of 6 μmol/L, the levels of both DHA and ASC reached respectively their maximum values (6.8 μg/g, 4.97 μg/g). The values of the ASC and DHA contents had a moderate positive correlation with the the H_2O_2 concentrations in media (R_{ASC}=0.727, R_{DHA}=0.640). The contents of DHA and ASC were increased by 1.9-fold and 2.4-fold with respect to control. The ASC / DHA ratio was gradually increased (R=0.929). Meanwhile, under the same oxidative stress condition, the content of DHA was higher than that of ASC.

One conclusion from these results was that H_2O_2-induced oxidative stress favored the accumulation of endogenous ascorbate in sclerotia of Q1 strain.

Effect of H_2O_2-induced oxidative stress on glutathione content in sclerotia of Q1 strain

Similarly, the contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) in sclerotia were strongly dependent on the oxidative stress growth condition (Table 3). GSH and GSSG contents in sclerotia showed increasing trends with the increase of H_2O_2 concentrations in media (R_{GSH}=0.888, R_{GSSG}=0.997) (P<0.01). The GSH/GSSG ratio decreased linearly with increasing H_2O_2 concentration (R=-0.933). Meanwhile, under the same oxidative stress condition, the content of GSH was higher than that of GSSG.

One conclusion from these results was that H_2O_2-induced oxidative stress favored the accumulation of endogenous glutathione in sclerotia of Q1 strain.

Effect of H_2O_2-induced oxidative stress on APX activity in sclerotia of Q1 strain

Peroxidases (APX and GPX) are distributed throughout the cell and catalyze the reduction of H_2O_2 to H_2O. APX uses ascorbate as electron donor in the first step of the AsA-GSH cycle and is considered the most important plant peroxidase in H_2O_2 detoxification [30]. As shown in Figure 3, under H_2O_2-induced oxidative stress, APX activity reached the maximum at the H_2O_2 concentration of 3 μmol/L (640 U/g DW/min). Under the H_2O_2 concentration from 6 μmol/L to 10 μmol/L, APX activity decreased. The value of the APX activities had a negative correlation with the H_2O_2 concentrations in media (R=-0.555). Compared with the control, APX activity decreased 1.54 fold. The values of the APX activities had a weak positive correlation with the value of the GR activiy (R_{GR}=0.057). Meanwhile, the values of the APX activities had a significant negative correlation respectively with the contents of ASC and DHA (R_{ASC}=-0.928, R_{DHA}=-0.952) (P<0.05), had also a weak negative correlation with the carotenoids content in sclerotia (R=-0.098), and had a moderate negative correlation with the contents of GSH and GSSG (R_{GSH}=-0.152, R_{GSSG}=-0.496).

Effect of H_2O_2-induced oxidative stress on GR activity in sclerotia of Q1 strain

As shown in Figure 4, GR activity decreased by almost 3.4-fold at 3 μmol/L with respect to control indicating higher enzyme activity either in response to the superoxide anions formed due to H_2O_2 toxicity or for more synthesis of glutathione. Although he GR activity increased from 6 μmol/L to 10 μmol/L H_2O_2, but still significantly lower than the control (P<0.05). This decrease in enzyme activity is indicative of drastic stress of higher H_2O_2 concentration on the test strain (R=-0.420). The values of the GR activities had a weak negative correlation respectively with the contents of ASC and DHA (R_{ASC}=-0.153, R_{DHA}=-0.036), had also a moderate negative correlation with the carotenoids content in sclerotia (R=-0.483), and had a negative correlation with the contents of GSH and GSSG (R_{GSH}=-0.058, R_{GSSG}=-0.423).

Effect of H_2O_2-induced oxidative stress on MDHAR activity in sclerotia of Q1 strain

Information on MDHAR activity in sclerotia of Q1 strain was shown in Figure 5. It was found that the MDHAR activity in sclerotia had a positive correlation with the H_2O_2 concentrations in media (R=0.448). Under the oxidative stress condition induced by H_2O_2 concentration of 3 μmol/L, the MDHAR activity in sclerotia reached its maximum value of 1699 U/g DW/min. Compared with the control, MDHAR activity increased 2.15 fold. The values of the MDHAR activities had a moderate negative correlation respectively with the contents of ASC and DHA (R_{ASC}=-0.311, R_{DHA}=-0.327), had also a weak negative correlation with the carotenoids content in sclerotia (R=-0.023), and had a moderate positive correlation with the contents of GSH and GSSG (R_{GSH}=0. 102, R_{GSSG}=0.128).

Effect of H_2O_2-induced oxidative stress on DHAR activity in sclerotia of Q1 strain

Information on DHAR activity in sclerotia of Q1 strain was shown in Figure 6. The results showed that enhancement of H_2O_2 concentration from 0 μM/L to 3 μM/L in media increased the DHAR activity of Q1 strain significantly. However, the enhancement of H_2O_2 concentration from 3 μM/L to 10 μM/L in media did not increase significantly the DHAR activity. Therefore, under the oxidative stress condition induced by H_2O_2 of 3 μM/L, the DHAR activity in sclerotia reached its maximum value of 817 U/g DW/min. The values of DHAR activities had a moderate positive correlation (R=0.603) with the H_2O_2 concentration. The values of the DHAR activities had a weak positive correlation respectively with the contents of ASC and DHA (R_{ASC}=0.227, R_{DHA}=0.099), had also a moderate positive correlation with the carotenoids content in sclerotia (R=0.675), and had a moderate positive correlation with the contents of GSH and GSSG (R_{GSH}=0. 414, R_{GSSG}=0.594).

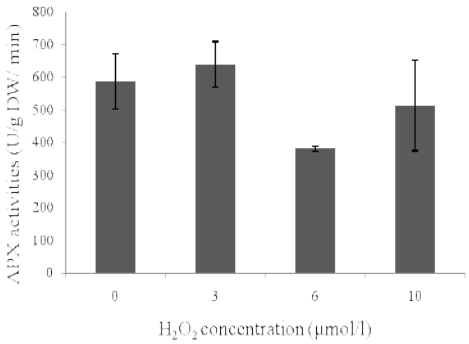

Figure 3: Effect of H_2O_2–induced oxidative stress on sclerotia's APX activities of *Penicillium thomii* Q1 strain. Error bars represent the standard deviation of triplicate samples.

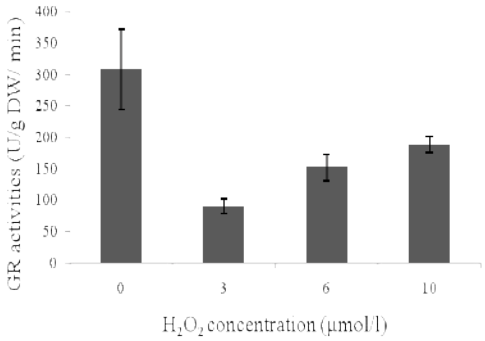

Figure 4: Effect of H_2O_2–induced oxidative stress on sclerotia's GR activities of *Penicillium thomii* Q1 strain. Error bars represent the standard deviation of triplicate samples.

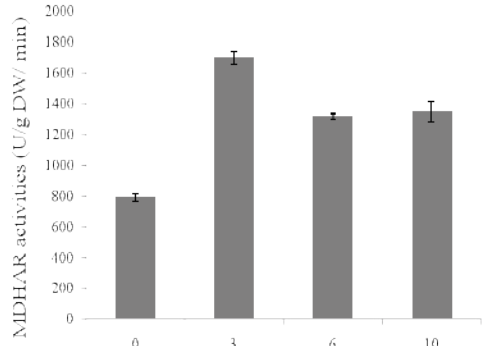

Figure 5: Effect of H_2O_2–induced oxidative stress on sclerotia's MDHAR activities of *Penicillium thomii* Q1 strain. Error bars represent the standard deviation of triplicate samples.

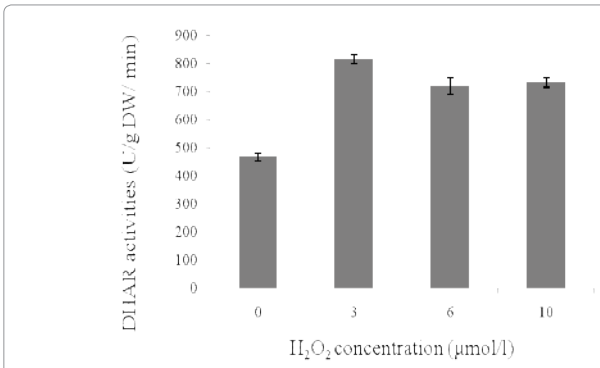

Figure 6: Effect of H_2O_2–induced oxidative stress on sclerotia's DHAR activities of *Penicillium thomii* Q1 strain. Error bars represent the standard deviation of triplicate samples.

Discussion

Georgiou [31] had advanced a theory which proposed that sclerotial differentiation in fungi was triggered by oxidative stress. Our previous experiment [19] showed that when Q1 strain was grown under copper induced higher oxidative stress condition, its sclerotial biomass was 1.39 times higher than that at low oxidative stress condition. The data of this study give additional evidence to support Georgiou's theory, and they are also in accordance with the general theory of microbial differentiation, which also postulates that this phenomenon is induced by oxidative stress. And our results indicate that H_2O_2 increases oxidative stress as is evident from increased lipid peroxidation, in accordance with previous findings showing that MDA accumulated greatly after H_2O_2 exposure and that the cell membrane is the primary site affected by H_2O_2 toxicity. This might be due to the overproduction of ROS under H_2O_2 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 work contained high H_2O_2 concentrations sufficient to activate ROS production and subsequently oxidative stress.

The protection to these ROS via the antioxidant system is complex and highly organized. AsA-GSH cycle was About APX has a higher affinity for H_2O_2, allowing for the scavenging of small amounts of H_2O_2 in more specific locations. In our experiments, H_2O_2 treatments induced APX activities in the sclerotia of Q1 strain. This means that APX activity decreased linearly with increasing H_2O_2 concentration. The highest concentration of H_2O_2 (10 μM/L) proved to be extremely toxic resulting in a decline of APX activity. The hyperactivity of peroxidase under H_2O_2 stress indicated 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 [32].

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 [10,12,33,34]. Ascorbate can directly scavenge ROS (including hydroxyl and superoxide radicals and hydrogen peroxide) either nonenzymatically or enzymatically [35]. In the latter case, it is used as a substrate for the H_2O_2-splitting enzyme ascorbate peroxidase [23]. 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 [36-44]. In our experiment, carotenoids, ascorbate and glutathione can be accumulated in sclerotia of Q1 strain. However, the effect of H_2O_2-induced oxidative stress on carotenoids content in sclerotia was different from that on ascorbate content and glutathione content. It could be found that the carotenoids content in sclerotia had a significant positive correlation with the H_2O_2 concentrations in media (R=0.941) (P<0.05), and the total ascorbate content in scelrotia had a positive correlation with the H_2O_2 concentrations in media (R=0.683). The GSH content in scelrotia had a significant positive correlation (P<0.05) with the H_2O_2 concentrations in media (R_{GSH}=0.977). The highest carotenoids content was obtained from the PDA plate supplemented with H_2O_2 of 10 μM/L. The total ascorbate content was the sum of ASC and DHA content.

The highest total ascorbate content was obtained from the PDA plate supplemented with H_2O_2 of 6 μM/L (11.77 μg/g dry sclerotia). The highest GSH and GSSG contents were separately obtained from the PDA plate supplemented with H_2O_2 of 6 μM/L and 10 μM/L (Table 3). These results indicated that the oxidative stress induced by a higher amount of H_2O_2 (about 10 μM/L) favored endogenous carotenogenesis of Q1 strain, whereas the oxidative stress induced by a lower amount of H_2O_2 (about 6 μM/L) favored the accumulation of ascorbate and glutathione in sclerotia.

Under the low oxidative stress, the APX and GR activities were increased. The values of APX and GR activities had negative correlation with the oxidative stress. The values of MDHAR and DHAR activities had positive correlation with the oxidative stress. It contributed to the reduction of MDHA and DHA for ASC. It can catalyze the regeneration of ascorbate and make ascorbate remained higher reduced state in the body. It is very important to adapt to the stress environment. Results showed that under the low oxidative stress condition, Q1 strain by AsA-GSH cycle in APX and GR these two kinds of antioxidant enzymes to survive adverse environment; while under the high oxidative stress condition, Q1 strain by AsA-GSH cycle in ASC and DHA, GSH and GSSG of non-enzymatic antioxidants and antioxidant enzymes MDHAR, DHAR to help strains through adverse environment.

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Citation: Zhao W, Han J (2016) Effect of H_2O_2-Induced Oxidative Stress on Sclerotial Differentiation and Asa-GSH Cycle of *Penicillium thomii* Q1 Strain. J Environ Anal Toxicol 5: 338. doi:10.4172/2161-0525.1000338