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Influence of the oxidative stress induced by the organophosphate pesticide bromopropylate on the mitochondrial respiratory chain in *Trichoderma harzianum*

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ABSTRACT

The present study was designed to investigate the effect of bromopropylate on its own transport rate, glycolysis and tricarboxylic acid cycle metabolite levels, adenine nucleotides, and membrane lipid peroxidation (LPO) as well as the activities of mitochondrial electron transport chain (ETC) enzymes in eukaryotic *Trichoderma harzianum*. The transport rate of bromopropylate reached a maximum level within the first 24 h of incubation for all studied concentrations. The succinate dehydrogenase (SDH) and cytochrome c oxidase (CCO) activities reached their maxima at 72 h for 2.5 and 10 mg/L of bromopropylate, respectively. In addition, the intracellular pyruvate levels increased for bromopropylate concentrations up to 2.5 mg/L. The maximum intracellular α-ketoglutarate level was determined at 5 mg/L, while the intracellular fumarate and citrate levels reached their maximums at 7.5 mg/L of bromopropylate. The variations in the adenine nucleotide levels showed a positive correlation with both α-ketoglutarate and fumarate levels. Nevertheless, the LPO levels increased with increasing bromopropylate concentrations. These results may indicate that the membrane becomes more damaged from an impaired respiratory chain, which may then cause an increase in electron leakage.

1. Introduction

In the aerobic respiratory system, the glycolytic pathway oxidizes glucose to pyruvate, which is then taken up into the mitochondria and converted to acetyl-CoA in the mitochondrial matrix. Then, acetyl-CoA is used by the tricarboxylic acid (TCA) cycle, which interconverts carbon skeletons in a series of anaplerotic reactions that provide NADH for the electron transport chain (ETC). The conventional paradigm for the mitochondrial ETC involves the transfer of electrons from reducing equivalents, such as NADH, $FADH_2$ or succinate, via four inner membrane protein complexes to oxygen with the concomitant translocation of protons into the intermembrane space. The resultant potential across the inner membrane drives ATP synthesis.

Metabolic homeostasis is disrupted by several environmental factors. One of the environmental factors is the pesticides that have been used in agriculture to enhance food production. Unfortunately, the spread and the uncontrolled use of pesticides have led to severe environmental pollution and potential health hazards from the toxic effects on non-targeted organisms. Among these pesticides, the organophosphate pesticides (OPs), such as bromopropylate, are widely used and are known to exert their toxic actions primarily by interference with the respiratory system [1]. These OPs affect the mitochondria by inhibiting key enzymatic activities and ATP generation [2,3]. These defects cause electron leakage during respiration, and this leakage leads to the formation of reactive oxygen species, which results in the destruction of proteins, membranes, unsaturated fatty acids, DNA, etc. Therefore, within the respiratory system, not only is energy generated but free radicals are also produced [4]. Some studies suggest that exposure to a wide range of pesticides could result in the progressive degeneration of membrane structures that are rich in polyunsaturated fatty acids (PUFAs), which is caused by the production of excessive free radicals and termed lipid peroxidation (LPO).

Soil microorganisms are the organisms most affected by the toxic effect of pesticides. The microbial diversity and the community structure within soil are largely influential in the ability of plants to resist environmental factors such as the uncontrolled use of pesticides [5,6]. Certain strains of *Trichoderma harzianum* have been shown to effectively control several pathogens from different

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host plant species. Hence, the differentiation of *T. harzianum* metabolism can cause the alteration of the soil microbial community and increase the level of phytopathogenic fungi. Therefore, in the present study, *T. harzianum* was used as a eukaryotic model to investigate the effect of toxicity caused by the OP bromopropylate on the respiratory system. Therefore, the activities of the ETC enzymes, SDH and CCO in the isolated mitochondria were measured, and the levels of the adenine nucleotides, which serve as a useful index of altered energy metabolism following exposure to toxicants, were investigated in *T. harzianum*. The ETC is involved in a dynamic relationship with the TCA cycle and glycolysis. Therefore, the variation of some glycolysis and TCA cycle metabolites levels were determined as well. Moreover, the toxicity of bromopropylate was evaluated by measuring the LPO levels as a marker of oxidative damage from free radical generation.

2. Materials and methods

2.1. Media and growth conditions

T. harzianum (63059) was obtained from Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH (DSMZ) and maintained at 25 °C for 7 days on malt yeast glucose agar [7] that contained 5 g malt extract, 2.5 g yeast extract, 10 g glucose and 20 g agar in 1 L of distilled water. Then, the cultures were grown in a liquid medium containing 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 6.9 g Na_2HPO_4, 0.3 g $MgSO_4{\cdot}7H_2O$, 1 g peptone, 0.3 g urea and 1% (w/v) glucose in 1 L of distilled water [8]. Before autoclaving, the pH was adjusted to 5.6. The cultures were inoculated with 1 mL spore suspensions (OD_{620} = 2.000) in 250 mL shaking flasks containing 100 mL of culture and incubated with agitation at 180 rpm and 28 °C. After the cultivation process, the cells were collected and washed with distilled water prior to storage at −20 °C.

2.2. Isolation of mitochondria

The thawed samples were resuspended in 10 mM Trizma-HCl buffer, pH 7.5, containing 1 mM EDTA, 300 mM sucrose and 0.1% bovine serum albumin using a buffer volume 8 times the weight of the sample. The cell samples were homogenized in tubes at 8500 rpm for 15 s on ice. The cell suspensions were ground in 1.5 mL plastic vials and centrifuged at 2000 rpm for 15 min, and then, the cell debris was removed. The supernatants were centrifuged at 15,000 rpm for 15 min. The final pellets contained the mitochondria. Before assaying, the pellets were resuspended in a BSA-free isolation buffer.

2.3. Enzyme activities in the isolated mitochondria

SDH activity was assayed by measuring the initial rate of decrease in the dichlorophenolindophenol (DCIP) absorbance at 600 nm. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1.0 mM EDTA, 20 mM sodium succinate, 3 mM sodium azide, 32 μM DCIP and the enzyme solution [9].

CCO activity was assayed by measuring the initial rate of decrease in the cytochrome c absorbance at 550 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 30 μM reduced cytochrome c. To reduce the cytochrome c, which was in 10 mM potassium phosphate buffer (pH 7.5), sodium borohydride was added and the absorbance was monitored at 550 and 565 nm until the absorbance ratio at 550/565 nm was greater than 10 [10].

2.4. Preparation of the extracts for high performance liquid chromatography (HPLC)

The samples were prepared using a modified procedure from Ganzera et al. [11]. The intracellular metabolites from the cell pellet were extracted in a solution of 50% (w/v) boiling ethanol for 15 min, where the boiling ethanol was directly transferred into the chilled sample. This solution was shaken for 15 min at 90 °C in a water bath and then centrifuged for 10 min at 12,000 rpm. The supernatant was quickly frozen and lyophilized. The lyophilyzate was redissolved in ultra-pure water (200 μL) and then filtered through a 0.20 μm filter before injection into the HPLC.

2.5. HPLC conditions

A Thermo ODS–2 Hypersil column (250 mm × 4.6 mm) was used to determine the adenine nucleotide (ATP, ADP and AMP) levels. The analysis was conducted by an Agilent HP 1100 HPLC system under the following conditions: mobile phase, 50 mM aqueous triethylamine buffer (adjusted to pH 6.5 with phosphoric acid; (A) and acetonitrile (B); flow rate, 1 mL/min; injection amount, 20 μL; detection wavelength, 254 nm; and column temperature, 20 °C. The gradient elution began with a gradient from 99A/1B to 95A/5B over 10 min and then, over the next 10 min, the ratio was changed to 92.5A/7.5B. Each run was followed by a 5 min wash with 70 parts B/30 parts 0.1% phosphoric acid [11]. The analysis of organic acids was conducted on an Agilent HP 1100 HPLC system equipped with an Alltech IOA–1000 column and UV detector. The conditions were mobile phase, 9.0 mM H_2SO_4 solution; flow rate, 0.4 mL/min; injection amount, 20 μL; detection wavelength, 210 nm; and column temperature, 40 °C [12].

2.6. Extraction of bromopropylate and analysis by gas chromatography–mass spectrometry (GC–MS)

The extracellular bromopropylate in the liquid culture was recovered by solid phase extraction using an Envocarb C18 solid phase extraction (SPE) cartridge. The method described by Gomis et al. was modified as follows: the SPE cartridge (100 mg) was activated with methanol, then distillated water and finally a methanol/water = 1:1 (v/v) mixture [13]. The extracellular liquid of the growth medium was loaded on the SPE cartridge, washed with methanol:water = 1:1 (v/v) and dried under vacuum. The elution of bromopropylate was performed with hexane. The pesticide concentration was measured by GC–MS. The extracts were injected into the GC–MS system under the following conditions: split injection mode; injection volume, 5 μL; injector temperature, 250 °C; detector temperature, 280 °C; and carrier gas, helium. The gas chromatograph was operated in constant flow mode (electronic pressure control) at 36.2 mL/min. The GC temperature program was as follows: hold for 0.75 min at 50 °C, 50–150 °C at 25 °C/min, 150–200 °C at 3 °C/min, 200–280 °C at 8 °C/min, and hold for 15 min at 280 °C. A 0.25 mm × 30 m × 0.25 μm HP-5MS column was used.

2.7. Lipid peroxidation (LPO)

The LPO was estimated based on thiobarbituric acid (TBA) reactivity. The samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient at 532 nm of 153,000 $M\,r^{-1}\,cm^{-1}$ for the chromophore was used to calculate the MDA-like TBA produced [14].