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ORIGINAL ARTICLE

Effects of three phenolic compounds on mitochondrial function and root vigor of cotton (*Gossypium hirsutum* L.) seedling roots

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Abstract

Continuous cropping of cotton causes accumulation of allelochemicals in soil that results in substantial crop yield and quality losses. To elucidate the physiological mechanism of the effects of allelochemicals on cotton root growth, and solve the problem of continuous cropping obstacles, hydroponics experiments were carried out to study the effects of three allelochemicals (*p*-hydroxybenzoic acid (PHBA), phloroglucinol, and ferulic acid) at different concentrations (0.8, 4.0, and 20.0 mmol L^{-1}) on the production of reactive oxygen species, antioxidant enzyme activities, and mitochondrial function of cotton seedling roots. All three phenolic compounds suppressed cotton root growth, decreased the activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase) and H^+-ATPase in root mitochondria, and increased generation of O_2^- and the content of H_2O_2. They also increased the degree of openness of mitochondria permeability transition pores, and decreased the membrane fluidity of mitochondria, and the ratio of cytochrome (Cyt) c/a, thus resulting in the damage of mitochondrial structure and overall function of the root system. Ferulic acid at 20.0 mmol L^{-1} inhibited cotton root growth more than the other treatments. Above all, all three kinds of allelochemicals inhibited antioxidant enzyme activity and mitochondrial function in cotton seedling roots, and the inhibition depended on the dose of phenolic compounds. Compared to PHBA and phloroglucinol, ferulic acid was a stronger inhibitor of cotton seedling root growth.

Keywords Cotton · Phenolic compounds · Mitochondrial function · Root vigor · Root

Introduction

As a major economic crop, cotton has an important position in China and national economy. However, due to current agricultural trends in China, continuous cropping presents very serious obstacles and restricts the sustainable development of modern agricultural facilities, thus result in substantial crop yield and quality losses (Jing et al. 2016; Liu et al. 2008), especially in Xinjiang Autonomous Region which is the biggest cotton area in China. The continuous cropping obstacle arises as a result of numerous interactions between the cotton plant and soil, including allelopathic autotoxicity caused by phenolic compounds (Li et al. 2009,2013). Therefore, to solve the continuous cropping obstacle, the damaging mechanisms of phenolic compounds on cotton plant growth should be elucidated. Phenolic compounds are one of the most important secondary metabolites implicated in allelopathy and have been detected in both natural and managed ecosystems. However, excessively accumulation of phenolic compounds in soil ultimately results in toxicity to the plant itself or neighbors (Saraf et al. 2014; Tian et al. 2015). For example, aqueous extracts (containing allelochemicals) of cotton straw with a concentration of 20–80 mg mL^{-1} have been reported to significantly inhibit the growth of cotton seedlings (Liu et al. 2008). 0.25 g L^{-1} ferulic acid apparently decreased the root vigor of cotton seedlings and antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), as well as increased the content of malonaldehyde (MDA) (Jiang et al. 2015). Vanillic acid, *p*-coumaric acid, caffeic acid, and syringic

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acid with a concentration of 1 mmol L^{-1} suppressed the growth of taproots and secondary roots of *Pisum sativum* L. (Vaughan and Ord 1990). Cinnamic acid at a concentration of 125 mg per kg soil inhibited the respiratory intensity and destroyed the respiratory mechanism of seedling roots of *Malus hupehensis* Rehd (Gao et al. 2010). 4 mmol L^{-1} phloridzin decreased the activity of tricarboxylic acid cycle enzymes (citrate synthase, *cis*-aconitase, and isocitrate dehydrogenase) in mitochondria of *Malus hupehensis* Rehd root systems (Wang et al. 2012). These results indicated that phenolic compounds affect not only the root morphological structure, but also the root mitochondrial function. The main allelochemicals in the rhizosphere of cotton were isolated and identified to be *p*-hydroxybenzoic acid (PHBA), ferulic acid, and phloroglucinol (Jiang et al. 2015). However, up to now, there is limited information about the effects of these three phenolic compounds on the growth of cotton.

Cotton roots are the first organ that directly exposed to the soil and subjected to allelochemicals. Root mitochondria are major metabolism sites, in which many physiological processes including the tricarboxylic acid cycle, generation of reactive oxygen species (ROS), modulation of redox status, oxidative phosphorylation as well as death receptor-mediated apoptosis were happened. In apoptosis triggered by many stimuli, more and more evidence supports the importance of mitochondria in coordinating caspase activation. Mitochondrial structure and function are significantly altered during the apoptotic process. Recent evidence indeed indicated that apoptosis can trigger the uncoupling of oxidative phosphorylation from ATP production and induce the burst of ROS and loss of mitochondrial membrane potential associated with decrease of the activity of antioxidant enzyme and H^+-ATPase (Norberg et al. 2010; Tsai et al. 2011). One of the major physiological mechanisms that involved in mitochondrial dysfunction during the apoptotic process is an alteration of mitochondrial permeability transition pores (MPTP).

Studies on animals indicated that the openness of MPTP is the central coordinator for programmed cell death (PCD), and is sufficient and necessary for apoptosis (Costantini et al. 1996; Xu et al. 2016). The over-opening of MPTP leads to a series of changes, including uncoupling of the respiratory chain, decrease of mitochondrial membrane potential ($\Delta\psi$), increase of ROS generation, Ca^{2+} in the cytoplasm flow to mitochondrial matrix, as well as release of cytochrome *c* into the cytosol (Paillard et al. 2009). Previous studies on plants also indicated that adverse stresses, such as drought, NaCl, and cadmium-affected mitochondrial function, disturbed energy metabolism, induced the increase of Ca^{2+} concentration in the cytoplast, and formed ROS in mitochondria (Li et al. 2003; Lin et al. 2005; Zhao et al. 2013). Moreover, aqueous extracts of *Galinsoga parviflora* Cav. containing allelochemicals also induced PCD in guard cells of *Vicia faba* (Zhou et al. 2016). Nonetheless, how PHBA, ferulic acid, and phloroglucinol affect the openness of MPTP requires further investigation.

In this study, the effects of three kinds of phenolic compounds (PHBA, phloroglucinol, and ferulic acid) on mitochondrial function and root vigor were examined in cotton. The aims of our present work were: (i) to investigate the responses of cotton growth to PHBA, phloroglucinol, and ferulic acid; (ii) to investigate whether MPTP openness was induced by these three phenolic compounds; and (iii) to assess the potential role of antioxidant ability and ROS levels in MPTP openness. This research will promote our understanding of the damaging mechanisms of allelochemicals to cotton roots, and provide the basis for solving continuous cropping obstacles in cotton fields.

Materials and methods

Experimental design

Hydroponics experiments were performed on 25 April 2017 in a phytotron at the Cotton Experimental Station of Jiangsu Academy of Agricultural Sciences, Nanjing (32°02′N and 118°50′E), Jiangsu Province, China. Cotton variety CCRI-50, widely planted in China, was used as material in this study. Seeds were sterilized with 3.0% (v/v) hydrogen peroxide for 10 min, then washed with deionized water for five times, then were sown in sterilized matrix. When the second true leaf of the cotton plant unfolded, seedlings of uniform growth were selected and transplanted into plastic pots with the size of 30 cm × 20 cm × 16 cm (length × width × height). The stem base of the seedling was wrapped with a sponge, then the seedlings were fixed in the plastic pot via a polyethylene foam board, with six seedlings in each pot.

Based on the analysis of allelochemical contents in leach liquor of cotton straw and the allelochemical concentration in the cotton field soil (Jiang et al. 2015; Liu et al. 2008), PHBA, phloroglucinol, and ferulic acid were selected in this study with three concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}, respectively. PHBA, phloroglucinol, and ferulic acid, purchased from the American Sigma-Aldrich, were first dissolved in absolute ethyl alcohol, and then diluted it with 1X Hoagland's nutrient solution to the required concentrations. A total of ten treatments were used for this study: control (CK) with 1X Hoagland's nutrient solution; A1, A2 and A3 with PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Each pot was filled with 5.0 L of nutrient solution. The experiment was arranged in a completely random design. Three replicates for each treatment were used with six seedlings in one pot representing a replicate.

The light source in the growth chamber was a high-pressure sodium lamp providing a PPFD of 450 ± 30 μmol m^{-2} s^{-1}. Plants were grown under a 12/12 h photoperiod with 30 ± 2 °C/20 ± 2 °C (day/night) temperature. Deionized water was added to the plastic pots every day to replenish the consumption of water caused by transpiration. An air pump was used for aeration, and the nutrient solution was renewed every 3 days. Samples were taken for analysis at the 20th day after treatment.

Growth analysis

Random plants of all treatments ($n = 5$) were separated into shoot and root fractions, and were placed in an oven at 70 °C for 72 h and used to determine dry weight.

Determination of root vigor

Root vigor was determined using the triphenyl tetrazolium chloride (TTC) method (Liu et al. 2008). The young root samples were washed with distilled water and blotted with tissue paper. Root samples of 0.5 g were fully immersed in the solution containing 5 mL of 0.4% TTC and 5 mL phosphate buffer (0.06 mol L^{-1}, pH 7.0) and incubated at 37 °C in the dark for 3 h before the addition of 2 mL of 1 mol L^{-1} sulfuric acid to stop the chemical reaction. The roots were removed, homogenized in a mortar filled with 4 mL of ethyl acetate. The red extract solution was transferred into a test tube, and ethyl acetate was added to 10 mL for spectrophotometric measurement at 485 nm. The absorbance was used to quantify the amount of tetrazolium which was reduced in the reaction with a standard curve, by which the root vigor was calculated.

Isolation of mitochondria

Mitochondria from fresh cotton root were isolated as described by Huang et al. (2014) with some modifications. Briefly, fresh cotton root samples (± 15 cm from root tip, approx. 5.0 g) were homogenized with a glass-homogenizer in 5 mL suspension buffer (1 mM EDTA, 0.4 M sucrose and 50 mM pH 7.4 Tris–HCl) and centrifuged at 1500 × *g* for 15 min at 4 °C to pellet the cellular debris. The supernatant was further centrifuged at 14,000 × *g* for 15 min at 4 °C in a new tube. The precipitate was then washed with the medium (0.4 M sucrose and 50 mM pH 7.4 Tris–HCl) by three times. The resulting pellets, containing intact mitochondria were resuspended in an appropriate volume suspension buffer. All the above operations were performed at 0–4 °C. To ensure the same number of mitochondria in the unit volume pellet, all the suspension process were operated carefully, and the mitochondrial extraction liquid were gently reversed and shaken before testing, then the unit volume of the mitochondrial extract were absorbed accurately. A total of 100 μL mitochondria supernatant was used to determine protein concentration according to the Bradford method (Bradford 1976).

Determination of generation rate of O_2^- and H_2O_2 content in mitochondria

The O_2^- generation rate was determined according to Panda et al. (2008) with slight modification. In brief, 0.1 mL of 10 mM hydroxylammonium chloride, 1.7 mL of 0.05 M phosphate buffer (pH 7.8) and 80 L of mitochondria supernatant were incubated at 25 °C for 20 min and then added to 1 mL of 7 mM α-naphthylamine sulfonic acid and 1 mL of 17 mM *p*-aminobenzene. The mixture was incubated at 25 °C for 20 min. The O_2^- generation rate was then determined by the absorbance at 530 nm. H_2O_2 content in isolated mitochondria was assayed using a modified non-enzymatic method of Panda et al. (2008). In brief, 20 μL mitochondrial suspensions, 100 μL titanium sulfate and 880 μL double-distilled water were incubated at 25 °C for 15 min, then the absorbance of filtered prior was detected at 410 nm against a blank which had been subjected to the same procedure but contained no plant material. Absorbance was converted into H_2O_2 concentrations using a standard curve made with the known concentrations of H_2O_2.

Determination of antioxidant enzyme activities in mitochondria

The nitro blue tetrazolium (NBT) method detailed by Tan et al. (2008) was used to assess the activity of SOD. One unit of SOD activity was defined as the volume of enzyme required to inhibit the rate of NBT reduction by 50% as observed. Total SOD activity was expressed as units mg·protein^{-1}. CAT activity was determined following the method of Liu et al. (2008) with slight modification. In brief, the reaction mixture contains 100 μL mitochondria supernatant and 2.9 mL of 50 mM phosphate buffer (pH 7.0), then the reaction was initiated by adding 1 mL of 50 μM H_2O_2, and stopped by adding 2 mL of 10% H_2SO_4, then the CAT activity was estimated using 10 mM potassium permanganate titration to pink. POD activity was determined by adopting the method of Tan et al. (2008), the rate of change of absorbance at 470 nm was recorded.

Determination of openness of the MPTP, value of cytochrome Cyt c/a, and mitochondrial membrane fluidity

The MPTP opening was determined according to De Marchi et al. (2004). Briefly, mitochondrial extraction solution was throughly shaken, and 0.5 mL solution was taken

for centrifugation at 10,000 × *g* for 10 min. The supernatant was removed, 3 mL of 5 mmol L^{-1} HEPES (pH 7.2, containing 220 mmol L^{-1} mannitol, 70 mmol L^{-1} sucrose, and 5 mmol L^{-1} succinic acid) was added, and incubated at 20 °C for 2 min. The absorbance at 540 nm was recorded using a spectrophotometer (UV2401, Shimadzu, Japan). The absorbance variation per minute caused by the mitochondria was used to represent the openness degree of MPTP.

The determination of Cyt c/a was essentially assayed as described by Tonshin et al. (2003). Briefly, the obtained mitochondria were suspended with 0.2% (W/V) BSA, and the mitochondrial protein concentration contained in the suspension liquid was adjusted to approximately 0.5 mg mL^{-1} in ice-base for 15 min. A spectrophotometer (UV-2401, Shimadzu, Japan) was used to determine the absorption value at 550 nm and 630 nm. The ratio of the absorption values between both wavelengths was calculated.

The determination of mitochondrial membrane fluidity followed the method of Yao et al. (2010), 2.85 mL of 0.3 mol L^{-1} mannitol, and 60 µL of 5 mmol L^{-1} 8-aniline-1-naphthalene sulfonic acid (ANS) were added to 0.3 mL mitochondrial extracting solution. After 1 min, the reaction was measured with a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan) at wavelengths of 480 nm emission and 400 nm excitation at 5 nm slit width. The fluorescence intensity caused by mitochondria in the root system per unit mass was used to represent membrane fluidity.

Determination of mitochondrial H^+-ATPase activity

The determination followed the method of Blumwald and Poole (1987) and was slightly modified. The reaction system (500 µL) contained 150 µL of 30 mmol L^{-1} Hepes–Tris (pH 8.0), 50 µL of 50 mmol L^{-1} KCl, 50 µL of 30 mmol L^{-1} $MgSO_4$, 50 µL of 0.1 mmol L^{-1} ammonium molybdate, 50 µL of 0.1 mmol L^{-1} Na_3VO_4, and 100 µL membrane protein. 50 µL of 3 mmol L^{-1} ATP-Tris (pH 8.0) was used to start the reaction. The suspension was incubated at 37 °C for 30 min, and 50 µL of 55% TCA was added to terminate the reaction. The suspension was incubated at 25 °C for 15 min, 2.5 mL inorganic phosphorus protective agent (containing 0.016 mol L^{-1} EDTA-Na_2, 4.0% ammonium molybdate, 0.001 mol L^{-1} PVP, 0.172 mol^{-1} hydroxylamine sulfate, and 0.0875 mol L^{-1} H_2SO_4) and 250 µL of 6.47 mmol L^{-1} NaOH were added. After 40 min, the OD value at 550 nm was determined, and the enzyme activity was represented as µmol Pi mg^{-1} protein h^{-1}.

Statistical analyses

Analysis of variance was performed using SPSS 11.0 (SPSS Software Inc., USA). The means were compared using the least significant difference (LSD) test at the $P \leq 0.05$ level. Figures were illustrated with Microsoft Excel 2010.

Results

Effects of different phenolic compounds on the dry weight of cotton

Figure 1 showed that the dry weights of shoots and roots gradually decreased with increasing phenolic concentrations, with a comparatively greater effect observed in the root. Compared to the control, the dry weights of the shoot and the root systems with treatments of PHBA, phloroglucinol, and ferulic acid at the concentration of 20.0 mmol L^{-1} decreased by 48.1%, 65.6%, 46.9%, and 67.3%, 57.2%, and 74.7%, respectively. At the same concentration, the dry weights of the shoot and the root with ferulic acid treatments were significantly lower than that of PHBA and phloroglucinol treatments, which indicated that ferulic acid inhibited cotton growth more than PHBA and phloroglucinol.

Effects of different phenolic compounds on root vigor of cotton

Root vigor is one of the key indicators for evaluating root function, with greater root vigor corresponding to stronger root physiological function (Liu et al. 2008). Root vigor gradually decreased with increasing phenolic compound concentrations (Fig. 2). At a concentration of 0.8 mmol L^{-1},

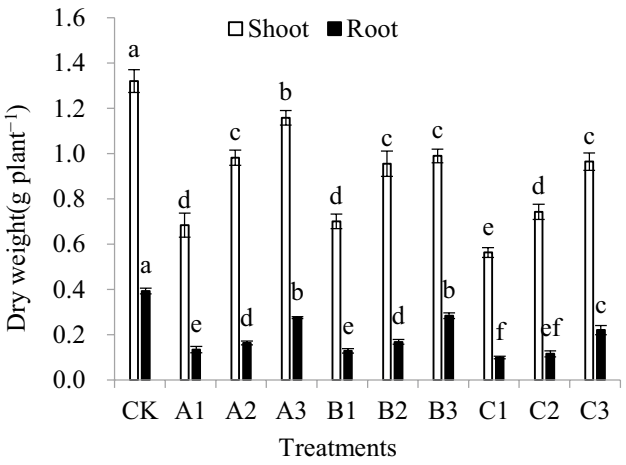


Fig. 1 Effects of different phenolic compounds on the dry weight of cotton shoots and roots. Plants grown under control conditions (CK); A1, A2 and A3 with PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 5$). Bars with different letters are significantly different at $P = 0.05$ probability level

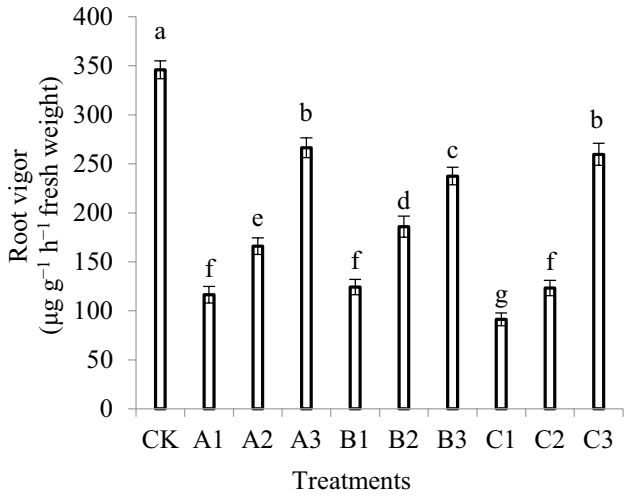


Fig. 2 Effects of different phenolic compounds on cotton root vigor. Plants grown under control conditions (CK); A1, A2 and A3 with the PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 3$). Bars with different letters are significantly different at $P = 0.05$ probability level

the root vigor with treatments of PHBA and ferulic acid was significantly higher than that of phloroglucinol. However, at concentrations of 4.0 and 20.0 mmol L^{-1}, the root vigor with ferulic acid treatment was lower than that of PHBA and phloroglucinol.

Effects of different phenolic compounds on generation of O_2^- and H_2O_2 content in cotton root mitochondria

O_2^- in the root system is mainly generated from the electron transport chain in mitochondria, while H_2O_2 is mainly generated by the disproportionation reaction of O_2^-. The increasing generation rate of O_2^- and H_2O_2 content indicate disturbed electron transfer in mitochondria. The generation rate of O_2^- and the H_2O_2 content in cotton root mitochondria gradually increased with increasing phenolic compounds concentration (Fig. 3). Ferulic acid treatment increased both the generation rate of O_2^- and the H_2O_2 content more than that of PHBA and phloroglucinol, which indicated that ferulic acid damage to electron transfer in root mitochondria is greater than that of PHBA and phloroglucinol.

Effects of different phenolic compounds on antioxidant enzyme activities in cotton root mitochondria

As shown in Fig. 4, with increasing phenolic compounds concentration, the SOD activity in cotton root

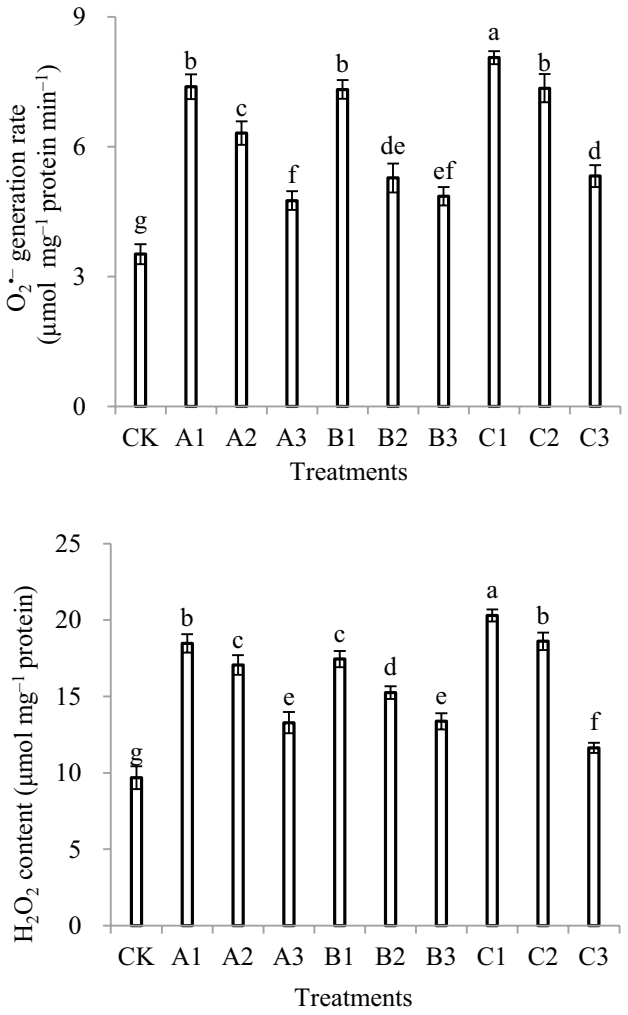


Fig. 3 Effects of different phenolic compounds on generation of O_2^- and H_2O_2 content in cotton root mitochondria. Plants grown under control conditions (CK); A1, A2 and A3 with the PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 3$). Bars with different letters are significantly different at $P = 0.05$ probability level

mitochondria decreased continuously. At the 0.8 mmol L^{-1} treatment level, the SOD activity with ferulic acid treatment was significantly higher than that with PHBA and phloroglucinol; however, at the 20.0 mmol L^{-1} treatment level, the result was opposite. For POD activity, 0.8 mmol L^{-1} PHBA increased POD activity compared to control, while other treatments decreased POD activity. With a treatment concentration of 20.0 mmol L^{-1}, POD activity under ferulic acid treatment was significantly lower than that of PHBA and phloroglucinol. Treatment with 0.8 mmol L^{-1} of phenolic compounds decreased CAT activity. With increasing treatment concentrations, the CAT activity steadily decreased in response to

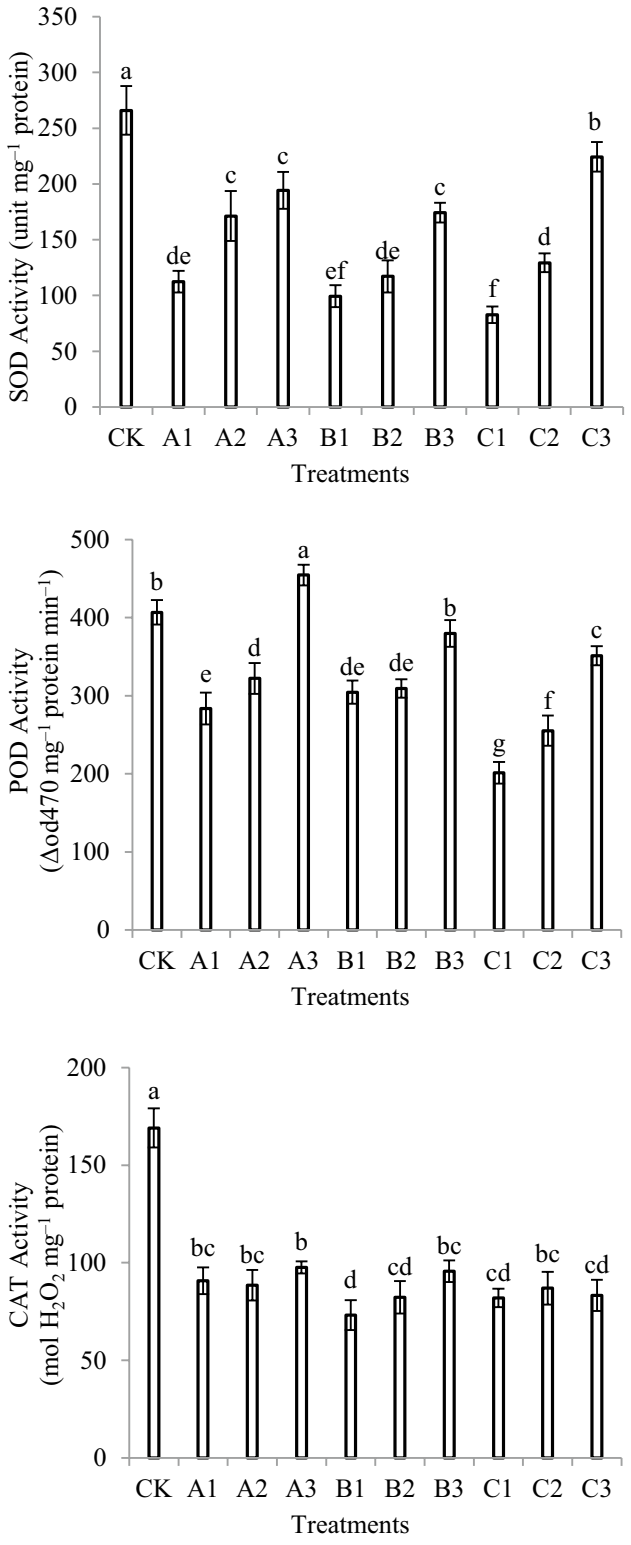


Fig. 4 Effects of different phenolic compounds on antioxidant enzyme activities in cotton root mitochondria. Plants grown under control conditions (CK); A1, A2 and A3 with the PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 3$). Bars with different letters are significantly different at $P = 0.05$ probability level

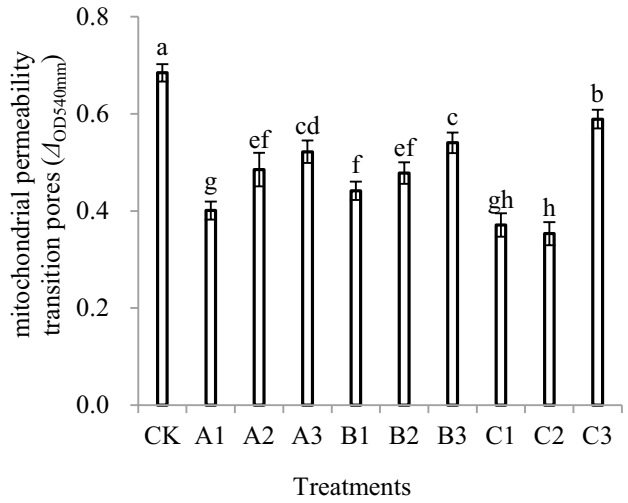
phloroglucinol; however, minor variations were observed with PHBA and ferulic acid treatments. With a treatment concentration of 20.0 mmol L^{-1}, CAT activity with PHBA was the highest, followed by ferulic acid, with the lowest observed with phloroglucinol.

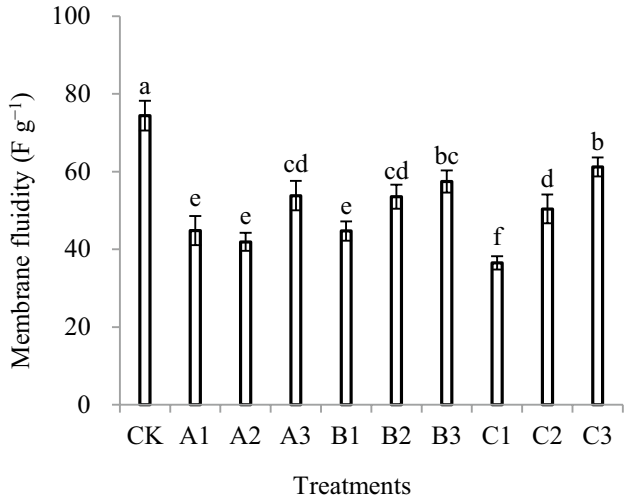
Effects of different phenolic compounds on MPTP, membrane fluidity, and value of Cyt c/a in cotton root mitochondria

The absorbance of mitochondria suspension liquid at 540 nm reflected the variation of MPTP; the lower the absorbance is, the higher the openness degree of MPTP. Excessive openness of MPTP destroys mitochondrial function, and resulted in cell injury or programmed cell death (PCD) (De Marchi et al. 2004). Figure 5 showed that the mitochondria membrane absorbance gradually decreased with increasing phenolic concentrations, which indicated that MPTP openness increased. With a treatment concentration of 0.8 mmol L^{-1}, ferulic acid treatment had a higher absorbance than that of PHBA and phloroglucinol, while with a treatment concentration of 4.0 and 20.0 mmol L^{-1}, the result was opposite.

Membrane fluidity is an important characteristic of the mitochondria, and a change in mitochondrial membrane fluidity will result in changes in mitochondrial structure and function (Muriel and Pérezrojas, 2015). Mitochondrial membrane fluidity decreased continually with increasing phenolic compound concentrations (Fig. 5). At the lowest level of treatment (0.8 mmol L^{-1}), the differences in mitochondrial membrane fluidity were small, while at the highest level (20.0 mmol L^{-1}) the mitochondrial membrane fluidity with ferulic acid treatment was significantly lower than that of PHBA and phloroglucinol.

Cytc and Cyta are the essential components of the electron transfer chain in mitochondrial inner membranes. Cytc is loosely attached to the surface of the inner mitochondrial membrane, while Cyta is tightly attached. As MPTP openness gradually increased, mitochondrial membrane is increasingly distorted and folded, then Cytc is released from the inner membrane. When MPTP is excessively open, Cytc may enter into the cytoplasm through the transition pores while Cyta is still attached to the inner mitochondrial membrane (Garofalo et al. 2015). Therefore, Cyt c/a was used to reflect the relative change of the Cytc value in mitochondria (Zhu et al. 2002). Figure 5 showed that, with a concentration of 0.8 mmol L^{-1}, all three phenolic compounds significantly decreased Cyt c/a. With increasing phenolic concentrations, the differences in the Cyt c/a value between phenolic compounds narrowed, and at a concentration of 20.0 mmol L^{-1}, there were no significant differences.





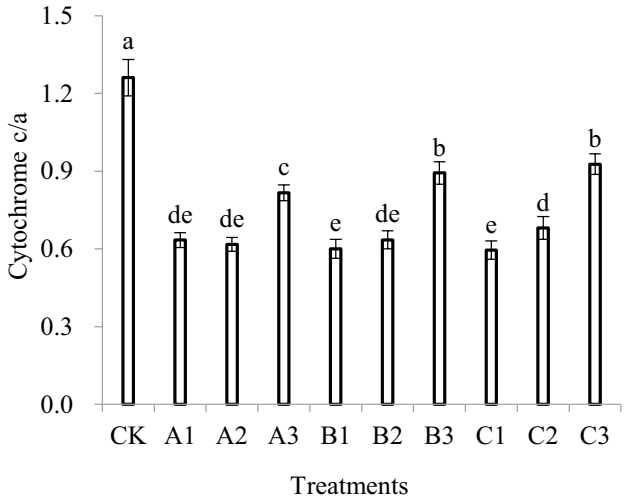


Fig.5 Effects of different phenolic compounds on MPTP, membrane fluidity, and value of Cyt c/a in cotton root mitochondria. Plants grown under control conditions (CK); A1, A2 and A3 with the PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 3$). Bars with different letters are significantly different at $P = 0.05$ probability level

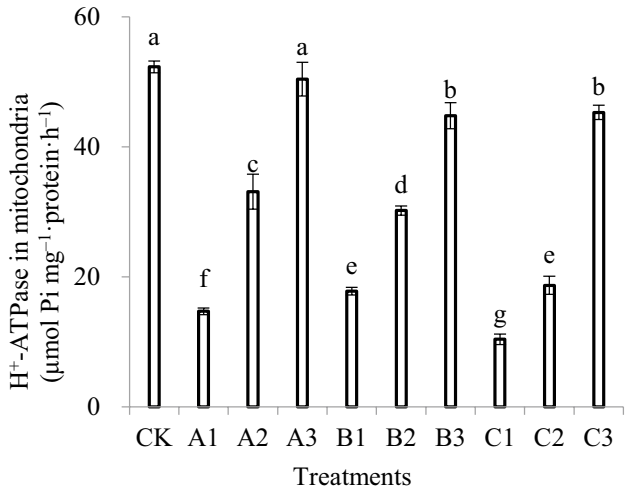


Fig. 6 Effects of different phenolic compounds on the mitochondrial H^+-ATPase activity in the cotton root. Plants grown under control conditions (CK); A1, A2 and A3 with the PHBA concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; B1, B2 and B3 with phloroglucinol concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}; C1, C2 and C3 with ferulic acid concentrations of 20.0, 4.0, and 0.80 mmol L^{-1}. Data are mean ± standard error ($n = 3$). Bars with different letters are significantly different at $P = 0.05$ probability level

Effects of different phenolic compounds on the mitochondrial H^+-ATPase activity in the cotton root

As shown in Fig. 6, 0.8 mmol L^{-1} PHBA had little effect on the H^+-ATPase activity; however, 0.8 mmol L^{-1} phloroglucinol and ferulic acid significantly decreased H^+-ATPase activity. With increasing concentrations, H^+-ATPase activity continuously decreased. Compared to control, 20.0 mmol L^{-1} PHBA, phloroglucinol, and ferulic acid treatments decreased H^+-ATPase activity by 71.8%, 66.1%, and 80.1%, respectively.

Discussion

Treatment with the three phenolic compounds reduced cotton seedling dry matter accumulation considerably, which conformed with previous reports that wheat straw returned to cotton fields inhibited cotton plant growth and decrease seed yield by releasing phenolic compounds into the soil (Liu et al. 2008). The studies of Gu et al. (2013) showed that the inhibitory effect of PHBA on root growth of rice seedlings was higher than that of ferulic acid, while the studies of Jung et al. (2001) showed that the inhibitory effect of ferulic acid on root growth of potato seedlings was higher than that of phloroglucinol. In this study, it was also indicated that ferulic acid had a much stronger negative effect on cotton root growth than that of PHBA and phloroglucinol at the

same concentration. In the field soil, content of ferulic acid and PHBA is higher than that of phloroglucinol (content of ferulic acid, PHBA and phloroglucinol in soil is about 1.32–5.3 mg kg^{-1}, 1.5–5.7 mg kg^{-1}, and 0.7–1.6 mg kg^{-1}) (Jiang et al. 2015), which confirmed that the inhibitory effect of ferulic acid and PHBA on cotton growth was higher than that of phloroglucinol. Since cotton roots directly contact the phenolic compounds, the inhibition of root growth was stronger than that of shoots (Iversen 2014).

The mitochondrion is the main organelle for cells to conduct metabolism and energy conversion, and its integrality plays an important role in the maintenance of the entire cell's structure and function. It is also the major organelle for the generation of oxygen radicals, and is also easily damaged by oxygen radicals (Li et al. 2013; Yamamoto et al. 2003). In this study, three different phenolic compounds induced increased H_2O_2 content and the generation rate of O_2^- in cotton roots, while activities of SOD, POD, and CAT showed a decreasing trend with phenolic compounds treatment. These results are in agreement with the report of Zeng et al. (2001), in which activities of SOD, POD, and CAT in roots of grape, cucumber, and sorghum were reduced by secalonic acid F (an allelochemical produced by *Aspergillus japonicus*) treatment. In this study, ferulic acid treatment induced more H_2O_2 content and a higher generation rate of O_2^-, and lower SOD, POD and CAT activity than that of PHBA and phloroglucinol, especially at concentrations of 4.0 and 20.0 mmol L^{-1}. The reason may be that with long-term high-concentration ferulic acid compounds treatment, ROS accelerates protein degradation, suppresses the synthesis of antioxidant enzymes, leads to enzyme inactivation, and finally reduces reactive oxygen scavenging activity, thus resulting in severe oxidative damage to the membrane system (Mandhania et al. 2006).

MPTP is a megachannel penetrating the mitochondria inner and outer membrane and is implicated as a mediator of cell injury and death (Rodriguez-Enriquez et al. 2004). Under normal physiological conditions, the transient and periodically opening of MPTP enables free passage into the mitochondria of ions and metabolites that < 1.5 kDa to maintain proper homeostasis. The over-opening or nonreversible-opening of MPTP leads to disturbed mitochondrial membrane function, cell injury and triggers programmed cell death. In this study, three kinds of phenolic compounds decreased mitochondria membrane absorbance, indicated that MPTP were opened and mitochondrial membrane function was disturbed. Additionally, the reduction of mitochondrial membrane fluidity indicated that unsaturated fatty acids in the membrane were oxidized, and the value of Cyt c/a indicated that Cytc was released from mitochondrial inner membranes into the cytoplasm. Cytc is loosely combined on the mitochondrial inner membrane, and ROS likely oxidized the sulfhydryl of MPTP-related proteins into a disulfide bond and promoted the opening of MPTP which allowed for the release of Cytc (Sun et al. 2015; Huang et al. 2014). In addition, as an electron transport carrier on the respiratory chain, the losses of mitochondrial Cytc lead to the blockage of electron transfer, thus generating more ROS, which in return results in further opening of MPTP. This was consistent with the reports of Ciniglia et al. (2015), in which mitochondrial function in maize roots was suppressed, and programmed cell death was induced by walnut green husk waste water containing allelochemicals.

Root vigor is the comprehensive reflection of the number of living cells and its metabolic intensity in the root (Cui et al. 2016). Under three kinds of phenolic compound treatments, cotton root vigor decreased, which was not only related to reduced root cell metabolism, but also to the decreased number of living cells. The reduction of living cell numbers equals a relative increase in the number of dead cells, which is consistent with the changes of Cyt c/a.

Mitochondrial H^+-ATPase is one type of inherent protein complex in the membrane system. It releases energy through ATP hydrolysis, conducts ion transport against a concentration gradient to stabilize ion concentrations inside and outside of cells, and maintains the regular physiological metabolism of living bodies (Liu et al. 2014). In this study, treatment with 4.0 and 20.0 mmol L^{-1} of phenolic compounds decreased the activity of H^+-ATPase in mitochondrial membranes, indicating that the internal environment of the mitochondria was disordered and the ion transport mechanism within the membrane was disturbed. This was consistent with changes of membrane fluidity, Cyt c/a and ROS, as well as the results published by Hejl and Koster (2004), in which 100 µmol sorgoleone [the main allelochemical in root exudates from grain sorghum (*Sorghum bicolor*)], impaired the processes of solute and water uptake of soybean seedlings and decreased plasma membrane H^+-ATPase activity of corn root.

In summary, the results indicated that after the three kinds of phenolic acid allelochemicals treatments, the internal environment of mitochondria in cotton seedling roots was disordered and the ion transport mechanism within the membrane was disturbed. This is because of the increased reactive oxygen content and the decreased antioxidant enzyme activities under allelochemical treatment, which could not remove the excessive ROS in time. ROS attacked the membrane system, started membrane lipid peroxidation, and increased mitochondrial membrane permeability. Finally, Cytc was released from the mitochondrial inner membrane and entered into the cytoplasm, Cyt c/a was decreased, and mitochondrial membrane fluidity and membrane potential were reduced, which resulted in damage to mitochondria and a declining H^+-ATPase activity on the membrane. Ultimately, the membrane ion transport mechanism was

disordered, affected the regular physiological metabolism in mitochondria, and resulted in the inhibition of plant growth.

In the field environment, straw returning inevitably increased phenolic acids level in soil, but straw returning still has the effect of promoting plant growth (Sui et al. 2015), which was not consistent with our study, the reason was that our study was carried out under hydroponic conditions, which was great different in the chemical composition of the soil, so the study in soil needed not only to consider the fertilizer effect of straw returning, but also to consider the effects of soil physical and chemical properties and soil microbial on the transformation and efficiency of allelochemicals. For example, some phenolic acids, such as ferulic can be readily metabolized by soil microorganisms and transformed into other smaller molecule phenolic acids, such as vanillic acid, PHBA and protocatechuic acid in soil and then affect the accumulation and magnitude of allelopathic interactions of phenolic acids. This study was only carried out in controlled laboratory experiments. Clearly, further work is needed to study the environmental behaviors of adsorption, desorption and retention of the identified phenolic compounds under real-field condition.

Author contribution statement Guowei Zhang conceived, designed and performed the experiments; analyzed the data and wrote the paper. Rruixian Liu conceived and designed the experiments. Changqin Yang analyzed the data. Wanchao Ni analyzed the data.

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