

Full Length Research Paper

Exploring the Impact of Antimicrobial Volatile Organic Compounds on the Antioxidative Pathways of *Guignardia citricarpa*

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Volatile organic compounds (VOCs) produced by the yeast *Saccharomyces cerevisiae* strain CR-1 are able to inhibit the development of the fungal phytopathogen *Guignardia citricarpa*, causal agent of the disease citrus black spot. Antimicrobial VOCs have potential applications to control plant pathogens; however, there is limited information on the action mechanisms. It is possible that VOCs trigger the accumulation of harmful levels of reactive oxygen species. Therefore, this work aimed to evaluate the effect of the artificial mixture of VOCs, constituted by alcohols and esters identified from *S. cerevisiae*, on the antioxidant system of *G. citricarpa*. The VOCs at 0.48 µl/ml of air space completely suppressed the mycelial growth and reduced the respiratory rate. Increased activity of the antioxidant enzymes superoxide dismutase and catalase in the first 24 to 48 h of exposure to the VOCs indicating intracellular accumulation $O_2^{\cdot -}$ and H_2O_2 , respectively was observed; however, the antioxidant glutathione pathway was not activated. In addition, increased lipid peroxidation was detected, confirming the oxidative stress process. Therefore, it was reported for the first time the disruption of the cellular redox homeostasis in microorganisms caused by VOCs. This knowledge is important to better understand the action mechanisms of antimicrobial volatiles and to develop safer fumigants to control postharvest diseases.

Key words: Antifungal, citrus, fumigation, stress response.

INTRODUCTION

The citrus black spot caused by the ascomycete fungus *Guignardia citricarpa* (Anamorph: *Phyllosticta citricarpa*)

is one of the most important diseases of citrus in producing areas of Africa, Asia, Australia and South

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America. Several fruit symptoms are associated to the disease and infected fruits can develop them at postharvest during transport or storage. Although the disease does not cause internal decay, fruits become aesthetically damaged. This scenario limits the exportation because citrus black spot is an A1 quarentenary disease in the European Community and the United States of America (OEPP/EPPO, 2009).

The chemical control is the main tool to control plant diseases; however, the use of carbendazim, one of the few effective fungicides to combat the citrus black spot, was banned (U.S. EPA, 2012). The impact of the chemical control on human health and environment increased the interest for alternative control methods. The use of microbial antagonists may interrupt some stage of the pathogen's life cycle by several mechanisms such as parasitism, competition for nutrients and colonization niches, and production of hydrolytic enzymes and antibiotic compounds (Sharma et al., 2009), including volatiles (Strobel, 2011).

Microbial volatile organic compounds (VOCs) are considered ideal info chemicals as their sphere of activity extends from proximal distances due to aqueous diffusion, to greater distances via aerial diffusion, affecting negatively the physiology of competitor microorganisms. Therefore, it is possible that volatile metabolites have played an important role during the microbial evolution in the context of community, population and functional dynamics (Wheatley, 2002). Potential applications of the fumigation employing safe natural VOCs include the control of pathogens in fruits, grains and seeds in closed chambers during transport and storage, and to replace the methyl bromide and others harmful fumigants to control soil-borne pathogens (Strobel, 2011). The use of VOCs is advantageous as the residual effect is limited and minimizes the product handling (Mercier and Jimenez, 2004). In addition, fungistatic VOCs can act as chemosensitizing agents by increasing the pathogen susceptibility to lower dose of fungicides; thus, reducing costs and risks of negative side effects such as acquisition of fungicide resistance and environmental impact (Kim et al., 2012).

The action mechanisms of antimicrobial VOCs are little known. There is evidence that VOCs cause DNA damage (Mitchell et al., 2010) and interfere with morphogenesis-related enzymes (Wheatley, 2002; Fialho et al., 2011). It is possible that VOCs trigger the accumulation of harmful levels of reactive oxygen species (ROS) resulting in oxidative stress. The ROS include free radicals such as superoxide anion ($O_2^{\bullet-}$), and non-radical reactive species such as hydrogen peroxide (H_2O_2). These potent oxidizing agents react specifically and rapidly with macromolecules, resulting in DNA mutations, protein oxidations and lipid peroxidation (Aguirre et al., 2005; Heller and Tudzynski, 2011).

The ROS are continuously produced during the aerobic metabolism but are maintained at basal and harmless

levels by antioxidant mechanisms. Superoxide dismutase (SOD, EC 1.15.1.1) is considered the first line of defense by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2 . Subsequently, catalase (CAT, EC 1.11.1.6) detoxifies the H_2O_2 producing water and molecular oxygen (Aguirre et al., 2005; Heller and Tudzynski, 2011). The thioltripeptide glutathione (GSH) is also involved in the ROS scavenging. In this antioxidant system, GSH is oxidized by H_2O_2 to water in the presence of glutathione peroxidases (GPx, EC 1.11.1.9), forming oxidized glutathione (GSSG). The NADPH-dependent glutathione reductase (GR, EC 1.6.4.2) reduces GSSG regenerating GSH (Pocsi et al., 2004).

Physical and chemical stressors such as UV radiation, extreme temperatures, desiccation, heavy metals and exogenous H_2O_2 can increase the intracellular levels of ROS and overcome the antioxidant mechanisms, resulting therefore in oxidative stress (Angelova et al., 2005). However, the relationship between exposure to VOCs and oxidative stress in microorganisms has not yet been studied. This knowledge is important to better understand the microbial interactions mediated by volatile metabolites, and to develop new strategies to control the citrus black spot and other postharvest diseases. Formulations based on microbial VOCs could be employed to fumigate fruits infected but asymptomatic. This procedure could avoid the development of symptoms during the transport or at the final destination.

The yeast *Saccharomyces cerevisiae* strain CR-1, isolated from fermentative processes for fuel ethanol production, exhibited fungistatic effect on *G. citricarpa*. The antagonism was attributed to production of a mixture of volatile alcohols and esters (Fialho et al., 2010). Therefore, this study aimed to investigate antioxidative response of *G. citricarpa* exposed to the artificial mixture of VOCs, originally identified from the yeast *S. cerevisiae*.

MATERIALS AND METHODS

Phytopathogenic fungus

Guignardia citricarpa was isolated from orange fruit lesions and maintained in Potato-Dextrose-Agar (PDA) (Difcolaboratories, Detroit, MI, USA) at 26°C. The fungus is deposited as isolate IP-92 in the mycological culture collection of the Laboratory of Plant Pathology in the Department of Crop Protection at FCAV/UNESP in Jaboticabal, SP, Brazil. The origin and DNA sequence of the fungus was reported by Wickert et al. (2012).

Antifungal activity

From previous information obtained by gas chromatography coupled to mass spectrometric detection (GC-MS) analysis of the gaseous atmosphere was produced by *Saccharomyces cerevisiae* strain CR-1 (Fialho et al., 2010). It produced an artificial mixture of VOCs using authentic standard chemicals (Sigma/Aldrich, St. Louis, MO, USA). The mixture contained the six compounds positively identified, and the proportion of each compound was calculated in relation to all other components of the mixture (Table 1).

Table 1. VOCs produced by *Saccharomyces cerevisiae* strain CR-1 on PDA medium (Fialho et al., 2010).

Compound	% Relative (v/v)
Ethanol	85.3
Unidentified	1.5
Ethyl acetate	1.8
3-methyl-1-butanol	6.9
2-methyl-1-butanol	2.4
Phenylethyl alcohol	0.7
Ethyl octanoate	1.4

As previously described by Fialho et al. (2011), it was employed for the bioassays of a two section-divided polystyrene plate (BD Falcon, Franklin Lakes, NJ, USA). On one side of the plate, containing 10 ml of PDA, a 5 x 5 cm sterile semi-permeable membrane was placed. A pathogen mycelium plug (5 mm) was added onto the membrane, and the plate was incubated at 26°C. After five days of growth, on the opposite side of the plate, 24 µl (0.48 µl/ml of air space) of the artificial mixture of VOCs were added on a sterile cotton wool. The plates were sealed with parafilm and incubated at 26°C. The free headspace of the plate was 50 ml and this was used to calculate the concentration of VOCs. The control consisted of plates containing the pathogen in the absence of the artificial mixture.

After 24, 48 and 72 h of exposure to the VOCs the mycelia were harvested from the membranes, weighed and stored at -80°C. The radial mycelial growth was also evaluated daily. Three replicates were used for each treatment.

Determination of the respiratory rate

Five days old cultures of *G. citricarpa* were exposed to the artificial mixture of VOCs at 0.48 µl/ml of air space, as described above. After 72 h of exposure, atmosphere samples were collected with a gas-tight syringe through a rubber septum adapted to the plates and injected into a gas chromatographer Trace 2000 (Thermo Fisher Scientific, Austin, TX, USA), equipped with flame ionization detector (FID). The CO₂ concentration was expressed in ml CO₂/g biomass per day of growth. Five replicates were used for each treatment.

Protein extraction

Antioxidant enzymes were extracted as described by Monteiro et al. (2011) with minor modifications. The frozen mycelia were grounded in a mortar with liquid nitrogen and added to 100 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L EDTA and 3 mmol/L dithiothreitol (DTT) in the proportion of 5 ml/g biomass. The homogenates were centrifuged at 15,000g for 20 min at 4°C and the supernatants were collected and kept at -80°C prior the enzyme analysis. The protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard.

Enzymatic assays

All the enzymatic activities were performed as previously described by Monteiro et al. (2011) with modifications. For superoxide dismutase (SOD) activity non-denaturing PAGE was carried out in gels containing 10% polyacrylamide with a 4% polyacrylamide

stacking gel (Laemmli, 1970). Equivalent to 20 µg total protein were loaded and the electrophoresis performed at constant current of 20 mA for 4 h at 4°C. The gels were washed with distilled water and incubated in the dark for 30 min in 100 mmol/L sodium phosphate buffer (pH 7.8), containing 1 mmol/L EDTA, 0.05 mmol/L riboflavin, 0.1 mmol/L nitrobluetetrazolium (NBT) and 0.3% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED). The gels were washed in distilled water and exposed to fluorescent light until the development of colourless bands in a purple-stained gel and fixed with 7% (v/v) acetic acid. Bovine liver SOD (2 units) was used as standard. The densitometric analysis was performed using ImageJ 1.47v analysis software (National Institute of Health, Bethesda, MD, USA).

The catalase (CAT) activity was determined by using a reaction mixture containing 1.450 ml 0.036% (v/v) H₂O₂ in 100 mmol/L sodium phosphate buffer (pH 6.8). After addition of 0.05 ml of protein extract the H₂O₂ decomposition was monitored at 240 nm for 1 min at 25°C. The specific enzyme activity was expressed in U/mg protein, where one unit (U) corresponds to the decomposition of 1 µmol of H₂O₂ per min under the assay conditions.

The glutathione reductase (GR) activity was determined by using a reaction mixture consisting of 1 ml 100 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), 1 mmol/L oxidized glutathione (GSSG) and 0.1 mmol/L NADPH. After addition of 0.05 ml of protein extract the GSSG reduction was monitored at 412 nm for 1 min at 30°C. The specific enzyme activity was expressed in U/mg protein, where one unit (U) corresponded to 1 µmol of reduced glutathione (GSH) per min under the assay conditions.

Determination of the lipid peroxidation

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid (TBA) reactive substances, estimated as malondialdehyde (MDA) equivalents (Heath and Packer, 1968). Mycelial material (500 mg) was homogenized in 5 ml 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000g for 20 min. The supernatant (0.5 ml) was added to 1 ml 0.5% (w/v) TBA in 20% (w/v) TCA, heated in boiling water bath for 30 min, and cooled in ice. After centrifugation at 10,000 g for 5 min, the absorbance of the supernatant was determined at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex was calculated using the extinction coefficient 155 mM⁻¹ cm⁻¹ and expressed as mmol/L of MDA/g biomass.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using Tukey's test at $P < 0.01$ to examine significant differences between treatments. All results were expressed as means ± standard deviation (SD).

RESULTS

The synthetic mixture of VOCs was prepared and its effect on the mycelial growth of *G. citricarpa* was evaluated (Figure 1). The fungal development was paralyzed after the first 24 h of exposure and at the end of 72 h the mycelial growth was approximately 30% lower when compared to the control. In addition, the respiratory rate of *G. citricarpa* decreased 54% (Figure 2). However, the fungus resumed normal development when transferred to new medium in the absence of the VOCs.

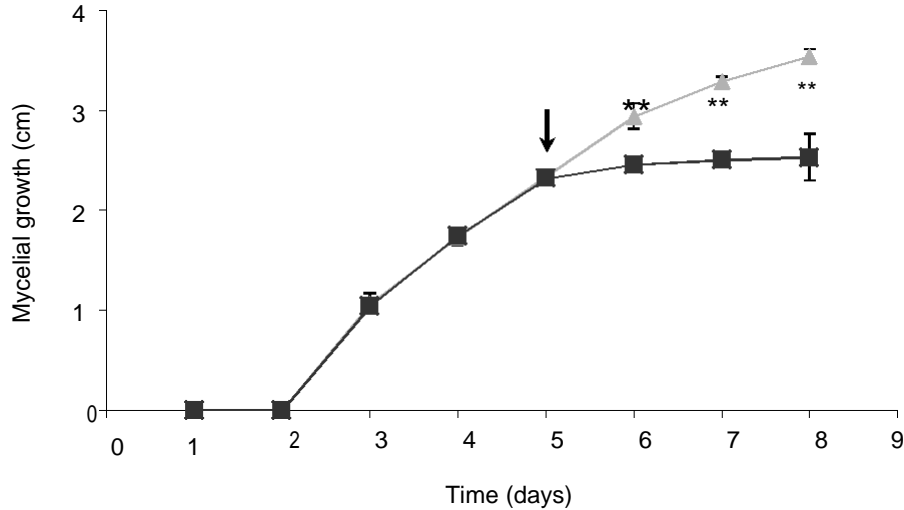


Figure 1. Effect of the artificial mixture of VOCs on mycelial growth of *Guignardia citricarpa*. The arrow indicates the addition of the VOCs. Values are means of three replicates (\pm SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (\blacktriangle) and Volatiles (\blacksquare).

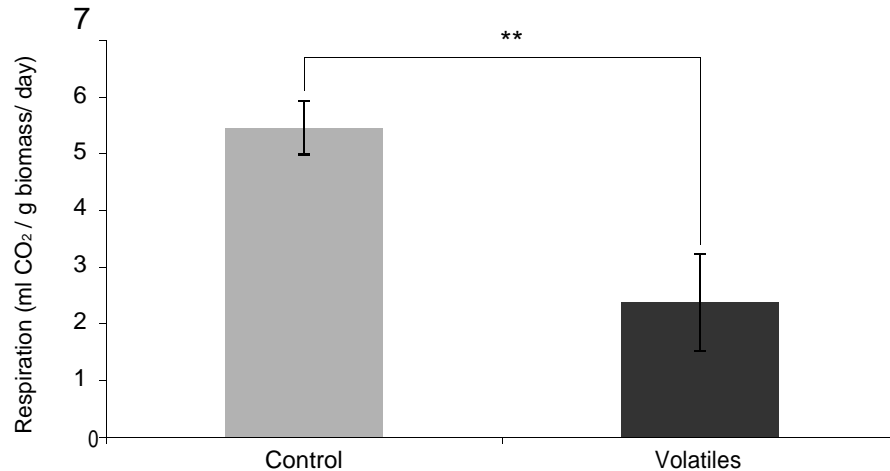


Figure 2. Effect of the artificial mixture of VOCs on respiratory rate of *Guignardia citricarpa* after 72 h of exposure. Values are means of five replicates (\pm SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (\square) and Volatiles (\blacksquare).

In order to study the antioxidative response of *G. citricarpa* to the antimicrobial VOCs, the activity of three key antioxidant enzymes (SOD, CAT and GR) were evaluated. As observed in the Figure 3, three SOD isoforms (I, II and III) were detected. The SOD isoform I did not show significant change, however, the activity of the SOD isoform II increased 37 and 110% after 24 and 48 h of exposure to the VOCs, respectively, while the activity of the SOD isoform III increased, 17 and 90%, respectively, in the same period. After 72 h of exposure both SOD isoforms were not significantly affected when

compared to the controls. Furthermore, no other change, including appearance or disappearance of isoforms was observed.

Under normal physiological conditions, *G. citricarpa* exhibited gradual increase in CAT activity during the culture ageing. The exposure to the VOCs increased the CAT activity by 32% in the first 24 h; however, after 72 h the activity was reduced by 24% (Figure 4A). In the present study, despite the effects observed on the SOD and CAT activities, GR activity was not affected by the VOCs (Figure 4B). Differently from CAT, under normal

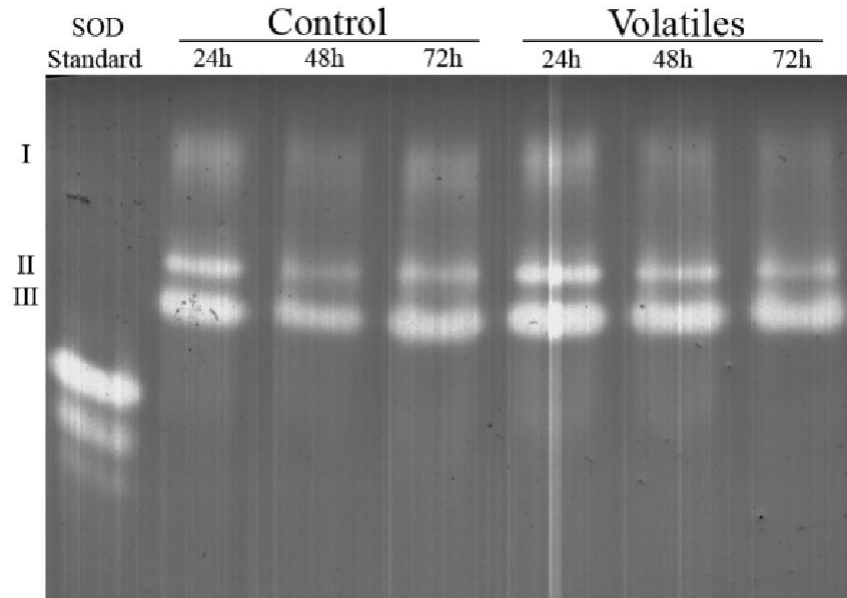


Figure 3. Effect of the artificial mixture of VOCs on superoxide dismutase activity of *Guignardia citricarpa* after 24, 48 and 72 h of exposure, determined by non-reducing SDS-PAGE. The numbers I, II and III indicates the SOD isoforms detected. Bovine liver SOD (2 units) was used as standard.

conditions the GR activity remained stable during the fungal development.

The MDA content in *G. citricarpa* was quantified aiming to evaluate the lipid peroxidation triggered by the VOCs (Figure 4C). The lipid peroxidation increased approximately 3 and 1.5 times after 24 and 48 h of exposure to the VOCs, respectively, however, after 72 h the lipid peroxidation was reduced to normal levels.

DISCUSSION

The mycelial growth of *G. citricarpa* stopped when the artificial mixture was added to the plates, mimicking the fungistatic effect of the *S. cerevisiae* atmosphere on the phytopathogen as previously reported by Fialho et al. (2010, 2011). In addition, reduction in the respiratory rate was observed, probably as a result of the low metabolism. Reports on the influence of VOCs on the microbial respiration are limited and the results available have been shown to vary. Some volatiles such as acetaldehyde can increase the respiration of soil microorganisms (Owens et al., 1969), while others did not show any effect (Ko and Chow, 1977). Humphris et al. (2001) evaluated the respiratory rate of wood degrading basidiomycetes exposed to antifungal VOCs identified from *Trichoderma* spp. The compound 2-methyl-1-butanol inhibited between 77 and 100% the respiration of all fungi at 2500 µg/ml, however, stimulated the respiration of *Postia placenta* at low concentrations (2.5 and 24 µg/ml). The VOCs octanal and hexanal inhibited

the respiration of the fungi while acetone produced a stimulating effect on *Trametes versicolor*.

The early response of SOD and CAT in *G. citricarpa* indicates that the VOCs triggered the formation of $O_2^{\bullet-}$ and H_2O_2 , respectively. The exposure of bacteria, yeasts and filamentous fungi to the herbicide paraquat, which generates specifically $O_2^{\bullet-}$, increases the synthesis of SOD, but not CAT (Bussink and Oliver, 2001; Moradas-Ferreira et al., 1996; Amo et al., 2002). Angelova et al. (2005) studying 12 species of filamentous fungi reported that the SOD activity in the fungi was increased by 2 times when exposed to paraquat while the CAT activity was increased by 2.5 times when exposed to H_2O_2 .

As expected, the results demonstrated that *G. citricarpa* contains antioxidant enzymes even under normal physiological conditions as ROS are generated during the respiratory metabolism. In addition, antioxidant enzymes are important for phytopathogens not only to neutralize their own ROS, but also to counteract the oxidative burst generated by their host cells during the initial phase of the infection process. The ROS produced by plants may have direct antimicrobial activity and/or may signal the activation of latent defense mechanisms. Therefore, antioxidant enzymes are considered an important virulence factor for fungal phytopathogens (Rolke et al., 2004; Heller and Tudzynski, 2011; Veluchamy et al., 2012). The cytoplasmic Cu/Zn-SODs, encoded by the genes *bcsod1* and *sssod1* in the plant pathogens *Botrytis cinerea* and *S. sclerotiorum*, respectively, were implicated in the $O_2^{\bullet-}$ detoxification and in host defense suppression (Rolke et al., 2004; Veluchamy

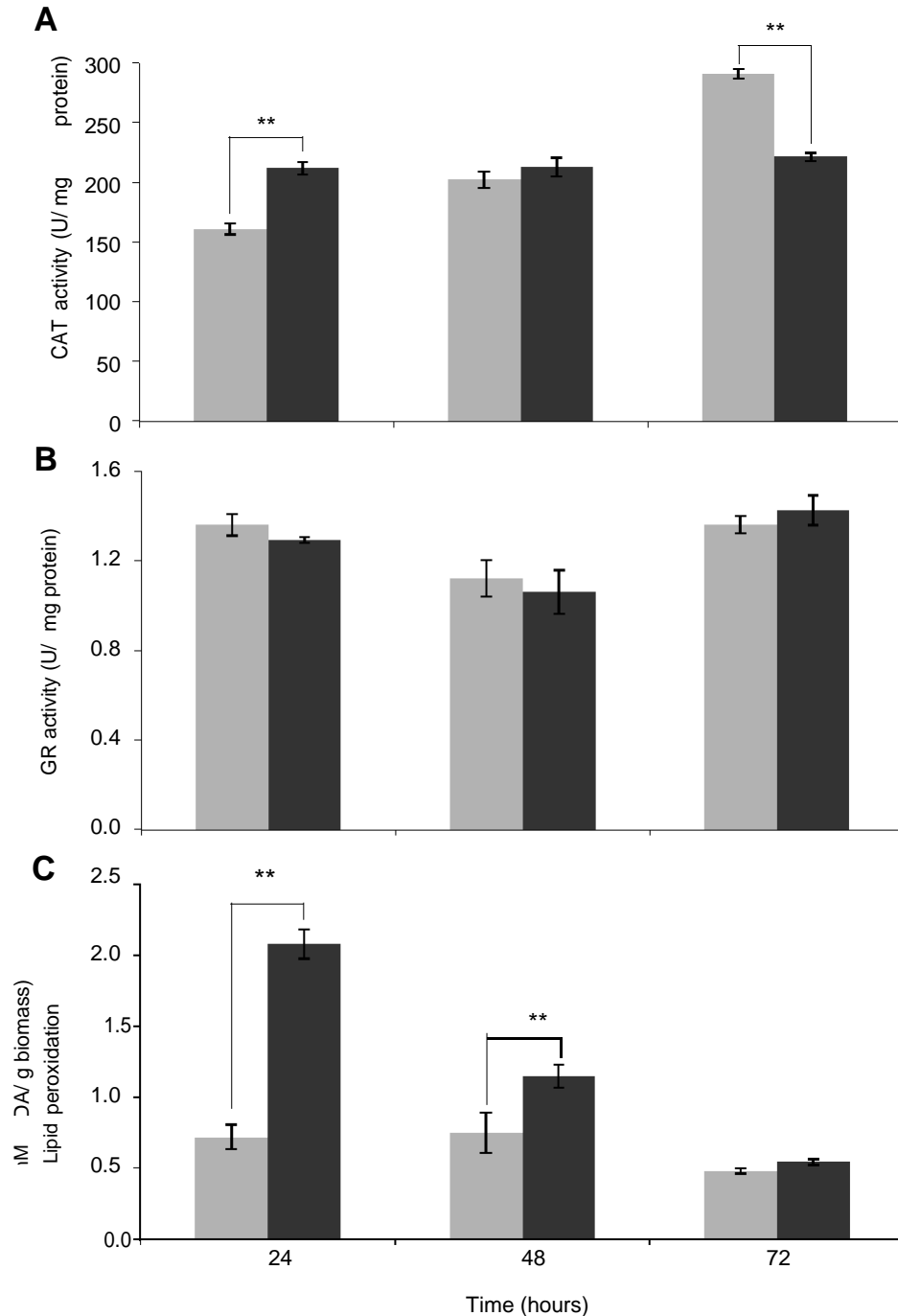


Figure 4. Effect of the artificial mixture of VOCs on catalase (A) and glutathione reductase (B) activities, and lipid peroxidation (C) of *Guignardia citricarpa* after 24, 48 and 72 h of exposure. Values are means of three replicates (\pm SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (■) and Volatiles (■).

et al., 2012). It is possible that SOD could also be a virulence factor in *G. citricarpa*.

The gradual increase in CAT levels during the culture ageing of *G. citricarpa* in normal conditions may constitute an adaptive response to the enhanced

generation of H_2O_2 in entering into the stationary phase, as also observed in *S. cerevisiae* (Jakubowski et al., 2000). In *A. nidulans* the genes *catA* and *catB* are expressed during processes of spore formation and in vegetative mycelium, respectively. Both genes provide

protection at different stages of development against exogenous H₂O₂ and other stress conditions. Homologues of the genes *catA* and *catB* have been found in other *Aspergillus* species, and in *Neurospora crassa* (Li et al., 2009; Hansberg et al., 2012). Malolepsza (2004) demonstrated that CAT is an important virulence factor in *B. cinerea* infecting tomato plants.

The antioxidant glutathione pathway in *G. citricarpa* was not activated by the VOCs. Therefore, it is proposed that SOD and CAT may play more important roles in the detoxification of ROS triggered by VOCs. Moreover, an antioxidant system other than glutathione could have been employed by *G. citricarpa* exposed to the VOCs. A complex system consisting of thioredoxins, which operates in parallel to the glutathione system, can also be important to maintaining the GSH redox state by regenerating the GSH from GSSG (Tan et al., 2010). Studies related to the glutathione pathway in filamentous fungi are scarce. In *A. niger* and *Penicillium chrysogenum* CAT played a more important role in H₂O₂ detoxification than enzymes belonging to the glutathione pathway (Emri et al., 1999; Li et al., 2008). More recently, Bento et al. (2013) reported increased GR activity in the basidiomycete *Pycnoporus sanguineus* growing in PDA medium supplemented with antifungal plant extracts of two species of *Casearia*, however, this effect was less evident in *Trametes villosa*.

The VOCs increased the lipid peroxidation in *G. citricarpa* mainly in the first 24 h of exposure. Some of the consequences of the lipid peroxidation are decreased membrane fluidity, increased permeability to H⁺ and other ions, and eventual cellular rupture. In addition, cytotoxic by-products, mainly MDA, are formed during the process, which has effects on DNA and proteins away from the area of their generation (Li et al., 2009). The lipid peroxidation is one of the most important parameters of oxidative stress, however, studies reporting lipid peroxidation in fungi exposed to antifungal compounds are limited, and the effect of VOCs on this oxidative stress biomarker had not been reported until now.

Although the mycelial development of *G. citricarpa* had been suppressed by the VOCs, the fungus was not killed (fungistatic effect). In part, the non-lethality may be due to the early response of SOD and CAT, enhancing the fungus ability to avoid continued exposure to high levels of ROS and associated deleterious intracellular events. This observation, associated to the decreased lipid peroxidation after prolonged exposure to the VOCs suggest that *G. citricarpa* was able to counteract at least in part the oxidative stress established by the VOCs. In addition to the initial unbalanced redox state, other inhibitory mechanisms may be involved. It was reported previously in *G. citricarpa* negative effect of the VOCs on the morphogenesis-related enzymes laccase, tyrosinase, β -1,3-glucanase and chitinase (Fialho et al., 2011).

Oxidative stress caused by several chemical and physical factors has been extensively studied in proka-

ryotes, animal and plant cells and yeasts (Lushchak, 2011). However, information about filamentous fungi, especially phytopathogens, is scarce and fragmented. Studies have shown that the activity of antimycotics such as amphotericin B and itraconazole are linked to oxidative stress (Kim et al., 2012); however, this is the first study reporting the relationship between exposure to VOCs and oxidative stress in microorganisms.

Conclusion

The results demonstrate that oxidative stress in microorganisms can be triggered not only by physical stress and water-diffusible substances, as already extensively reported, but also by VOCs. Information on the action mechanisms is important to better understand the microbial interactions mediated by volatile metabolites in nature, and to develop safer fumigants to control the citrus black spot caused by *G. citricarpa* and other postharvest diseases.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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