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RESEARCH PAPERS

Antioxidant response in *Chenopodium album* elicited by *Ascochyta caulina* mycoherbicide phytotoxins

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Summary. Antioxidant defence responses were evaluated in *Chenopodium album* plants treated with a mixture of the phytotoxins ascaulitoxin, 2,4,7-triamino-5-hydroxyoctandioic acid (ascaulitoxin aglycone) and *trans*-4-aminoproline, produced by the pathogenic fungus *Ascochyta caulina*, previously proposed as mycoherbicide for this noxious weed. The enzymatic and non-enzymatic effects of these phytotoxins on the ascorbate system and on catalase activity were assessed by evaluating their biological and specific activities through spectrophotometric and electrophoretic analyses. In addition, the oxidative status was monitored through evaluating H_2O_2 content during the time-course. The mixture of toxins induced high levels of H_2O_2 accumulation resulting in an oxidative burst in the plant cells. Ascorbate peroxidase and catalase had crucial roles in detoxifying H_2O_2. The persisting metabolic perturbations, however, led to severe necrosis and death of *C. album* plants. The induced H_2O_2 production may be generated by the fungus as part of its necrotrophic nature. This study explains the defence responses in *C. album* to the mycoherbicide, in particular, the ascorbate systems' components and H_2O_2 as an index of oxidative stress.

Key words: Chenopodiaceae, mycoherbicide, weed biology, oxidative stress, defence response.

Introduction

Chenopodium album L. (common name lambsquarters or fat-hen) is one of the most successful colonizing weeds, growing in most arable cropping systems and many soil types over a wide range of pH values (Holm *et al.*, 1977). It is a troublesome weed in crops of sugar beet, potato, maize, cereals, and vegetables throughout the world (Holm *et al.*, 1977), and is considered one of the ten worst weeds in Europe. It is currently controlled with herbicides in most crops, but in maize and some vegetables it is relatively tolerant or resistant to many herbicides (Heap, 2016).

Ascochyta caulina (P. Karst.) Aa & Kesteren is the fungal causal agent of a disease of *C. album*. This pathogen has been studied and proposed as a possible agent for the biological control of *C. album* (Kempenaar *et al.*, 1996; Vurro *et al.*, 2001), as the post emergence application of fungal pycnidiospores to young plants caused rapid appearance of necrotic spots. Depending on the amount of necrosis developed, this disease retarded growth or resulted in plant death (Kempenaar *et al.*, 1996). In previous studies, three main low-molecular-weight hydrophilic phytotoxins from liquid culture filtrates of the fungus, namely ascaulitoxin, ascaulitoxin aglicone (AscA) and *trans*-4-amino proline, were isolated and chemically and biologically characterized (Evidente *et al.*, 1998, 2000, 2001). Their use in mixture as a natural and environmentally friendly herbicide with broad spectrum of action was proposed, based on a series of relevant biological and technological properties (i.e., small molecular size, broad-spectrum phytotoxicity to different plant species, lack of antibiotic and zootoxic activities, and full solubility in water) (Vurro *et al.*, 2012).

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Despite the interesting applicative and biotechnological potential, however, very little is known about the mechanism of action of this powerful herbicide mixture. Recently, the mode of action of AscA was studied on amino acid metabolism in *Lemna paucicostata* (Duke *et al.*, 2011). In this system, the toxin proved to be a potent growth inhibitor, having an I_{50} of less than 1 μM, and an almost complete inhibitory effect at about 3 μM. Its action is slow, starting with growth inhibition, followed by a darkening of the fronds, and then chlorosis and death. These authors hypothesized a unique mechanism of action related to amino acid transport or amino acid metabolism, based on their findings.

Fungal toxins have important roles in pathogenesis and induce oxidative stress in some plant-fungal pathogen interactions (Paciolla *et al.*, 2014). These are often characterized by the presence of reactive oxygen species (ROS) that can act as mediators of the induction of stress tolerance and especially as cytotoxic compounds. Hydrogen peroxide (H_2O_2) is considered a ROS having a dual role in plants: at low concentrations, it acts as a secondary messenger involved in the signalling pathways in various biotic and abiotic stress responses, whereas it triggers cell death at high concentrations (Quan *et al.*, 2008). ROS accumulation induced by oxidative stress results in reinforcing the host defence systems. The ascorbate system, reduced glutathione, α-tocopherol, carotenoids, flavonoids, and enzyme scavengers such as superoxide dismutase (SOD), glutathione peroxidases (GPX), catalase (CAT), and generic peroxidases (POD), are involved and can have important roles (Mittler *et al.*, 2004). The ascorbate system, which is found in chloroplasts and cytosol, involves the oxidation and re-reduction of ascorbate (ASC) through the action of the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Apel and Hirt, 2004). APX removes the hydrogen peroxide utilizing ascorbic acid as electron donor and gives monodehydroascorbate (MDHA). When MDHA is not reduced to ascorbate by MDHAR or by ferredoxin, then dehydroascorbate (DHA) is produced. The DHAR then reconverts DHA to ASC. There is little knowledge of the effects of the simultaneous treatment of multiple phytotoxins in plants, whereas effects of single toxins having phytotoxic activity have been reported (Siriwardana and Lafont, 1978; Chang and Xue, 1990; Sagakuchi *et al.*, 2000; Paciolla *et al.*, 2008). There may be synergistic or antagonistic effects between different chemical signals in triggering syntheses of plant defence components (Vidhyasekaran, 2008).

Our aim was to investigate how a mixture containing the three main toxins produced by *A. caulina* affects the defence responses in the host plant. Particularly, the enzymatic and non-enzymatic components of the ascorbate system (namely ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, ascorbate, dehydroascorbate) were studied, together with catalase. H_2O_2 was monitored as an index of oxidative stress. This study aimed to improve the understanding of the molecular mechanisms responsible for the phytotoxicity of the compounds, and for the efficient biocontrol action of this mycoherbicide.

Materials and methods

Production of toxins

A strain of the fungus *Ascochyta caulina* (ITEM 1058) stored in the Culture Collection of the Institute of Sciences of Food Production (CNR, Bari, Italy) was grown in liquid culture. The culture filtrates obtained were purified as described by Avolio *et al.* (2011), giving the mixture of metabolites containing almost exclusively the three toxins, ascaulitoxin, ascaulitoxin aglycone and *trans*-4-amino-D-proline, at amounts, respectively, of 80, 240, and 30 mg L^{-1} of culture filtrate (Evidente *et al.*, 2001). Briefly, the lyophilized material was dissolved in 1 M of formic acid and then loaded on a Dowex-50 resin, previously packed in a chromatography column. The column was washed with distilled H_2O and then eluted with 1 M NH_4OH. The basic eluate was fluxed by a N_2 stream and then lyophilized (Avolio *et al.*, 2011). The toxin mixture appeared as a yellow-brownish fully water-soluble powder, which was dissolved in distilled water at the concentration of 1 mg mL^{-1} for further use in the experiments. A silicone-based surfactant (Sylgard, Dow Corning Europe) at 0.05% was added to the solution to improve its adhesion on leaf surfaces.

Plant material and treatments

Chenopodium album seeds were sown in pots. After seedling emergence, they were thinned to a sin-

gle plant per pot, and grown in a greenhouse to the fourth leaf stage at 20–25°C, under natural light conditions with photoperiod of 8–10 h. The toxin solution was applied with a hand sprayer and 1 mL (containing 1 mg of toxin mixture) was sprayed on each plant. At intervals of 24, 48 and 72 h, treated and control (untreated) leaves were collected and used for analyses vs. a time zero control. Control plants were each sprayed with the same quantity of distilled water and surfactant.

Measurement of H_2O_2

Two g of leaves were homogenized in 6 mL of 100 mM sodium phosphate buffer, pH 6.8. The homogenate was filtered through four layers of cheesecloth to remove cellular debris and then centrifuged at 18,000 *g* for 20 min at 4°C. The H_2O_2 content was measured as reported by Lanubile *et al.* (2012). A supernatant aliquot of the reaction mixture was read at 436 nm and its absorbance was compared to the extinction coefficient of an H_2O_2 standard.

Preparation of plant extract and enzyme assay

Three g of leaves were homogenized in 6 mL solution containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$ and 0.05% cysteine. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1,000 *g* for 5 min. The supernatant was re-centrifuged at 20,000 *g* for 20 min at 4°C. The resultant supernatant was desalted by dialysis against 50 mM Tris-HCl, pH 7.8, collected and used for enzymatic activity assay and for native-PAGE. Total protein content was measured according to Bradford (1976) with serum albumin as a standard. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the procedure of Paciolla *et al.* (2008). Briefly, the reaction mixture contained 50 mM phosphate buffer, pH 6.5, 50 μM ASC, 90 μM H_2O_2, and 0.1 mL desalted enzyme extract in 3 mL final volume. The consumption of ASC was recorded as the decrease in absorbance at 265 nm (extinction coefficient 14 $mM^{-1}cm^{-1}$). The activity of monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) was assayed according to the method of Mastropasqua *et al.* (2012). The reaction mixture containing 100 mM Tricine–NaOH buffer, pH 8.0, 0.2 mM NADH, 1 mM ASC, 0.2 units ascorbate oxidase, and 0.1 mL desalted enzyme extract in 3 mL final volume. The reaction was monitored by the decrease in absorbance at 340 nm due to NADH oxidation (extinction coefficient 6.22 $mM^{-1}cm^{-1}$). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed by measuring the rate of increase of the absorbance of ASC at 265 nm (extinction coefficient 14 $mM^{-1}cm^{-1}$) in 3 mL of 0.1 M citrate buffer, pH 6.2, containing an aliquot of supernatant, 2 mM DHA and 4 mM reduced glutathione (Paciolla and Tommasi, 2003). Catalase (CAT, EC 1.11.1.6) activity was assessed according to Lanubile *et al.* (2015). The measure of H_2O_2 oxidation rate at 240 nm was made in a reaction mixture containing 0.1 mL desalted enzyme extract, 0.1 M phosphate buffer, pH 7.0, and 18 mM H_2O_2 (extinction coefficient 23.5 $mM^{-1}\,cm^{-1}$).

Ascorbate and dehydroascorbate measurements

Two grams of each sample were homogenized in 10 mL of cold 5% metaphosphoric acid in a porcelain mortar with quartz sand. The homogenate was centrifuged at 20,000 *g* for 15 min and the supernatant used to determine ASC and DHA contents as reported by Paciolla *et al.* (2008).

Activity of enzymes on native gel

Native-PAGE was performed according to the method of Paciolla and Tommasi (2003), using a Protean IIxi cell electrophoresis unit (Bio-Rad Laboratories Inc.). The same amount of total proteins of each sample was loaded on each lane. After the electrophoretic runs, the gels were washed with distilled water and then incubated in the appropriate buffer (as described below) in order to visualize the bands of activity on the gels. For APX localization, each gel was incubated for 15 min in 0.1 M Na-phosphate buffer, pH 6.2, containing 4 mM ASC and 4 mM H_2O_2 as substrates. The gel was then washed and stained for 15 min in the dark with a solution of 0.125 N HCl containing 0.1% (w/v) potassium ferricyanide and 0.1% (w/v) ferric chloride. The APX isoenzymes were identified as achromatic bands on a Prussian blue background because of the reaction between ferric chloride and ferrocyanide, the latter having been produced by the reduction of ferricyanide with unreacted ASC. Visualization of catalase was performed utilizing 0.05% H_2O_2 as substrate and a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride for stain. Catalase

isoforms appeared as achromatic bands on a dark Prussian blue background. For DHAR proteins, each gel was incubated in 0.1 M Na-phospate solution, pH 6.2, containing 2 mM DHA and 4 mM reduced glutathione. The same procedure was carried out for APX stain, with the isoforms appearing as blue bands on light Prussian blue background.

Densitometric analysis

After staining, the gels were acquired utilizing the Gel/ChemiDoc and Quantity One software (Bio-Rad Laboratories Inc.) to obtain information on the changes in the activity of each band due to treatment for 24 h. A relative value of 100 was assigned to the intensity of the bands present in the control.

Statistical analyses

All the experiments were repeated at least four times and the values reported are the means ± s.d. Analysis of variance was applied to the data and means were compared using the Duncan test to identify significant differences (P<0.05).

Results and discussion

Chenopodium album leaves treated with toxin solutions were severely dehydrated with loss of cell turgour 72 h after treatment, and appearance of necrotic and chlorotic areas (data not shown). Previous studies report that a suspension of pycnidiospores of *A. caulina* sprayed onto young *C. album* plants caused rapid appearance of necrotic spots; and a correlation was found between necrosis development and loss of photosynthetic efficiency (Kempenaar *et al.*, 1996).

Greenhouse experiments have shown that the use of toxin solutions in conjunction with spores of *A. caulina* strongly improved the biocontrol efficacy of this fungus by more than 30%. Furthermore, the simultaneous application of toxins or fungal spores, together with low doses of herbicides (metribuzin and rimsulfuron at 1/5 of the recommended rates), were more phytotoxic than single agent treatments (Vurro *et al.*, 2001). Our results indicate that the biological activity of the toxin mixture is involved in necrosis development, whose effects consequently hamper leaf photosynthesis. The mixture of three toxins applied to *C. album* seedlings was highly phytotoxic, triggering loss of leaf turgor. The observed strong dehydration was a similar effect to those caused by different toxins produced by other fungal species (Paciolla *et al.*, 2014, 2008).

The mixture of toxins induced changes in H_2O_2 levels in *C. album* leaves (Figure 1). A statistically significant (P<0.05) increase of H_2O_2 levels was detected already at 24 hours after treatment (HAT). After longer periods, an additional increase occurred that was significantly greater than the control.

In some plant-pathogen interactions, toxins play important roles in pathogenesis, and may raise levels of H_2O_2 and other reactive oxygen species (ROS) (Paciolla *et al.*, 2014, 2008). Although H_2O_2 is continually produced during normal metabolism and is considered an important signal molecule (Orozco-Càdenas *et al.*, 2001), at high concentrations it is very toxic for cells (Guan *et al.*, 2000). The H_2O_2 increase observed after treatment with toxin mixture may be due to augmented dismutation of anion superoxide in H_2O_2 by superoxide dismutase, rather than photorespiratory processes (Noctor *et al.*, 2002). On the other hand, a strong decrease of glyoxylate has been observed in the presence of ascaulitoxin aglycone (Duke *et al.*, 2011). This suggests that the toxin may inhibit the photorespiratory pathway (Duke *et al.*, 2011), although the decrease in the glyoxylate can also be due to its use in aminotransferase reactions (Liepman and Olsen, 2003, 2001).

Plants treated with the toxin mixture showed significantly (P<0.05) increased ascorbate peroxi-

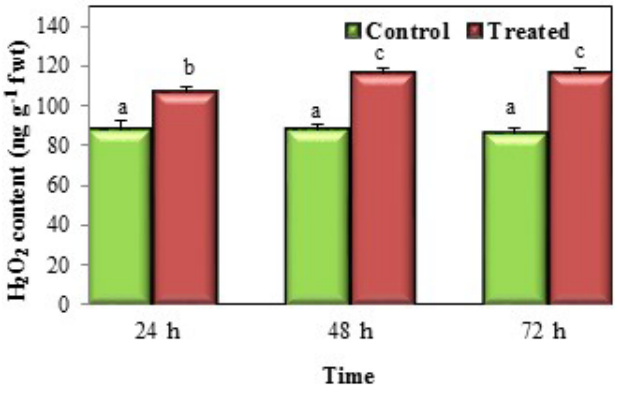

Figure 1. H_2O_2 content in treated and untreated plants of *Chenopodium album* at different times. Data are mean values ± SD of four experiments. Bars with different letters were significantly different (P<0.05) from each other. f wt = fresh weight.

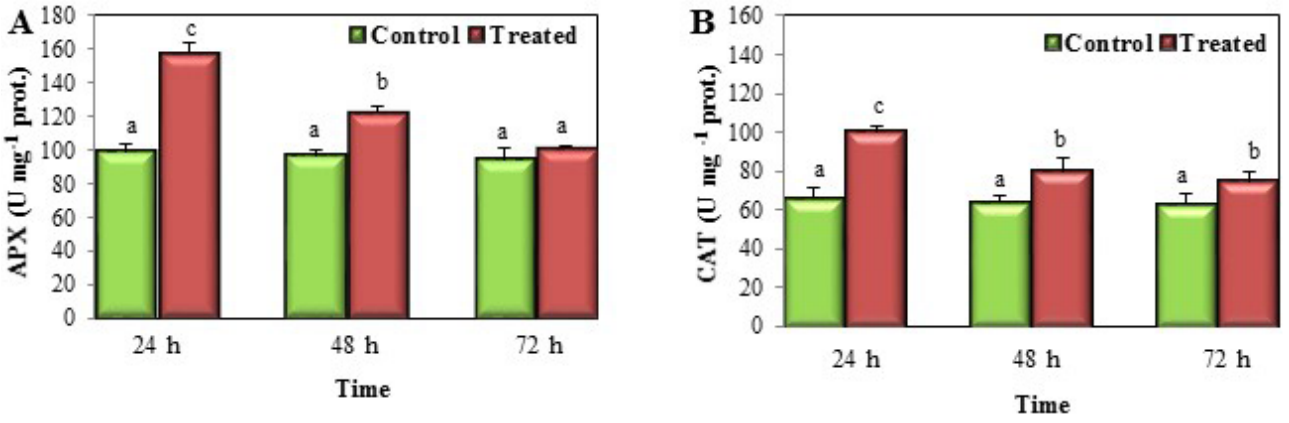

Figure 2. Specific activity of APX (A) and CAT (B) enzymes in leaf tissues of treated and untreated *Chenopdium album* plants at 24 h intervals. Data are mean values ± SD of four experiments. Bars with different letters were significantly different ($P<0.05$) from each other. For APX, 1 U = 1 nmol of ascorbate oxidized min^{-1}; for CAT, 1 U = 1 nmol of H_2O_2 dismutated min^{-1}.

dase activity at 24 and 48 HAT, in comparison to the control. By 72 HAT the activity decreased reaching a value similar to the control (Figure 2A). The change of the electrophoretic pattern on the activity observed in native gels at 24 HAT (Figure3A) correlated with the increase of specific APX activity. One band of APX activity in control and two APX bands in the treated sample were seen by native-PAGE at 24 HAT. The densitometric analysis of band intensity indicates a doubling of APX after treatment (217 vs. 100). The bands of the treated sample had different electrophoretic mobilities than the control (Figure 3A).

Catalase specific activity also significantly ($P<0.05$) increased after treatment, with the greatest value at 24 HAT (Figure 2B). As shown in Figure 3B, there was 50% greater catalase activity at 24 HAT in the treated plants (151 vs. 100), with one native-PAGE band with similar electrophoretic mobility visualized in both control and treated plants. Increases of H_2O_2 and other ROS induce the activity of defence systems, including antioxidative enzymes and molecules (Smirnoff, 2005). The ascorbate system and other antioxidants such as catalase may be directly involved in these defence mechanisms. Together with augmented H_2O_2 level, we observed an increase of ascorbate peroxidase and catalase, which are both important enzymes in H_2O_2 removal from cells. The APX family consists of at least five different isoforms including thylakoid and glyoxysome membrane forms, as well as a chloroplast stromal

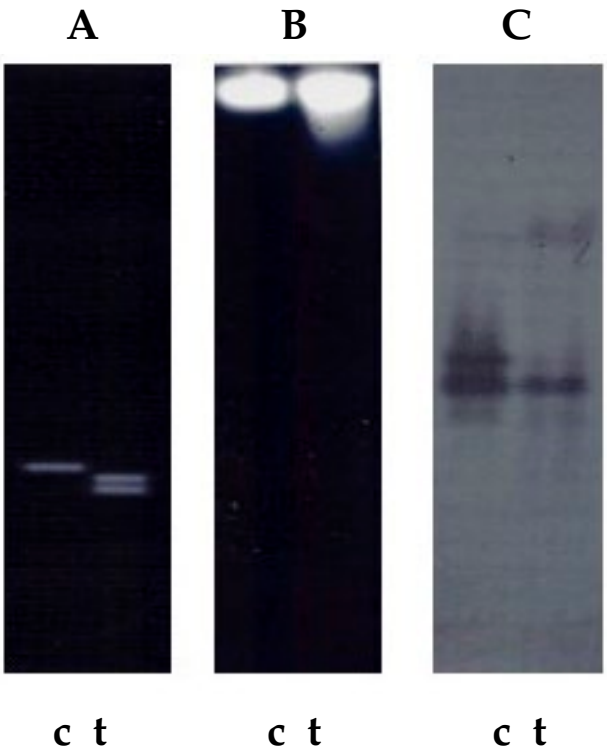

Figure 3. Isoenzyme pattern and activity staining of APX (A), CAT (B) and DHAR (C) enzymes after native electrophoresis, for *Chenopodium album* plants treated with *Ascochyta caulina* toxin mixture (t), or untreated (c), 24 h after treatment. In each lane, 200 μg total proteins were loaded. The gels were analysed utilizing the Gel Doc (see Materials and methods).

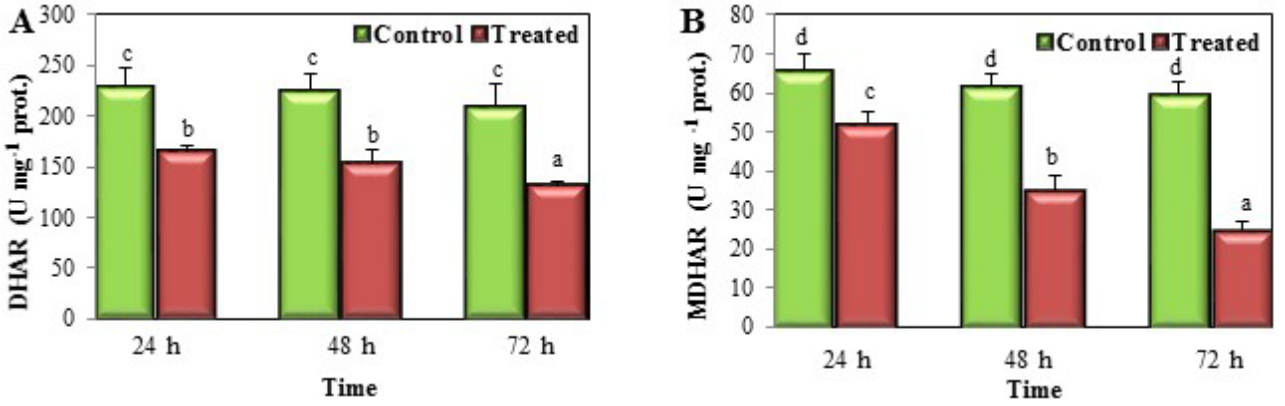

Figure 4. Changes in enzymes of ASC reconversion, namely dehydroascorbate reductase -DHAR- (A) and monodehydroascorbate reductase –MDHAR- (B), in treated and untreated *Chenopodium album* leaf tissue during the time course. Data are mean values ± SD of four experiments. Bars with different letters were significantly different (P<0.05) from each other. For DHAR, 1 U = 1 nmol of dehydroascorbate (DHA) reduced min^{-1}; for MDHAR, 1U = 1 nmol of NADH oxidized min^{-1}.

soluble form and a cytosolic form (Noctor and Foyer, 1998). APX has been studied in many species of angiosperms (Conklin, 2001) and gymnosperms (Tommasi and Paciolla, 2000). H_2O_2 is reported to increase the activities of leaf CAT and APX with the induction of related-isoform(s) under stress conditions (Gill and Tuteja, 2010). The changed isozymatic pattern characterized by appearance of new APX isoforms in treated sample at 24 HAT, could suggest the presence of oxidative stress and consequent cell damage. Additional and specific isoforms useful in removal of accumulated H_2O_2 and/or alternative splicing of messenger RNA may be due to further cell compartments involved in stress responses. In addition, APX can eliminate low and toxic concentrations of H_2O_2 more efficiently than CAT and POD, due to its higher affinity for H_2O_2 (μM range) compared to CAT and POD (mM range) (Asada, 1992). The greater band intensity in treated samples, as well supported also by the enzyme activity, underlined also for CAT a greater capacity of cell detoxification versus H_2O_2. The increased activity could be explained by differences in the enzyme's kinetic features and/or in CAT gene expression; the action of unknown effectors and/or post-translational regulation may alter the kinetic properties (Gibon *et al.*, 2004). This scenario showed that APX and CAT enzymes counteracted the oxidative status due to enhanced presence of H_2O_2. However, the persisting high levels of H_2O_2 in treated plants at longer times highlighted an insufficient counter-action to oxidative stress. The production of H_2O_2 may be generated or induced in the plants by the presence of the toxins, favouring necrosis.

The specific activity of enzymes involved in the regeneration of ascorbate, namely MDHAR and DHAR, are shown in Figure 4. A significant (P<0.05) decrease of their activity during the time-course in the treated plants occurred, reaching at 72 HAT values less than half the respective controls (-57 % for MDHAR and -34% for DHAR). Native-PAGE of DHAR at 24 HAT had a different pattern, with a different number of bands and relative mobility in control and treated samples. The bands of the control were more evident as compared to those for the treated samples, with the band intensity of the treated samples having total values less than half of the control (100 vs. 232) (Figure 3C). A gradual and significant (P<0.05) decrease in ASC during the time-course was observed in treated tissues, in comparison to the control, reaching the lowest content at 72 HAT (-57 %) (Figure 5A). The content of DHA, i.e. the oxidized form of ASC, followed a different trend. A significant (P<0.05) decrease occurred up to 48 HAT, whereas at 72 HAT the DHA level was significantly (P<0.05) greater with respect to the control (Figure 5B). The control contents of both ASC and DHA were constant during the time course, with no changes in the ascorbate pool (ASC + DHA) and in the redox state (ASC/ASC + DHA). Conversely, the ascorbate pool strongly decreased at the various times after the treatment with the toxin mixture, reaching nearly half at 72 HAT with respect to the control (315 vs.

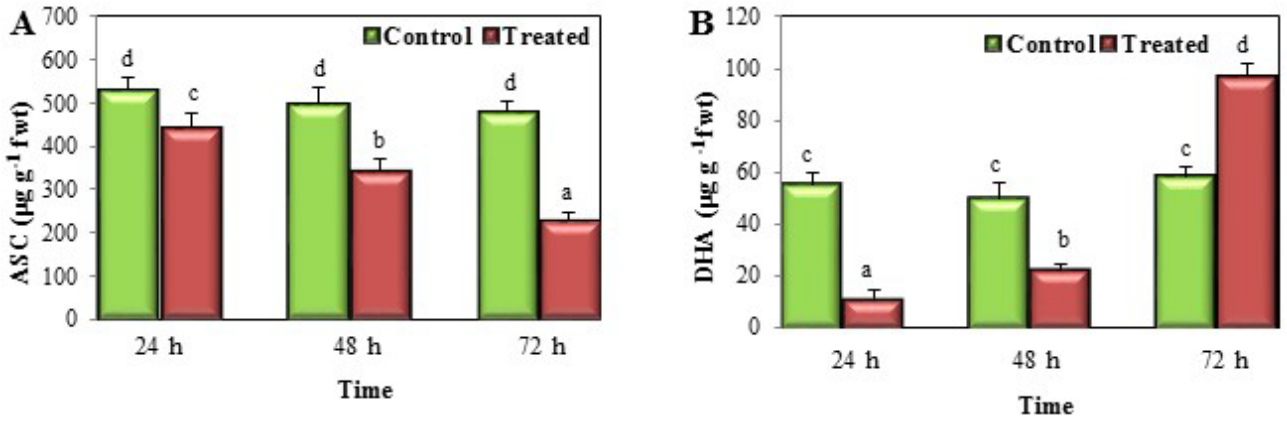

Figure 5. Changes in ASC (A) and DHA (B) in *Chenopodium album* leaf tissue treated with *Ascochyta caulina* toxin mixture, or untreated, at various times. Data are mean values ± SD of four experiments. Bars with different letters were significantly different (P<0.05) from each other. f wt = fresh weight.

585). However, the trend of the redox state did not significantly change until 48 HAT whereas at the longer time (72 HAT) there was a strong decrease due to the large increase of DHA, in comparison to the control (0.70 vs. 0.91).

Accumulation of DHA is also considered toxic for plant cells. If accumulated in plants, DHA can negatively interfere with plant growth (Cordoba-Pedregosa *et al.*, 1996). DHA also inhibits the activity of several enzymes *in vitro*, including malate dehydrogenase, fructose 1,6-bisphosphatase (Morell *et al.*, 1997) and hexokinase (Fiorani *et al.*, 2000). Increase of DHA may partly be due to the decreased reduction rate of both oxidative products of ASC, namely MDHA and DHA, by enzymes MDHAR and DHAR. The lower specific activity of DHAR after treatment was confirmed by its disappearance in native gels of isoforms having DHAR activity, thus indicating reduced reconversion of DHA in ASC. Moreover, proteins that have DHAR activity, including thioredoxins, thioredoxin reductase, GSH peroxidase, and Kunitz-type trypsin inhibitor, are involved in antioxidant processes (Trumper *et al.*, 1994). Ascorbic acid is the most abundant, powerful and water soluble antioxidant and, in plants, it prevents or minimizes the damage caused by the ROS (Smirnoff, 2005). Conversely, the strong decrease in ASC with a corresponding large increase in DHA at later times resulted in decreased ASC/DHA ratio. A shift in cell redox state toward the oxidized form may alter normal cellular homeostasis (Foyer and Noctor, 2005). Only moderate changes in metabolite levels were measured in *L. paucicostata* plants 72 h after treatment with 10 mM of AscA, and no phytotoxic symptoms on plants were observed (Duke *et al.*, 2011). Most pronounced and consistent effects on metabolite levels included distinct changes in aminoacids, enhanced levels of citrate cycle intermediates and reduced amounts of tocopherols and increased levels of ascorbic acid antioxidants (Duke *et al.*, 2011). This trend, however, is in accordance with a previous study (Paciolla *et al.*, 2014) reporting that the effect of two or more toxins alone are not predictive of their combined effects.

Conclusions

Among the defence responses activated by *C. album* treated with the a mixture of toxins, there is the involvement of antioxidant systems including the ascorbate system and catalase. However, the toxic action of H_2O_2 is probably favoured by the decreased activity of H_2O_2 scavenging systems. The mixture of toxins, therefore, lowers the ROS detoxification capability of cells. The consequent degenerative process of diffused necrosis, appearing in the leaf tissues 72 HAT, eventually triggers plant death. The same symptoms could occur in other non-host plants, as the toxins appear to be non-specific, despite the specificity shown by the producer fungus. The rapid appearance of wide necrosis was reported also when the pure toxins were applied to many other

weedy plant species (Vurro *et al*., 2001). Moreover, these findings on the possible specific mechanisms of action in plants do contradict some other recent findings on the eco-toxicological profile of those toxins, that showed low or nil activity against aquatic (algae, *Daphnia*, fish) and terrestrial (earthworms) non-target organisms (Fumagalli *et al*., 2013). This evidence further supports the possible use of these metabolites as safe and environmentally friendly herbicides, in particular for controlling broadleaf weeds. Considering their characteristics, and in particular their non-specificity, these toxins could be developed as post-emergence herbicides, to be applied by spraying directly onto the weeds in absence of crop plants. Localized toxin applications could also be used where crop plants were present.

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