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ORIGINAL PAPER

Bioremediation of dry olive-mill residue removes inhibition of growth induced by this waste in tomato plants

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Abstract The disposal of dry olive-mill residue, the waste product from olive oil production, is a serious environmental issue. Dry olive-mill residue, being rich in organic and inorganic nutrients, could be used as fertilizer; however, it contains phenolic compounds that can inhibit plant growth. In order to clarify whether bioremediation of this waste could be a valuable strategy for its reuse, the effect of aqueous extract of dry olive-mill residue, untreated or bioremediated by the saprobe fungi *Coriolopsis rigida* and *Penicillium chrysogenum*-10, has been analyzed in relation to some physiological parameters of tomato plants. The data show that aqueous dry olive-mill residue significantly reduces the biomass of roots and shoots. In particular, it causes a dramatic reduction in root length, area, and volume as well as in the number of root tips. At an early stage, aqueous dry olive-mill residue also reduces the content of chlorophyll *a* and *b* and the efficiency of PS II. The inhibition of growth seems to be due to the increase in phenolic compounds that induce oxidative stress. Interestingly, when plants are treated with aqueous dry olive-mill residue bioremediated by saprobe fungi a decrease in phenolic content and an alleviation of oxidative stress occur. In conclusion, the results show that bioremediation of aqueous dry olive-mill residue is a useful tool to remove most of the inhibiting effects of this waste on plant growth.

Keywords Oxidative stress · Phenolics · Reactive oxygen species · Saprobe fungi

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Introduction

Olive oil production is one of the most important agro-industrial activities in the Mediterranean region. In addition to oil, this activity produces large quantities of waste whose disposal represents a serious environmental problem. Two-phase centrifugation, one of the most widely used olive-mill extraction systems, generates a wet waste product commonly called "alpeorujo". A second centrifugation of alpeorujo results in an extra oil yield and a new by-product that is dried and extracted with solvents, producing a final solid waste called dry olive-mill residue (DOR). Due to its high organic and inorganic nutrient content, DOR has great potential as fertilizer (Sampedro et al. 2007a). However, DOR also contains phytotoxic compounds capable of inhibiting the growth of microorganisms and thus germination and growth of plants (Sampedro et al. 2004; Saparrat et al. 2010). Therefore, before being applied to agricultural soils as an organic fertilizer, DOR needs to be remediated. Optimal removal of bio-recalcitrant phenols from DOR can be achieved using a well-designed sequential treatment consisting of various chemical, physical, and biological processes (Aranda et al. 2007). Bioremediation with saprobe fungi represents one of the most promising approaches for DOR detoxification (Sampedro et al. 2004, 2007a). Saprobe fungi are known to be capable of degrading a wide variety of pollutants, including phenolic compounds, which are the main

determinants of the phytotoxic effects of DOR (Sampedro et al. 2004, 2007a). The ability of saprobe fungi to bioremediate DOR is due to the presence of extracellular enzymes, namely laccase and peroxidase (Aranda et al. 2006, 2007; Sampedro et al. 2004). It has been demonstrated that a relationship exists between the production of laccase by the saprobe fungus *Coriolopsis rigida* and degradation of phenolic compounds in aqueous extracts of DOR (ADOR) (Aranda et al. 2006, 2007; Saparrat et al. 2010). In addition, previous studies show that the production of hydrolytic enzymes by the saprobe fungus *Penicillium chrysogenum*-10 leads to a decrease in ADOR phytotoxicity (Aranda et al. 2004). Phenolic compounds are considered to be powerful allelochemicals and can interfere with several physiological processes associated with seed germination as well as plant growth and development (Inderjit 2003; Weir et al. 2004). Phenolic allelochemicals are known to cause oxidative damage by increasing membrane leakage and inducing overproduction of reactive oxygen species (ROS) (Romero-Romero et al. 2005).

Reactive oxygen species, very low levels of which are generated under optimal growth conditions, dramatically increase under biotic and abiotic stress conditions. ROS overproduction induces lipid peroxidation, affecting membrane permeability, causes oxidative damage to DNA and proteins and can ultimately lead to cell death (Apel and Hirt 2004). ROS, apart from being toxic by-products of aerobic metabolism, are also key regulators of plant growth, development, and defense mechanisms (Mittler 2002). Plants possess several enzymatic and non-enzymatic mechanisms that protect cells against excessive oxidative damage caused by ROS (Mittler 2002). Among non-enzymatic antioxidants, ascorbate (ASC) and glutathione (GSH) play a pivotal role in ROS removal by acting as major redox buffers in plant cells (Noctor and Foyer 1998). Moreover, various antioxidant enzymes are directly involved in ROS removal. Superoxide dismutase (SOD) removes superoxide anion producing hydrogen peroxide (H_2O_2). The two enzymes catalase (CAT) and ascorbate peroxidase (APX) are directly responsible for H_2O_2 scavenging. H_2O_2 can also be removed by other peroxidases (POD) which play various roles in plant cells, including the metabolism of phenolic compounds (Almagro et al. 2009).

To date, despite research interest in the possibility of using ADOR as fertilizer, there is very little data on the impact of this waste on the physiology of plants. The aim of this study was to analyze the effect of ADOR, untreated or bioremediated by the saprobe fungi *C. rigida* and *P. chrysogenum*-10, on some physiological parameters related to growth of tomato plants. In addition, it has been studied whether untreated and bioremediated ADOR were capable of inducing oxidative stress and, consequently, defense responses mediated by ROS-scavenging systems.

Materials and methods

Obtainment of untreated and bioremediated ADOR

DOR was collected from an alperujo manufacturer (Sierra Sur S.L., Granada, Spain). ADOR was obtained by incubation of DOR with distilled water in a 1:2 (w:v) ratio for 8 h in an orbital shaker. The suspension obtained was filtered through several layers of cheesecloth and stored at −20 °C until use. ADOR showed the following characteristics: pH 5.3, total organic carbon 55.7 g l^{-1}, P 0.39 g l^{-1}, Ca 0.87 g l^{-1}, K 7.7 g l^{-1}, Mg 0.22 g l^{-1}, Na 0.043 g l^{-1}, S 0.16 g l^{-1}, B 8.3 mg l^{-1}, Cu 1.5 mg l^{-1}, Fe 25 mg l^{-1}, Mn 3.4 mg l^{-1}, and Zn 4.6 mg l^{-1}.

Strains of saprobe fungi *C. rigida* (CECT 20449) and *P. chrysogenum* 10 (EEZ 10) were maintained at 4 °C on malt extract agar plates. Saprobe fungi were grown on malt extract for 7 days at 28 °C with orbital shaking. Mycelia from these cultures were homogenized, inoculated in 70 ml of MB medium in an Erlenmeyer flask and incubated for 15 days. The flasks were successively supplemented with 70 ml of ADOR. After 15 days, the culture liquid was separated from the mycelium by filtration through a filter paper disk and the supernatant used for phytotoxicity and biochemical assays.

Plant growth conditions

Tomato (*Solanum lycopersicum*, var. Muchamiel) seeds were surface-sterilized for 20 min in 20 % (v/v) NaClO and, after rinsing with distilled water, were incubated for 5 h in sterile distilled water under shaking. Seeds were placed in plastic trays with a 5 cm-deep layer of vermiculite, while adding 500 ml Hewitt solutions every 2 days. Seeds were germinated under controlled conditions (75 ± 5 % relative humidity; 25–15 °C temperature; 16–8 h photoperiod and 460 μmol m^{-2} s^{-1} irradiance). After 15 days, plants with three fully expanded leaves were transferred to 300 ml pots containing a mixture of soil and sand in a 2:3 (v:v) ratio. Plants were grown in a greenhouse under the same controlled conditions described above. After 2 days, plants were treated with 5 % ADOR unbioremediated and bioremediated with the saprobe fungi *C. rigida* and *P. chrysogenum*-10. After 4, 10, and 30 days of treatment, roots and leaves were separately collected, frozen in liquid nitrogen, and kept at −80°C until use.

Morpho-physiological parameters of tomato plants

Dry weight (DW) was obtained by drying roots and leaves in an oven at 100 °C for 24 h.

To obtain information on morphological parameters (total length, area, volume, and number of root tips), the

root system was digitalized using a scanner (EPSON V700/V750 1.8V.3.4) and analyzed using WinRhizo software.

To determine photosynthetic pigments, tomato leaves were homogenized with 80 % acetone and centrifuged at 20,000×*g* for 20 min. The absorbance of supernatant was measured at 663, 648, and 470 nm for chlorophyll *a*, chlorophyll *b* and carotenoids, respectively. Content of chlorophylls and carotenoids was determined on the basis of equations described in Zhang and Kirkham (1996).

The efficiency of photosystem II was measured by Fluor Pen II FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive estimation of plant photosynthetic performance. The quantum yield of photosystem II was expressed as the ratio between the actual fluorescence yield in the light-adapted state (F'_V) and the maximum fluorescence yield in the light-adapted state (F'_M) according to the method described by Oxborough and Baker (1997). Measurements were taken in the second youngest leaf of four different plants for each treatment.

Markers of oxidative stress

H_2O_2 content was measured following 4-aminoantipyrine oxidation, as described by Frew et al. (1983). Briefly, roots and leaves were homogenized with 50 mM potassium phosphate buffer pH 7.8 and centrifuged at 20,000×*g* for 15 min. 5 µl of supernatant were mixed with 400 µl of 0.1 M potassium phosphate pH 6.9 containing 0.234 % (w/v) phenol, 0.1 % (w/v) 4-aminoantipyrine, 20 nM horseradish peroxidise. The mixture was incubated at room temperature for 5 min, and absorbance was measured at 505 nm. H_2O_2 concentration was calculated with reference to a standard curve obtained within the range 0–19 µM H_2O_2 and expressed as µmol mg^{-1} protein.

Lipid peroxidation was measured as malondialdehyde (MDA) content determined by the thiobarbituric acid reaction as described by Paradiso et al. (2008).

Total phenolics and non-enzymatic antioxidants

For the determination of phenolic content, roots and leaves were homogenized with 80 % methanol and centrifuged at 10,000×*g* for 20 min at 4 °C. 150 µl supernatant was incubated with 100 µl HCO_3Na_2, 500 µl distilled water, and 50 µl Folin reagent for 60 min in the dark. Absorbance of the mixture was read at 730 nm. Total content of phenolic compounds was expressed as µmol g^{-1} fresh weight with reference to a calibration curve obtained with gallic acid.

To determine ASC and GSH, tomato roots and leaves (0.5 g) were homogenized with cold 5 % metaphosphoric acid at 4 °C in a 1:4 ratio (w:v) to obtain deproteinized extracts. After centrifugation at 20,000×*g* for 15 min, the supernatants were collected and used for the analysis of total ASC and GSH levels as described by Paradiso et al. (2008).

Enzyme assays

Tomato roots and leaves were ground to a fine powder in a mortar in the presence of liquid nitrogen and mixed with an extraction buffer containing 50 mM Tris–HCl (pH 7.8), 1 % BSA and 0.5 % cysteine. Homogenates were centrifuged at 20,000×*g* for 20 min at 4 °C, and supernatants used for enzymatic determinations.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to the method described by McCord and Fridovich (1969). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 25 µg protein extract, 0.1 mM EDTA, 1 mM xanthine, 1 mM cytochrome c, and sufficient xanthine oxidase to obtain a reduction rate for ferrocytochrome *c* at 550 nm of 0.025 absorbance min^{-1}. One unit of SOD activity was defined as the amount of enzyme required to obtain 50 % inhibition of ferrocytochrome c reduction. Catalase (CAT EC 1.11.1.6) and ascorbate peroxidase (APX EC 1.11.1.11) activities were determined according to Paradiso et al. (2012) following H_2O_2 consumption at 240 nm ($\varepsilon = 23.5$ M^{-1} cm^{-1}) and ASC oxidation at 290 nm ($\varepsilon = 2.75$ mM^{-1} cm^{-1}), respectively.

Glutathione-S-transferase activity (GST EC 2.5.1.8) was determined following the formation of CDNB (1-chloro-2,4-dinitrobenzene) conjugate at 340 nm ($\varepsilon = 9.6$ mM^{-1} cm^{-1}) as described by Habig et al. (1974). Peroxidase activity (POD EC 1.11.1.7) was measured following the oxidation of 3,3′,5,5′-Tetramethylbenzidine (TMB) at 652 nm ($\varepsilon = 26{,}9$ mM^{-1} cm^{-1}), according to Ferrer et al. (1990).

All enzymatic activities were measured at 25 °C with a Beckman (Fullerton, CA, USA) DU 7000 spectrophotometer. Proteins were measured according to the method described by Bradford (1976) using bovine serum albumin as standard.

Statistical analyses

The experiments were conducted in a completely randomized design, with three replicates used for each independent experiment. Data were analyzed by one-way analysis of variance (ANOVA) followed by the separation of the treatments from controls as well as from each other by applying post hoc the Dunnett and Tukey tests, respectively.

Results and discussion

ADOR effects on morpho-physiological parameters of tomato plants

To verify whether ADOR treatments could affect plant biomass, the DW of roots and shoots was analyzed (Fig. 1a, b). In plants exposed to ADOR, a drastic decrease in root biomass was observed, starting after 10 days and remaining significant 30 days after treatment. Tomato shoots were also negatively affected by ADOR, since a significant reduction in biomass was observed after 10 and 30 days of treatment (34 and 54 %, respectively). It is known that ADOR contains soluble phenolics, mainly vanillic, hydroxybenzoic, ferulic, *p*-cumaric, gallic, and caffeic acid that are the principal cause of its phytotoxicity (Aranda et al. 2007). It has been investigated whether the negative impact of ADOR treatments on tomato growth was due to changes in the endogenous content of phenolics (Fig. 1c, d). Fig. 1c shows that ADOR caused a significant increase in the phenolic content of tomato root during the first 10 days of treatment. Interestingly, after 10 days of exposure to ADOR, phenolic content also increases in leaves, indicating a possible translocation of these compounds from roots to leaves (Fig. 1d). The increase in phenolics in leaves could be a useful defense strategy for alerting plants of stress situations and for permitting an induction of defense responses in this organ.

It is known that saprobe fungi possess extracellular laccase and peroxidase that can degrade phenolic compounds (Aranda et al. 2006, 2007; Sampedro et al. 2004; Saparrat et al. 2010). Consistently, when plants were grown in the presence of ADOR bioremediated with *P. chrysogenum*-10, the increase in phenolics in roots was appreciably reduced and only showed significant differences 4 days after treatment. Moreover, when tomato plants were exposed to ADOR bioremediated with *C. rigida,* phenolic content remained similar to that observed in control roots. Interestingly, the root biomass reduction in plants cultivated in the presence of ADOR bioremediated with both saprobe fungi was very transient, being only observed after 10 days and returning to values comparable to those for the control after 30 days (Fig. 1a). When plants were treated with ADOR bioremediated with both saprobe fungi, in

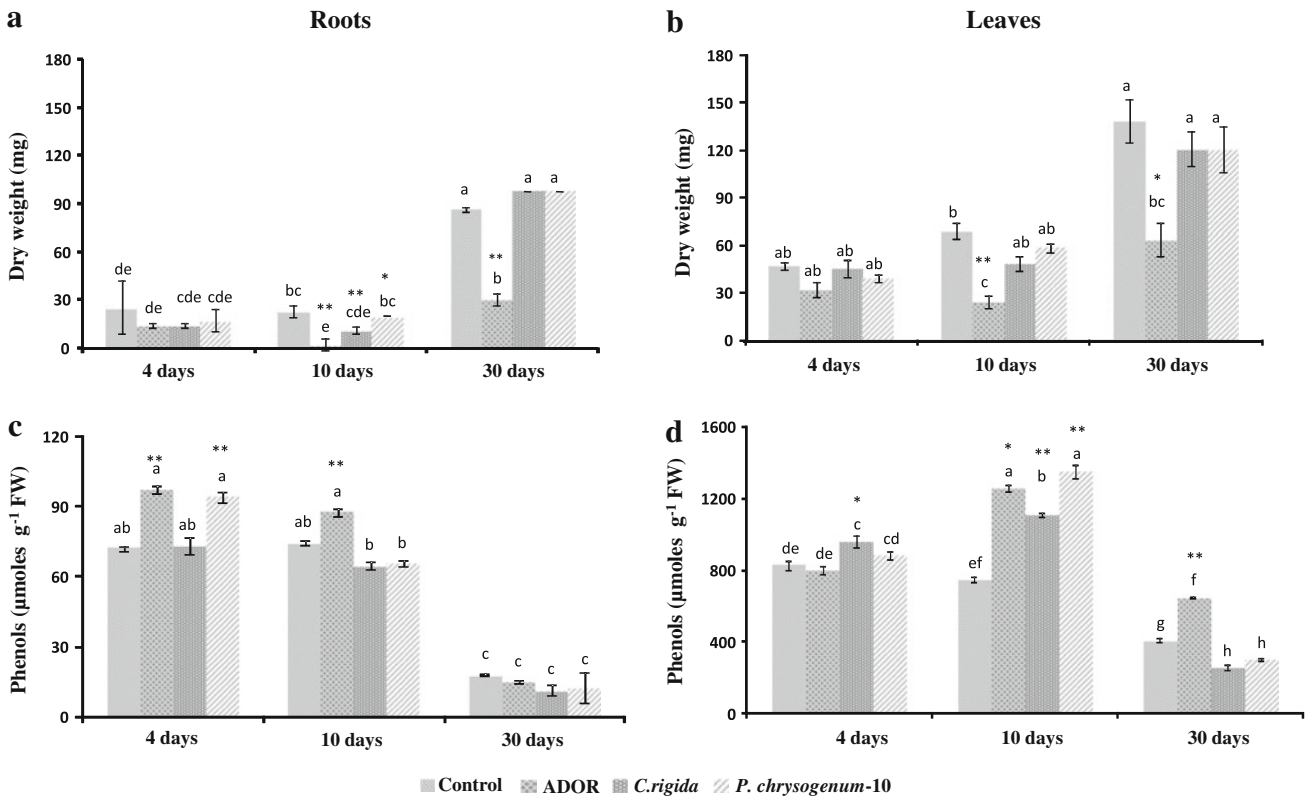

Fig. 1 Effect of untreated and bioremediated ADOR on biomass and phenolic content of tomato plants. Dry weight (**a**, **b**) and phenolic content (**b**, **c**) of roots and shoots in control and ADOR-treated tomato plants. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates ($n = 9$). In each data sample, (*, **) represent statistically different values with respect to the control according to the Dunnet test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

leaves phenolic content returned to values comparable with the control after 30 days (Fig. 1d) and shoot biomass was not affected (Fig. 1b).

The morphological characteristics of the total root system (length, area, volume, and number of root tips) after treatment with untreated and bioremediated ADOR were also analyzed (Fig. 2). Length, area, and number of root tips decreased significantly between 4 and 10 days after exposure to ADOR and then returned to values comparable to those for the control after 30 days (Fig. 2a, b, d). On the other hand, total root volume decreased significantly 30 days after the treatment with ADOR (Fig. 2c). These results indicate a high susceptibility of tomato roots to phenolic compounds and are consistent with the negative effect of caffeic acid on the early root growth of mung beans (Singh et al. 2009). When plants were exposed to ADOR bioremediated with *P. chrysogenum*-10 root development was less affected, since only a transient reduction in length and area was observed 10 days after treatment. Moreover, all the morphological characteristics of roots remained similar to those of the control after the plants were treated with ADOR bioremediated with *C. rigida* (Fig. 2), thus highlighting the greater capacity of this saprobe fungus to reduce phenolic compounds.

Many physiological processes of plants, such as transpiration, nutrient uptake, ATP synthesis, and phytohormone metabolism, can be altered in the presence of phenolic compounds (Inderjit 2003; Weir et al. 2004). Phenolics can also affect membrane permeability, reduce water uptake, and disrupt electron-transfer chains during mitochondrial respiration and photosynthesis (Weir et al. 2004). In order to determine whether ADOR treatments affect the photosynthetic efficiency of leaves, the content of chlorophylls and carotenoids and the efficiency of photosystem II (PSII) were analyzed (Fig. 3). There was a clear decrease in chlorophyll *a* after 10 days of exposure to untreated and bioremediated ADOR, even though the level of reduction was higher in plants grown in the presence of untreated ADOR (Fig. 3a). In addition, only the treatment with untreated ADOR caused a transient decrease in chlorophyll *b* (Fig. 3b), whereas no changes in carotenoids were observed after exposure to untreated and bioremediated ADOR (Fig. 3c). Consistently, phenolics have previously been reported to have an inhibitory effect on the accumulation of photosynthetic pigments in rice seedlings (Yang et al. 2002). Plants grown in the presence of untreated ADOR also showed a reduction in the efficiency of PS II during the first 10 days of exposure (Fig. 3d). Phenolics are known to alter PSII efficiency and inhibit electron transfer between plastoquinone *A* and *B*; moreover, phenolic compounds, acting as plastoquinone analogs, can interfere with plastoquinone binding at the DI protein of PS II (Inderjit 2003; Weir et al. 2004).

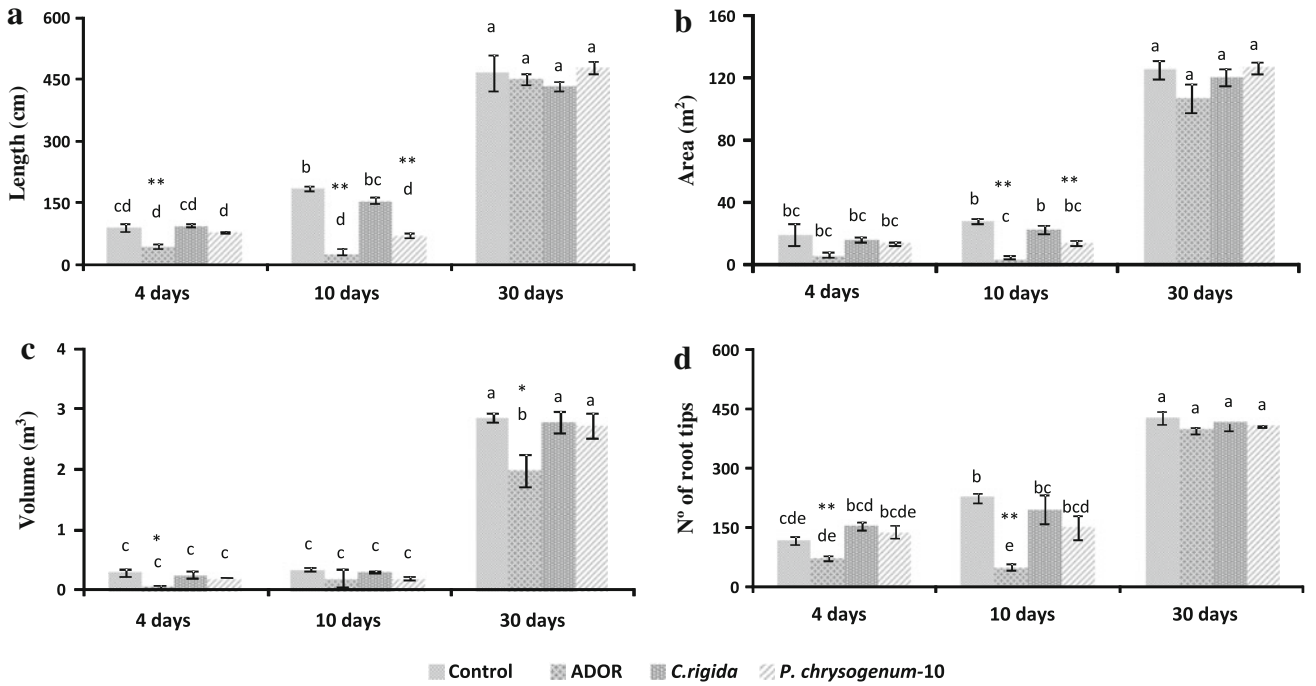

Fig. 2 Effect of untreated and bioremediated ADOR on the root morphology of tomato plants. Total length (**a**) area (**b**), volume (**c**), and number of root tips of root system in control and ADOR-treated tomato plants. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates. In each data sample, (*, **) represent statistically different values with respect to the control according to Dunnet's test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

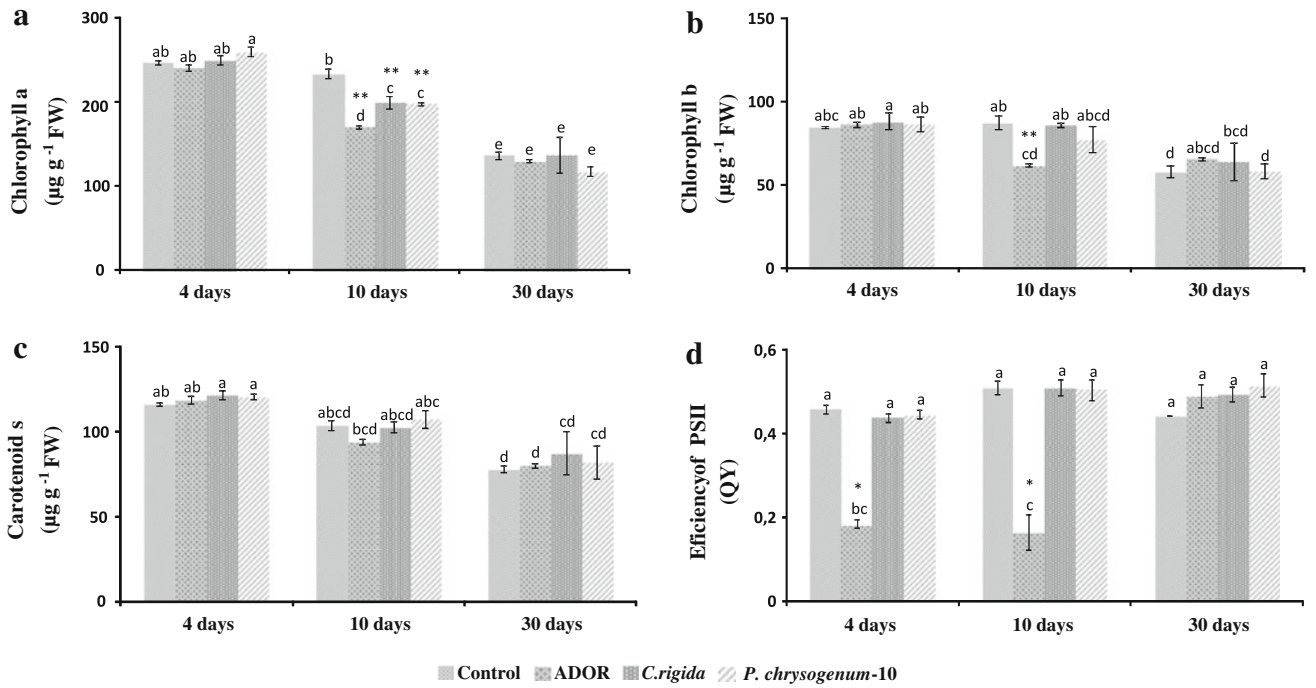

Fig. 3 Photosynthetic pigments and efficiency of PSII in tomato plants grown in the presence of untreated and bioremediated ADOR. Chlorophyll *a* (**a**), chlorophyll *b* (**b**), carotenoids (**c**), and efficiency of PSII (**d**) in control and ADOR-treated tomato plants. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates. In each data sample, (*, **) represent statistically different values with respect to the control according to the Dunnet test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

The results reported so far show that bioremediation of ADOR with saprobe fungi is an effective strategy for removing the phytotoxicity of this waste.

Changes in redox environment caused by ADOR treatments

Phenolic compounds are known to induce ROS overproduction causing oxidative stress (Weir et al. 2004). In order to verify whether the presence of ADOR induced oxidative stress in tomato plants, H_2O_2 production and lipid peroxidation were determined (Fig. 4a–d). In roots, ADOR caused an increase in H_2O_2 content at all the times analyzed. A transient increase in H_2O_2 was also observed in roots treated with ADOR bioremediated with *P. chrysogenum*-10, while no change occurred when ADOR was bioremediated with *C. rigida* (Fig. 4a). This dissimilar behavior is coherent with the different effect of ADOR bioremediated by the two fungi on root morphology and could be due to the diverse enzymatic systems utilized by *C. rigida* and *P. chrysogenum*-10 for ADOR degradation. Indeed, in *C. rigida* the oxidation of phenols by oxidative enzymes, such as ligninolityc enzymes, generates oxyradicals that can undergo to non-enzymatic coupling reactions with the formation of polymers that stabilize the reactivity of these molecules (Sampedro et al. 2007b). On the other hand, filamentous fungi have different pathways of phenol conversion and produce secondary metabolites that could have pro-oxidative activity (Isebaert et al. 2005; Iwasaki et al. 2011).

In leaves, a transient rise in H_2O_2 was observed in the presence of untreated and bioremediated ADOR, with H_2O_2 content returning to levels comparable to those for the control after long-term exposure (30 days).

The toxicity of many phenolics can be largely attributed to the formation of semiquinone radicals that donate electrons to molecular oxygen, thus forming superoxide anion (O_2^{-}). This molecule can undergo a whole series of further reactions to become the more reactive hydroxyl or hydroperoxyl radicals that, by inducing lipid peroxidation, can affect membrane permeability (Weir et al. 2004). An increase in lipid peroxidation has been reported in response to a variety of abiotic and biotic stress and can be considered an effective indicator of oxidative damage in cells (Apel and Hirt 2004; Paradiso et al. 2008). Various phenolics, such as benzoic and cinnamic acids, have been shown to be able to alter membrane permeability and to induce oxidative stress (Ding et al. 2007). Our data consistently show that ADOR induced oxidative stress in tomato plants, causing an increase in lipid peroxidation. Indeed, MDA content increased in roots after 10 days of exposure to untreated and bioremediated ADOR (Fig. 4c).

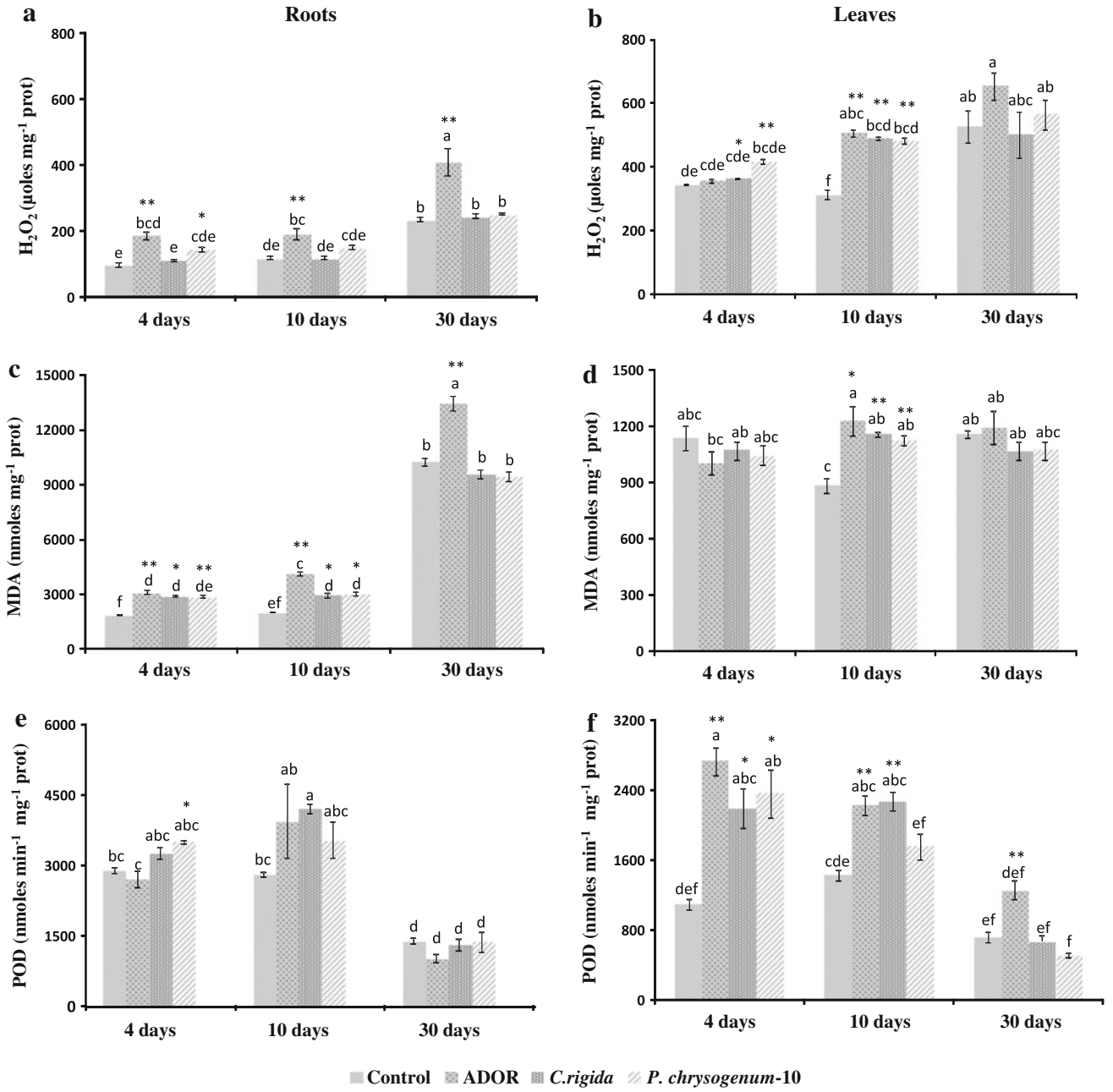

Fig. 4 Changes in oxidative markers induced by untreated and bioremediated ADOR in tomato plants. H_2O_2 content (**a**, **b**), lipid peroxidation (**c**, **d**), and peroxidase activity (**e**, **f**) in roots and leaves of tomato plants grown in the presence of untreated and bioremediated ADOR. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates ($n = 9$). In each data sample, (*, **) represent statistically different values with respect to the control according to the Dunnet test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

Lipid peroxidation in leaves increased during the first 10 days of exposure to untreated and bioremediated ADOR. However, after long-term exposure, MDA content returned to values comparable to those for the control only in the presence of bioremediated ADOR (Fig. 4d). It should be noted that lipid peroxidation increase was more precocious in leaves than in roots (Fig. 4c, d). This finding could be explained by the fact that POD activity was different in the two organs (Fig. 4e, f). In roots, POD activity did not change significantly following all treatments, with the exception of a slight and transient increase detectable after 4 days of treatment with ADOR bioremediated with *P. chrysogenum*-10 (Fig. 4e). On the other hand, in leaves, exposure to ADOR induced an enhancement of POD at all

the times analyzed. The increase in POD activity in leaves was more transient when plants were treated with bioremediated ADOR. POD activity actually returned to levels comparable to those for the control after 10 and 30 days in the presence of ADOR bioremediated with *P. chrysogenum*-10 and *C. rigida*, respectively (Fig. 4f). Phenolic compounds are known to be metabolized by POD, thus leading to the formation of phenoxyl radicals (Takahama and Oniki 1992), which could be directly responsible for lipid peroxidation (Ritov et al. 1996). In leaves, the increase in POD activity, observed 4 days after treatment, indicates that phenolics have a more active metabolism, which is responsible for the induction of the oxidative damage to lipids. In the leaves of plants treated with bioremediated ADOR, the reduction in lipid peroxidation consistently correlates with the lower level of POD activity.

To date, there is little data on ADOR's impact on the antioxidant metabolism of plants (Garcia-Sanchez et al. 2012). However, the effect of many phenolic compounds on antioxidant systems in a variety of plants has previously been described (Bai et al. 2009; Batish et al. 2006). In order to verify whether changes in the cellular redox state occurred after ADOR treatment, the two major hydrophilic antioxidants, namely ASC and GSH and stress-related enzymes (GST, SOD, CAT, and APX) were studied both in roots and leaves (Figs. 5, 6).

Following ADOR treatment, the two major non-enzymatic antioxidants, ASC and GSH, behaved differently in tomato roots. An increase in the ASC content of roots was observed after just 4 days of exposure of plants to ADOR untreated or bioremediated with *P. chrysogenum*-10 (Fig. 5a). On the other hand, GSH showed a dramatic and prolonged decrease up to day 10 with all treatments; however, after long-term exposure, the decline persisted only in plants grown in the presence of untreated ADOR (Fig. 5b). The decrease in GSH could be explained by the activation of detoxification processes. In actual fact, GSH can be conjugated to xenobiotic compounds by GST (Cummins et al. 2011). An increase in GST activity has consistently been observed 4 days after ADOR treatment (Fig. 5c). In addition, being GST capable of behaving like a peroxidase, an increase in its activity can remove H_2O_2 overproduced during the stress caused by ADOR, as already reported in relation to exposure to stress induced by other xenobiotics (Cummins et al. 2011).

Many phenolic compounds are known to be capable of increasing antioxidant enzymes (Bai et al. 2009; Batish et al. 2006; Singh et al. 2009). In roots grown in the presence of ADOR, an increase in all antioxidant enzymes was observed 4 days after treatment (Fig. 5 c–e). The increase observed in SOD activity in roots (Fig. 5d) indicates that excessive production of O_2^- has been triggered by ADOR treatment and that, consequently, SOD activity was up-regulated to mitigate oxidative damage. The increase in SOD activity also caused an increase in H_2O_2 content (Fig. 4a) and a parallel enhancement of the CAT and APX enzymes involved in H_2O_2 removal (Fig. 5 e, f). However, while the increase in CAT was transient, a more persistent activation of APX was observed. This difference could be due to the different kinetic characteristics of the two enzymes (Willekens et al. 1997). CAT is actually a very efficient tool for the gross removal of H_2O_2, but is less appropriate for fine-tuning sensitive redox balance. In contrast, APX has a high affinity to H_2O_2 and plays an important role in controlling the steady state level of this ROS required for cellular signaling (Mittler and Poulos 2005). After exposure to bioremediated ADOR, no significant changes were observed in GST and SOD activities (Fig. 5c, d), whereas CAT behaved in a similar way to that observed after treatment with untreated ADOR (Fig. 5e). On the other hand, APX activity increased during the first 10 days, though to a lesser extent than plants exposed to untreated ADOR, and returned to levels comparable to those of the control after long-term exposure (Fig. 5f). The removal of phenolic compounds by saprobe fungi reduced H_2O_2 production (Fig. 4a), indicating an alleviation of oxidative stress, which was also shown by a more rapid decrease in APX activity (Fig. 5f).

Changes in non-enzymatic and enzymatic antioxidants also occurred in leaves after ADOR treatment (Fig. 6). ASC increased after 10 days of exposure to untreated and bioremediated ADOR (Fig. 6a). On the other hand, GSH content increased after just 4 days of exposure to both untreated and bioremediated ADOR. The increase in GSH persisted until day 10 only in the leaves of plants exposed to untreated ADOR (Fig. 6b). The rise in these two metabolites underlines the possible involvement of the ASC-GSH cycle in the removal of H_2O_2, in line with the behavior observed in plants subjected to stress caused by other phenolic compounds (Hong et al. 2009; Martì et al. 2009). In tomato leaves, GST activity was not affected by exposure of plants to untreated and bioremediated ADOR (Fig. 6c). On the other hand, SOD activity increased at all the times analyzed after treatment with untreated ADOR, whereas its increase was transient in the leaves of plants treated with bioremediated ADOR (Fig. 6d). Finally, CAT and APX behaved in an opposite fashion, with CAT decreasing after 30 days of exposure to both untreated and bioremediated ADOR and APX, increasing after 10 and 30 days of exposure to all treatments (Figs. 6e, f).

This finding indicates that SOD acts as a first line of defense by regulating the intracellular concentration of O_2^-. The subsequent increase in H_2O_2, probably mediated by SOD activity, enables APX to be enhanced. The enhancement of antioxidant enzymes in leaves suggests that there is an improvement in the whole plant's defense

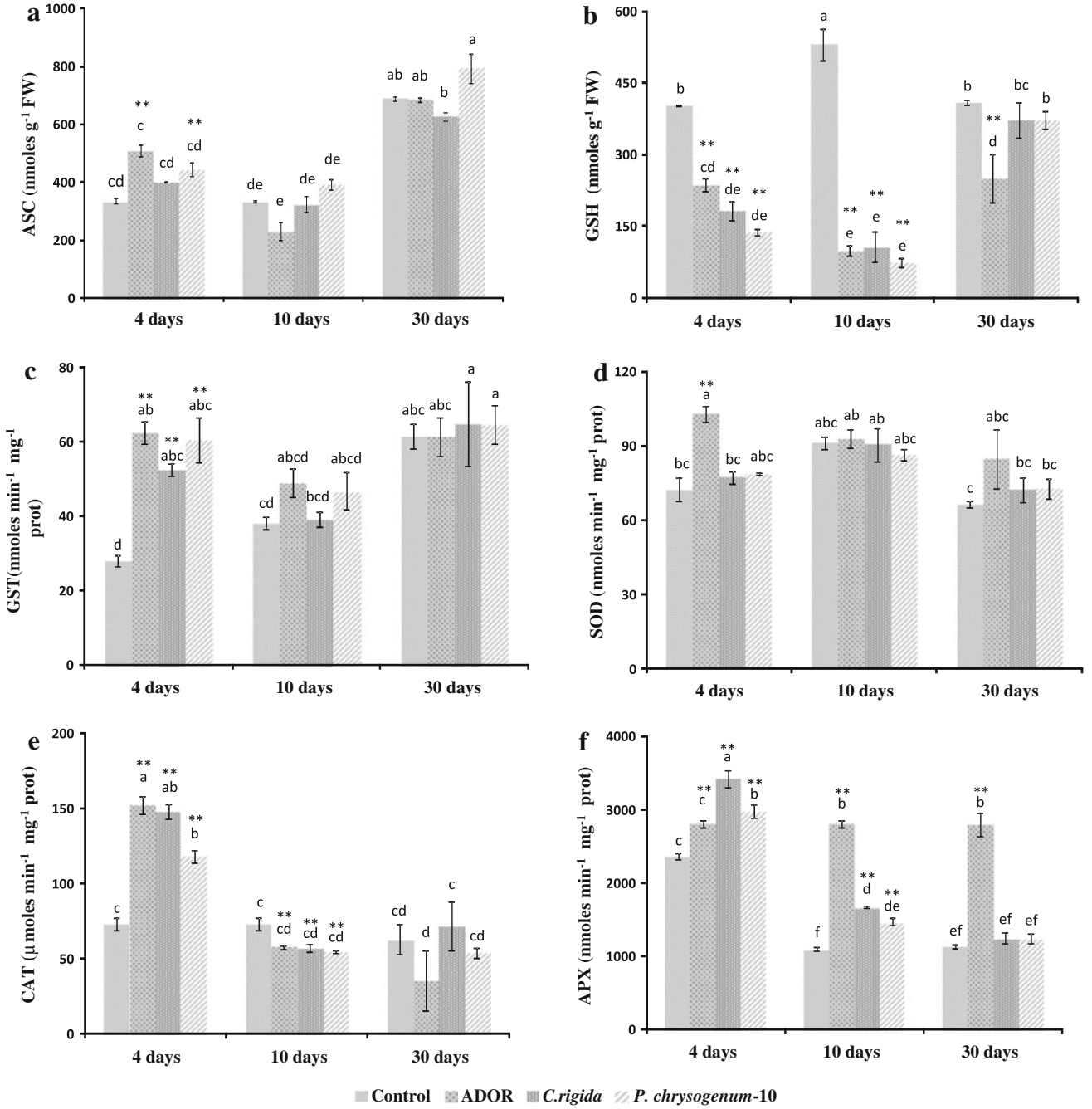

Fig. 5 Effect of untreated and bioremediated ADOR on antioxidants and stress-related enzymes in roots. Total contents of ASC (**a**) and GSH (**b**) and specific activities of GST (**c**), SOD (**d**), CAT (**e**), and APX (**f**) in roots of control and ADOR-treated tomato plants. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates ($n = 9$). In each data sample, (*, **) represent statistically different values with respect to the control according to the Dunnet test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

mechanisms against oxidative stress caused by ADOR. It should be noted that the improvement in some antioxidant mechanisms (GSH content and SOD activity) was transient in the leaves of plants grown in the presence of bioremediated ADOR, suggesting an alleviation of oxidative stress in these growth conditions.

Conclusion

Our findings show that ADOR significantly reduces the growth and development of tomato plants by inducing an oxidative stress. Consequently, tomato plants enhance antioxidant systems to avoid irreparable damage. Through

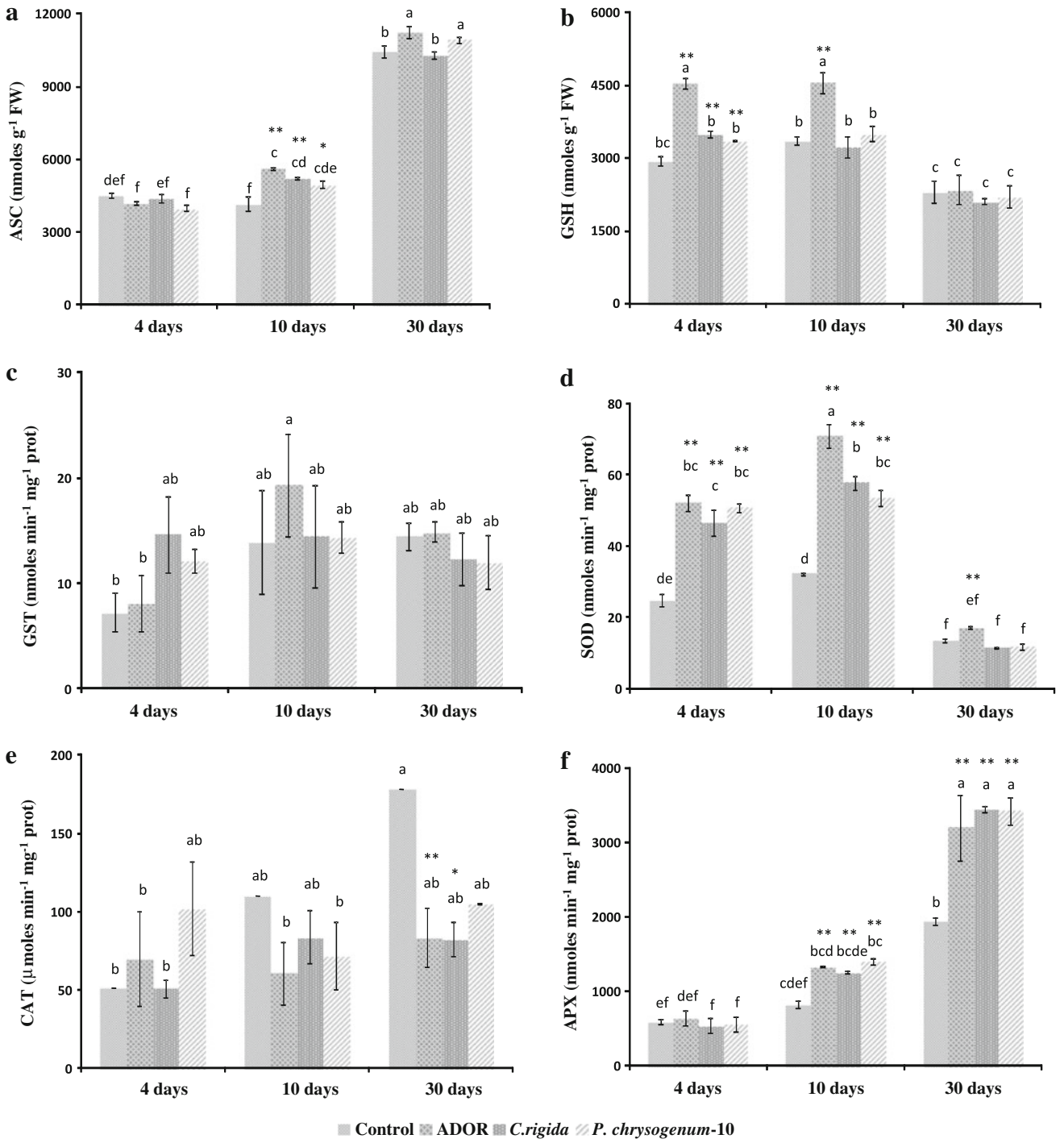

Fig. 6 Effect of untreated and bioremediated ADOR on antioxidants and stress-related enzymes in leaves. Total contents of ASC (**a**) and GSH (**b**) and specific activities of GST (**c**), SOD (**d**), CAT (**e**), and APX (**f**) in roots of control and ADOR-treated tomato plants. *C. rigida* and *P. chrysogenum*-10 indicate ADOR bioremediated with the respective fungi. Values represent the mean ± SE of three independent replicates ($n = 9$). In each data sample, (*, **) represent statistically different values with respect to the control according to Dunnet's test ($P \leq 0.05$ and $P \leq 0.01$, respectively). *Different letters* represent significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$)

the removal of phenolic compounds, bioremediation of ADOR by saprobe fungi significantly alleviates oxidative stress and eliminates most of the inhibiting effects of this waste on growth.

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