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Oxidative damage and cell-programmed death induced in *Zea mays* L. by allelochemical stress

**Claudia Ciniglia · Francesco Mastrobuoni ·
Marco Scortichini · Milena Petriccione**

Abstract The allelochemical stress on *Zea mays* was analyzed by using walnut husk washing waters (WHWW), a by-product of *Juglans regia* post-harvest process, which possesses strong allelopathic potential and phytotoxic effects. Oxidative damage and cell-programmed death were induced by WHWW in roots of maize seedlings. Treatment induced ROS burst, with excess of H_2O_2 content. Enzymatic activities of catalase were strongly increased during the first hours of exposure. The excess in malonildialdehyde following exposure to WHWW confirmed that oxidative stress severely damaged maize roots. Membrane alteration caused a decrease in NADPH oxidase activity along with DNA damage as confirmed by DNA laddering. The DNA instability was also assessed through sequence-related amplified polymorphism assay, thus suggesting the danger of walnut processing by-product and focusing the attention on the necessity of an efficient treatment of WHWW.

Keywords Allelochemical stress · *Zea mays* L. · ROS · Cell death · Antioxidant enzymes · SRAP assay

C. Ciniglia
Department of Environmental, Biological and Pharmaceutical Science and Technology Second University of Naples, Via Vivaldi 43, 81100 Caserta, Italy

F. Mastrobuoni · M. Scortichini · M. Petriccione (✉)
Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Fruit Trees Research Unit, Via Torrino 3, Caserta 81100, Italy
e-mail: milena.petriccione@entecra.it

Introduction

Allelopathy is a phenomenon involving beneficial or adverse effects through the release of secondary metabolites, defined as allelochemicals, in the environment (Inderjit 2003; Farooq et al. 2010). Allelochemicals interfere with the membrane permeability (Galindo et al. 1999), ion uptake (Lehman and Blum 1999), photosynthesis (Qian et al. 2009) and production of reactive oxygen species (Lara-Nunez et al. 2006; Singh et al. 2006); allelochemical stress can induce an imbalance in the oxidative status of cells, through changes in enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and non-enzymatic antioxidants including glutathione (GSH) and ascorbic acid (Lara-Nunez et al. 2006).

Previous studies in our laboratory have shown that the walnut green husk waste waters (WHWW), a by-product of the walnut production, possesses strong allelopathic properties (Ciniglia et al. 2012). Phytotoxicity tests and morphological investigations on representative crops such as lettuce, radish and maize indicated that WHWW can elicit a dilution-dependent stimulating effect on the growth of radish, lettuce cv. Cavolo Napoli, while an opposite inhibitory effect was observed on spinach, lettuce cvs. Gentilina and Canasta and maize (Ciniglia et al. 2012; Petriccione et al. 2013). This effect were exerted by the organic extractable fraction of WHWW and by the main phenolic component of such extract, 4,8-dihydroxy-1-tetralone, commonly known as regiolone (Ciniglia et al. 2012). The genotoxic and proaponecrotic potential of WHWW have been also assessed through DNA instability and chromatin modifications, thus suggesting the danger of walnut processing by-product and focusing the attention on the necessity of an efficient treatment of WHWW (Petriccione et al. 2013).

The purpose of the present study was to deepen on allelochemical stress caused by WHWW on roots of maize seedlings at biochemical and molecular level. The activity of some antioxidant enzymes such as CAT, APX, SOD, was evaluated. The membrane damage, the alteration of plasma membrane NADPH oxidase activity along with the levels of H_2O_2 have been also investigated. DNA integrity has been concurrently assessed, with multiple endpoints, including SRAP assay to confirm the presence of genetic damage and DNA laddering assay. The results were correlated with the expression of the metacaspase ZmMCII-1 and the mitogen-activated protein kinase ZmSIMK1, which are both involved in various abiotic stress.

Materials and methods

Plant material, growth conditions and WHWW treatment

Maize (*Zea mays* L. cv. Arkam) seeds were surface sterilized for 5 min in 5 % (v/v) commercial bleach. After rinsing several times with distilled water, seeds were soaked overnight at 4 °C.

The main by-product of walnut processing is the green husk which is removed from walnut shells by use of husk machines with abrasive metal elements operating while the walnuts are shaken under the pressure of water jets. This work-up results in the production of large amounts of wastewater (WHWW) containing the water extractable components together with hull residues.

Experiments were conducted in hydroponic growth systems in according to Conn et al. (2013). Maize seeds were germinated on a lid filled with an agarized germination medium immersed in non agarized germination medium. Following germination, 10-day-old maize seedlings were transferred to larger aerated hydroponics tanks with Hoagland solution where they remained until experimentation. In order to assess the toxicity of different treatments, maize roots were exposed directly in undiluted WHWW; Hoagland solution was used as a negative control. In each treatment, 80 maize seedlings were used and maize roots were sampled from plants 3, 6, 12 and 24 h after treatment. Twenty seedlings were scored each timing: three replicates of five plantlets were used for enzymatic assays while 5 seedlings were used for genomic analysis.

Enzyme extraction and assays

Maize roots were ground to a fine powder in liquid N_2 and then homogenized in ice-cold potassium phosphate buffer (100 mM pH 7.8) containing 2 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.0), 1 mM phenylmethylsulfonyl fluoride (PMSF); 0.2 % (v/v) Triton X-100; 5 % (w/w) insoluble polyvinylpolypyrrolidone with the addition of 5 mM ascorbate in the case of APX assay. The homogenate was centrifuged at $27.000 \times g$ for 20 min (4 °C). The supernatant was used as to determine protein concentration and enzyme activity measurement. The total soluble protein contents of the root extracts were determined according to Bradford (1976), with bovine serum albumin (BSA) as a standard. The soluble protein content was expressed as mg g^{-1} fresh weight (FW).

CAT (EC 1.11.1.6) activity was assayed according to Aebi (1984), with minor modifications. The H_2O_2 decrease was determined after the reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction was monitored measuring the decrease in the absorbance at 240 nm for 300s. The CAT activity was calculated according to the molar extinction coefficient of H_2O_2 (39.4 mM^{-1} cm^{-1}) and expressed as nmol H_2O_2 min^{-1} mg^{-1} protein.

APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada (1981). The oxidation of ascorbate was determined using the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA-Na$_2$, 0.5 mM ascorbic acid and 50 μl of crude enzyme extract. The reaction was started by the addition of 0.1 mM H_2O_2, and monitoring the decreasing absorbance at 290 nm for 300s. The APX activity was calculated according to the molar extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) and is expressed as nmol di H_2O_2 min^{-1} mg^{-1} protein.

SOD (EC 1.15.1.1) activity was assayed by the photochemical inhibition nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich 1971). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA and 30μl of enzyme extract and 2 mM riboflavin. The reaction was started by switching on the light (two 15 W fluorescent lamps) for 15 min, and the absorbance was measured at 560 nm. Two samples without the enzymatic extract and illumination were used as controls. One SOD unit was defined as the amount of enzyme corresponding to 50 % inhibition of the NBT reduction. The activity of the enzyme was expressed as units per 1 mg of protein (U mg^{-1} protein).

NADPH-oxidase (EC 1.11.1.7) activity was determined according to Tewari et al. (2013). The reaction mixture contained 10 μl of enzyme extract, 0.1 mM NBT, 0.2 mM NADPH, 0.1 mM MgCl$_2$, and 1.0 mM CaCl$_2$ in 1 ml of NADPH, and the change in absorbance at 530 nm was recorded. Rates of reduction of NBT were calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}. NADPH oxidase activity was expressed as μmol reduced NBT min^{-1} mg^{-1} protein.

Deoxyribonuclease (DNase) activity was assayed by measuring the release of acid-soluble material from denatured calf thymus DNA (Blank and McKeon 1989). The assay was prepared according to Tewari et al. (2013). DNase activity was expressed as U mg^{-1} protein.

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS), according to Heath and Packer (1968) with minor modifications. 1 g of roots were homogenized with 10 ml of TBA-TCA-HCl reagent (thiobarbituric acid 0.37 %, 0.25 N HCl and 15 % TCA). The mixture was incubated in boiling water for 30 min and the reaction was stopped by placing the sample in ice. The samples were centrifuged at 10,000 × g for 10 min and the absorbance of the supernatant was registered at 445, 532 and 600 nm. The amount of MDA–TBA complex (pink pigment) was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1} FW.

Hydrogen peroxide content

Hydrogen peroxide was measured spectrophotometrically after reaction with KI as described by Alexieva et al. (2001). The content of H_2O_2 was given on a standard curve with known concentrations of H_2O_2 and the values were expressed as μmol g^{-1} FW.

In situ localization of ROS

In situ localization of ROS was visualized using the highly sensitive, cell-permeable probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) with a fluorescence microscope. Root tips were incubated in 50 μM H2DCF-DA dye buffer (0.1 m NaCl/Pi, pH 7.0) for 30 min at 25 °C in the dark. The samples were washed three times with NaCl/Pi(pH 7.0) (Moschou et al. 2008). The images were taken (excitation: 480 nm, emission: 525 nm) with a fluorescence microscope (Nikon Eclipse E800 1, Tokyo, Japan) using a digital camera. To compare the density of H2DCF-DA, samples from different experimental conditions were set at the same time and analyzed under a fluorescence microscope using the same experimental parameters.

DNA laddering

Genomic DNA (10 μg) was loaded on 2 % agarose gel at 70 V for 2.5 h. DNA was stained by adding ethidium bromide to the agarose (0.5 g ml^{-1}) and were visualized under UV light with the ChemiDoc^{TM} XRS + System (BioRad, Milano) imaging system and analyzed by the ImageLab 3 software (BioRad, Milano) to estimate the internucleosomal fragmentation of DNA (Petriccione et al. 2013).

DNA extraction and SRAP analysis

Roots were used for DNA extractions. DNA was extracted using the procedure by Petriccione and Ciniglia (2012). The SRAP analysis was performed as described by Li and Quiros (2001). Thirteen random SRAP primer combinations were selected for SRAP analysis (Table S1). SRAP reactions were conducted in a 25 μl volume consisting of 2.5 μl of 10× PCR buffer, 1.5 mM MgCl$_2$, 1 U of Taq DNA polymerase, 200 mM of dNTPs, 0.3 mM of each primer, and template DNA approximately 50 ng. Polymerase chain reaction (PCR) was performed on BioRad Thermal Cycler (BioRad, Milan, Italy). The amplification reactions were carried out for 2 min at 94 °C as an initial denaturation. The PCR program comprised 5 cycles of 1 min at 94 °C for denaturation, 1 min at 35 °C for annealing, and 1 min at 72 °C for extension and 35 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing, and 1 min at 72 °C for extension; a final extension was performed at 72 °C for 7 min. A negative control, without genomic DNA, was run with every set of samples to confirm that no contaminating DNA was present in the reactions.

SRAP products were loaded onto 2 % (w/v) agarose gels (Sigma, USA) in 1x TAE buffer with 0.5 μg/mL ethidium bromide at 80 V constant voltage. For evaluating the base pair length of bands, DNA ladder 1 Kb (Fermentas, Italy) was loaded on first lane of each gel. Gels were visualized under UV light with the ChemiDoc^{TM} XRS+System (BioRad, Milano) imaging system and analyzed by the ImageLab 3 software (BioRad, Milano) to estimate automatically the length of bands into amplification products.

In order to confirm the reproducibility of the amplifications and the length of the fragments, the amplifications for each primer were repeated three times. The SRAP banding patterns were scored as appearance (1) and lost (0) of bands and only reproducible and clear bands were scored for the construction of the data matrix.

Changes in the SRAP profiles were expressed as Genomic template stability (GTS, %) calculated by the equation (Körpe and Aras 2011):

$$GTS = \left(1 - \frac{a}{n}\right) \times 100$$

where a is the average number of polymorphic bands in each treated sample and n the number of all bands in the

untreated sample. The difference of the value of GTS compared to the value 100 indicates the degree of polymorphism (P).

Real-time quantitative PCR analysis

Total RNA was isolated of treated and untreated maize roots by using the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total RNA was treated with DNase (Invitrogen Life Technologies, Carlsbad, CA, USA) and the concentration of RNA determined by UV/visible spectroscopy, while its structural integrity was checked on a non-denaturing agarose gel, followed by ethidium bromide staining. First-strand cDNA was synthesized from 3 μg of total RNA using oligo(dT)20 primers and the ThermoScript RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA), according to manufacturer's recommendations. Primer oligonucleotide pairs were designed by using Primer3 software and reported in Table S2. Quantitative Real time-PCR was performed using a CFX Connect Real-Time PCR Detection Systems (Bio-Rad) to analyze the specific expression patterns of maize MAP Kinase 1 (SIMK1) and Metacaspase type II (MCII-1) gene. cDNA was amplified in 96-well plates using the SsoAdvancedTM SYBR® Green Supermix (Bio-Rad), 25 ng of cDNA and 300 nM specific sense and anti-sense primers in a final volume of 20 μl for each well. Each sample was analysed in triplicate. A sample without template, was used as negative control, and a sample with not retro-transcribed mRNA instead of template cDNA, was used as control for genomic DNA contamination. Cycling parameters were: denaturation at 95 °C for 10s and annealing/extension at 60 °C for 30s (repeated 40 times). In order to verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol. The reference control gene (actin) was measured with three replicates in each PCR run, and its average Ct value was used for relative expression analysis. Relative fold changes in gene expression was calculated using the comparative $2^{-\Delta\Delta C_t}$ method and normalized to the corresponding reference gene levels (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Three biological and three technical repetitions were performed for each treatment and time point.

All the experiments were replicated thrice and all data were expressed as mean ± standard deviation. Significant differences between means were determined by ANOVA and subsequent multiple pairwise comparison by least significant differences (LSD) test. All statistical tests were carried out using SPSS (v. 20.0) software. Differences at $p < 0.05$ were considered significant.

Results

Effect of WHWW on H₂O₂ content and antioxidant enzyme activities

A progressive increase in H_2O_2 content was registered in maize roots under WHWW, thus confirming the oxidative stress of the by-product (Fig. 1). The highest values was recorded after 12 h (115.15 ± 5.7 μmol H_2O_2/g FW), although a statically significant amount was observed in early-stages of experiment (97.23 ± 3.5 μmol H_2O_2/g FW; at 6 h).

As a response to oxidative stress, the enzymatic activities of CAT, APX, SOD were measured in order to analyze the defense response triggered in maize roots under WHWW treatment. CAT activity increased in maize root of untreated and treated plants on a time-course basis. The activity of this enzyme dramatically increased in treated roots after 3 h (85 % compared to mock treatment) reducing to 20 % from 6 to 24 h (Fig. 2a). APX activity trend in maize roots was comparable between untreated and treated plants; all values were significantly ($p < 0.05$) lower than the mock treatment except after 6 h (Fig. 2b). SOD activity showed not statically significant difference between WHWW and mock treatments roots up to 3 h from the onset of WHWW exposure while from 6 to 12 h the SOD activity in treated roots was much higher than the mock treatment, reaching an increase of 3.0-fold to 24 h (Fig. 2c).

Lipid peroxidation and membrane damage

MDA is a marker of oxidative damage by ROS due to peroxidation of unsaturated lipids in biological membranes. To verify the potential ROS damage induced by WHHW treatment during 24 h, MDA content was
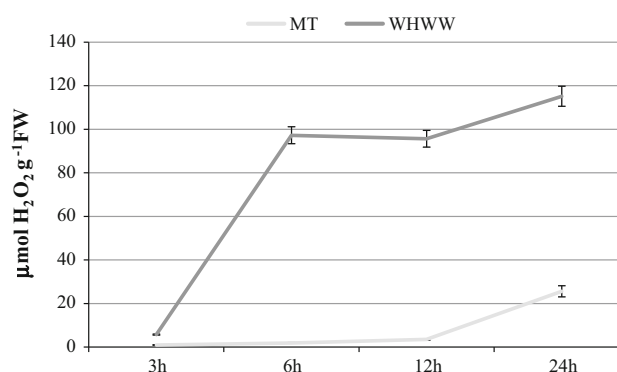

Fig. 1 H₂O₂ content in maize roots treated by WHWW and mock treatment (MT) for 24 h
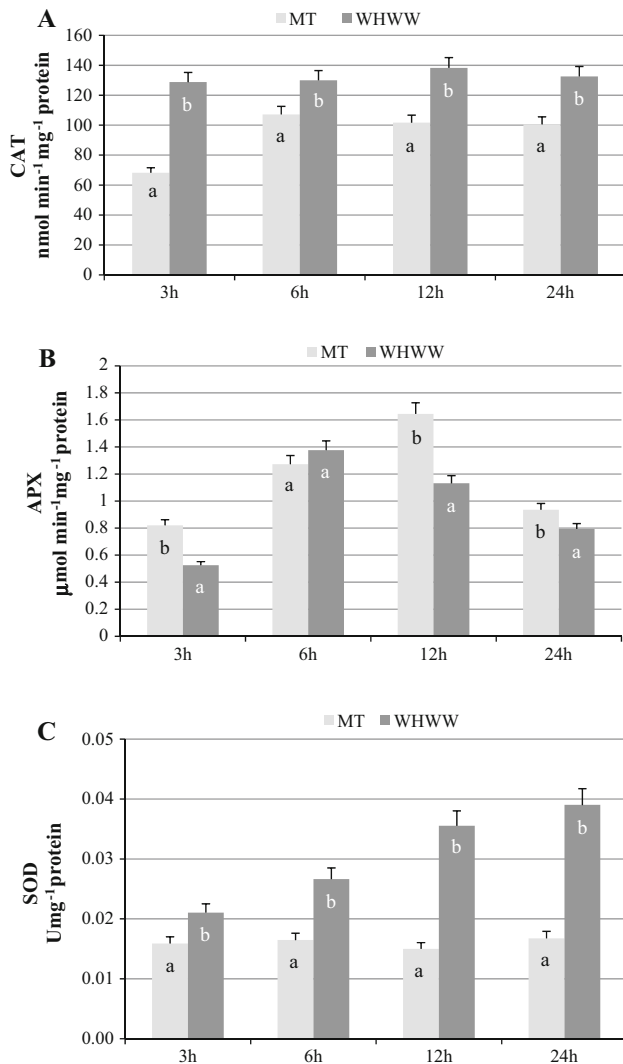
Fig. 2 Activity of catalase **a** ascorbate peroxidase **b** and superoxide dismutase **c** in maize roots treated by WHWW and mock treatment (MT) for 24 h. Mean (±SD) was calculated from three replicates. *Bars* with *different letters* are significantly different at $p < 0.05$

Fig. 3 MDA content in maize roots treated by WHWW and mock treatment (MT) for 24 h. Mean (±SD) was calculated from three replicates. *Bars* with *different letters* are significantly different at $p < 0.05$

Fig. 4 Activity of NADPH-oxidase in maize roots treated by WHWW and mock treatment (MT) for 24 h. Mean (±SD) was calculated from three replicates. *Bars* with *different letters* are significantly different at $p < 0.05$

In situ localization of ROS

The level of ROS in maize root tips exposed to WHWW treatments were evaluated by using the dye 2′,7′-dichlorodihydrofluorescin-diacetate that is particularly sensitive to hydrogen peroxide, forming the highly fluorescent 2′,7′-dichlorofluorescein compound. ROS accumulation in maize roots treated for 24 h was detected in vivo under a fluorescence microscope (Fig. 5). The images revealed that the roots treated with WHWW had higher fluorescence intensity throughout the root and apex than the mock treatment reflecting the accumulation of ROS, strictly correlated to H_2O_2 content (Fig. 1).

Alteration of DNA integrity

WHWW activated DNase inducing DNA fragmentation into oligonucleosomal units in roots of maize seedlings.

determined on maize roots (Fig. 3). MDA content clearly indicated that the levels of lipid peroxidation increased significantly owing to WHWW treatment in maize roots. A significant increase in MDA content was observed in WHWW-treated roots compared to untreated ones after 6 h exposure (68 %; $p < 0.05$); thereafter it remained at constant values until the end of the experiment. Furthermore, MDA content was significantly correlated with generation of H_2O_2 in maize root ($R^2 = 0.997$; Fig. 1). NADPH-oxidase activity did not show statistically significant differences compared to the mock treatment after 3 h from WHWW exposure. 28, 37 and 67 % reductions in NADPH-oxidase activity were observed after 6, 12 and 24 h, respectively (Fig. 4).
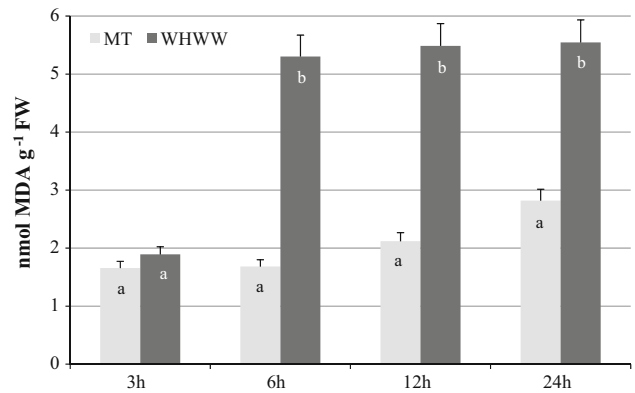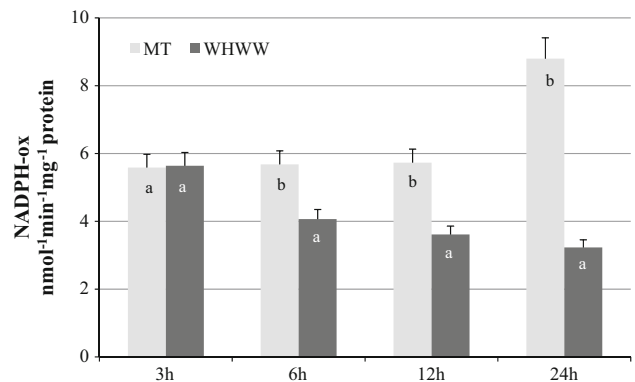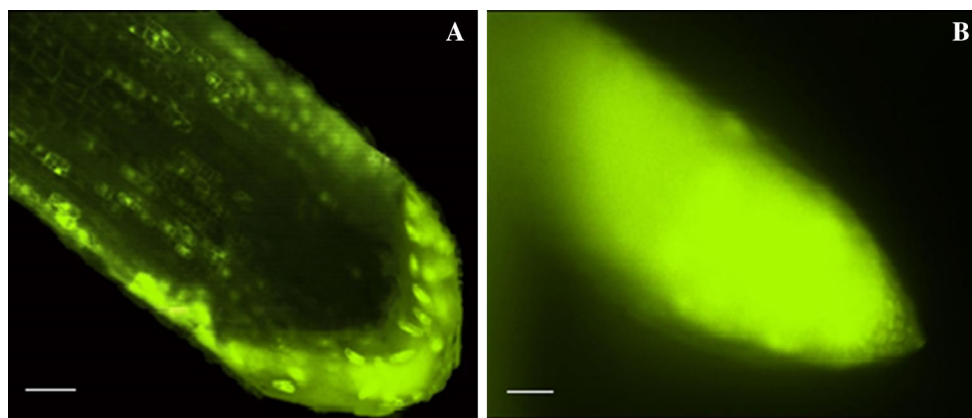
Fig. 5 Intracellular ROS detection by H2DCF-DA fluorescence microscopy in roots on maize seedlings untreated **a** or treated by WHWW **b** after 24 h on the exposure. *Bar* = 1 mm
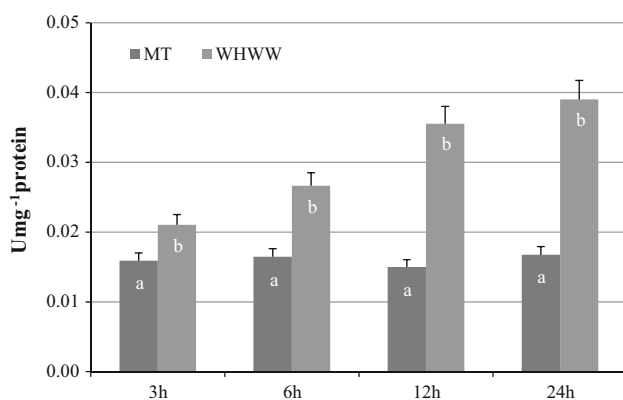

Fig. 6 Activity of DNAse in maize roots treated by WHWW and mock treatment (MT) for 24 h. Mean (±SD) was calculated from three replicates. *Bars* with *different letters* are significantly different at $p < 0.05$

These alterations were correlated to the time of WHWW exposure. DNase activity showed higher levels in WHWW-treated roots compared with untreated at all sampling hours, although the maximal inductions were obtained after 12 and 24 h with 50 ($p < 0.05$) and 57.7 % ($p < 0.05$), respectively (Fig. 6).

Integrity of the total DNA at different times after the treatment was analyzed by agarose gel electrophoresis. DNA isolated from untreated root cells from all maize seedlings was never fragmented into oligonucleosomal units and formed a single, high molecular weight band, while DNA isolated from treated root cells showed the characteristic "ladder" pattern of discontinuous DNA fragments. DNA laddering revealed significant increase of DNA fragmentation after 24 h of exposure to WHWW treatment with 71.6 % of DNA degradation compared to mock treatment (Fig. 7).

SRAP assay

SRAP assay has been tested as a valuable marker of genotoxicity on maize roots affected by WHWW in a time dependent manner. A total of 30 primer combinations were screened, 24 of them gave stable and reproducible amplification patterns.

Table 1 shows a summary of all SRAP profile modifications and products of selected primers. Total number of bands produced in untreated and treated maize roots were 228, (152 in mock treatment), with a molecular weight from 100 to 3283 bp and an average number of 9.5 amplified bands for each primer pair. Among 228 bands, 140 were polymorphic bands, with an average of 5.8 polymorphic bands per primer pair. SRAP profile in treated roots showed substantial differences between mock treatment and treated plants, in a time-dependent manner. The polymorphisms were caused by the appearance (a) and/or the loss (b) of amplified bands into treated samples compared with the mock treatment and the obtained results are listed in Table 1. Total number of bands increased with time (103 after 24 h). The number of disappeared SRAP bands was in all cases higher than the number of appeared ones. Changes in SRAP profiles, expressed as genomic template stability (GTS, %) were calculated (Table 1). GTS values strongly decreased in treated plantlets with increasing time (from 66.49 to 46.07).

A calculation of distances between WHWW and mock treatments were performed by clustering analysis method, in order to obtain similarity values and an accurate estimate of the level of DNA polymorphisms; for each amplification pattern, a similarity matrix and a dendrogram was realized. Dendrogram showed two major clusters (Fig. S1); the first clustering included only mock treatment. The second clustering included all the other treatments; thus confirming the genotoxic time-related effect of the WHWW.

Fig. 7 Image analyses of nucleosomal fragmentation in maize roots induced by exposure to WHWW after 3, 6, 12, and 24 h

Control

3h

6h

12h

24h
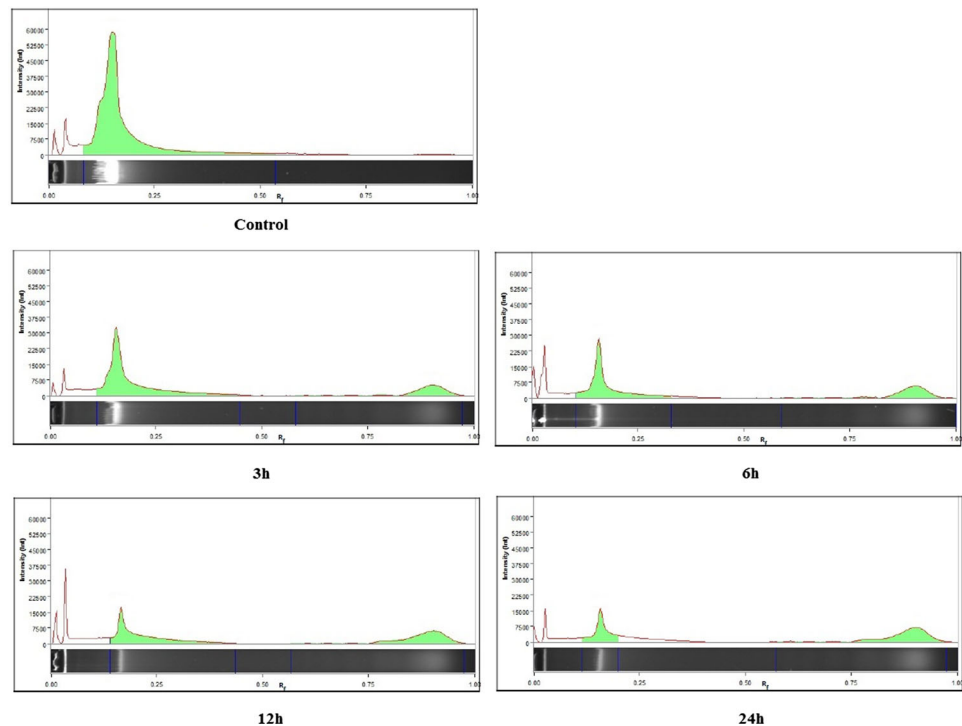
Gene expression analysis

Quantitative data of SIMK1 and MCII-1 gene expression in roots of WHWW treated maize seedlings are showed in Fig. 8. The absence of non-specific PCR products and primer dimer artefacts was checked by melting curves for each gene. WHWW significantly up-regulated the relative expression of SIMK1 and MCII-1. The transcript level of SIMK1 was elevated after 3 h (tenfold), reaching more than 45-fold, clearly detected after 6 h of treatment (Fig. 8), whereas no expression was detected in stressed maize roots after 24 h. The same trend was observed in MCII-1 gene expression, after 3 and 6 h of stress the expression of this gene was up regulated approximately 127-fold and 275-fold over the mock treatment, respectively.

Discussion

The walnut husk washing waters (WHWW) are a typical by-product of Mediterranean walnut production, which, according to our previous papers, possesses a strong allelopathic potential and a phytotoxic effect on maize and lettuce plants (Ciniglia et al. 2012; Petriccione et al. 2014). Since it is known from literature that allelochemicals can trigger oxidative stress (Lara-Nunez et al. 2006; Abenavoli et al. 2006), in this paper we investigated the effects of WHWW on the antioxidant system of maize. High level of

hydrogen peroxide has been detected in maize roots thus suggesting that this compound is one major contributor to oxidative damage induced by WHWW. Antioxidant enzymes are the most basic defense systems to environmental stress (Gill and Tuteja 2010); plants increase the activity of some antioxidant enzymes in an attempt to counteract the harmful effects of ROS generated either by the various oxidative states of allelochemicals themselves or by a plant signaling cascade that is induced by the allelochemical. In other cases, the allelochemical might directly inhibit oxidizing enzymes in some way, leaving the plant vulnerable to oxidative damage (Weir et al. 2004). CAT and APX are involved in detoxification of hydrogen peroxide in plant cell, utilizing the same substrate with different affinities because they belong to two different classes of H_2O_2-scavenging enzymes (Racchi 2013). High H_2O_2 registered in WHWW treated roots could have triggered the increase of CAT observed after 6 h. CAT is characterized by very high turnover number but low affinity to its substrate, implying an extremely fast rate of H_2O_2 decomposition. This enzyme is an effective tool involved in H_2O_2 detoxification when intracellular H_2O_2 concentration dramatically increases (Lara-Nunez et al. 2006; Ben Ahmed et al. 2009). Our results are in agreement with previous studies that showed the increase of the CAT activity in response to allelochemical stress induced by aqueous leachate of *Sicyos deppei* on tomato roots (Lara-Nunez et al. 2006), or to allelochemical compounds such as ferulic and benzoic acid tested on maize seedlings and

Table 1 Total bands in mock treatment (MT) and polymorphic bands in maize treated radicles

Primers	MT	3 h		6 h		12 h		24 h	
		a	b	a	b	a	b	a	b
EM1/M2	8	0	6	1	6	1	2	1	6
EM1/M5	3	0	1	1	3	0	3	1	3
EM2/M1	9	2	5	3	6	2	6	2	6
EM2/M3	4	0	3	1	2	1	3	0	4
EM2/M4	4	1	1	0	2	1	2	0	2
EM2/M5	6	1	1	0	2	0	1	0	4
EM3/M1	5	0	1	1	1	1	2	2	2
EM3/M2	4	0	3	0	1	0	1	0	3
EM3/M3	4	0	1	1	1	1	2	1	3
EM3/M4	4	0	4	2	3	0	2	0	3
EM3/M5	9	0	1	0	8	2	5	0	8
EM4/M1	8	0	1	1	2	1	3	0	3
EM4/M2	8	0	0	3	3	0	1	1	3
EM4/M3	7	1	1	2	1	2	1	2	1
EM4/M4	4	0	1	0	2	0	2	0	2
EM4/M5	9	0	4	0	7	0	6	0	7
EM5/M1	4	2	1	3	0	4	1	1	2
EM5/M2	8	0	6	1	7	1	7	1	7
EM5/M5	4	1	0	0	1	1	1	0	2
EM6/M1	7	0	1	1	1	1	1	2	1
EM6/M2	11	0	3	0	5	0	8	0	6
EM6/M3	6	5	2	1	2	4	2	2	2
EM6/M4	10	0	3	0	3	0	4	0	4
EM6/M5	6	0	1	0	2	2	3	0	3
TOT$_{control}$	152	13	51	22	71	25	69	16	87
TOT$_{(a+b)}$		64		93		94		103	
P		33.51		48.69		49.21		53.93	
GTS (%)		66.49		51.31		50.79		46.07	

GTS genomic template stability, *a* appearance of new bands, *b* disappearance of normal bands, *P* degree of polymorphism

Fig. 8 Expression of MAP Kinase 1 (SIMK1) **a** and Metacaspase type II (MCII-1) **b** gene in maize roots after WHWW and mock treatments for 24 h. Values are means of three replicates ±SD, normalized to internal control gene. Samples without visible *bars* have expression level below the threshold of detection

cucumber cotyledons, respectively (Devi and Prasad 1996; Maffei et al. 1999). APX is another important enzyme involved in the ascorbate–glutathione cycle, a major hydrogen peroxide detoxifying system, in which, it plays a key role catalyzing the reduction of H_2O_2 into H_2O, using ascorbate as a specific electron donor (Asada 1992; Shigeoka et al. 2002; Caverzan et al. 2012). WHWW inhibits the APX activity, an enzyme with a high H_2O_2 affinity but its activity depends on ascorbate as a reductant. This latter decreases in abiotic stress as demonstrated by Jin et al. (2003) in some conifers. SOD is a key enzyme in the detoxification of O_2^- which is converted in H_2O_2 (de Azevedo Neto et al. 2005; Foyer and Noctor 2005) and its activity is the first line of defense against ROS. In this study, SOD activity showed a significant increase during
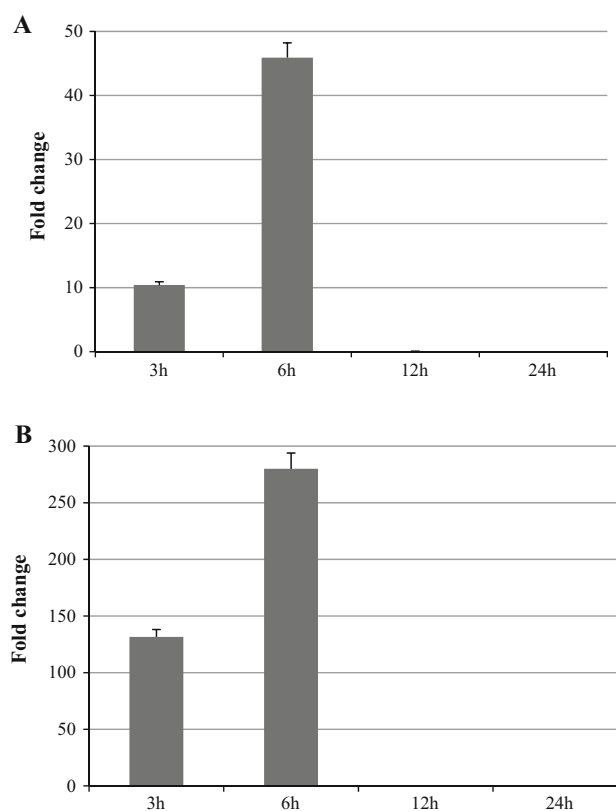
WHWW treatments as a signal of a high production of superoxide anion, followed by H_2O_2 accumulation. Previous studies have demonstrated that allelopathic cucumber root exudates/extracts and phenolic acids significantly increased SOD activity (Yu et al. 2003). Conversely, it was also reported that some allelochemicals reduce SOD activity. For example, the phytotoxic allelochemical secalonic acid F, from the fungus *Aspergillus japonicus*, significantly reduced SOD and POD activity in rape seeds, cucumber, corn and sorghum (Zeng et al. 2001). Likewise, aqueous extracts from rice reduced SOD activity in barnyard grass (Lin et al. 2000). In other studies, Sánchez-Moreiras and Reigosa (2005) showed that 2(3*H*)-benzoxazolinone (BOA) severely inhibited SOD activity in lettuce leaves and roots, and similar effects were reported for the aqueous leachate of *Sicyos deppei* on tomato roots (Lara-Nunez et al. 2006). The balance between SOD and APX or CAT activities in cells is crucial as well for determining the steady-state level of superoxide radicals and hydrogen peroxide.

WHWW in maize seedlings also induced ROS generation in the root meristematic region and into the central

elongation zone; other allelochemicals are known to induce ROS production as demonstrated in roots of *Arabidopsis thaliana* treated with (−)-catechin, an allelochemical from the invasive weed *C. maculosa* (Bais et al. 2002, 2003). ROS are produced through different metabolic pathways (Apel and Hirt 2004) and can affect membrane permeability, cause damage to DNA and protein, induce lipid peroxidation, and ultimately lead to programmed cell death (PCD) (Demidchik 2014).

Allelopathic compounds, including phenols can induce changes in the structure and permeability of plasma membranes in several plant species (Glass and Dunlop 1974; Macri et al. 1986; Politycka 1996; Zeng et al. 2001). This might be attributed to significant increase of enzyme involved in peroxidation of lipids, with the formation of hydroxyperoxides. One of them, malonyldialdehyde (MDA), is one of the final products of polyunsaturated fatty acids peroxidation in the plant cells and is known to be an indicator of peroxidation (Cruz-Ortega et al. 2002; Lara-Nunez et al. 2006). The MDA content markedly increased by WHWW treatment in roots of maize seedlings in time dependent manner. Our results are in agreement with previous studies that showed the increase of the MDA in response to allelochemical stress induced by aqueous leachate of *Sicyos deppei* on tomato roots (Lara-Nunez et al. 2006), and by benzoic acid on wheat seedlings (Yadav and Singh 2013). A prolonged contact of maize roots with WHWW would allow the phytotoxic substances (Ciniglia et al. 2012) to modify root membrane structure and its functions, as well as demonstrated by Asfi et al. (2012) on spinach plant treated with olive oil mill wastewater.

It is known that NAD(P)H-oxidoreductases are responsible of ROS production in the regulation of defense strategies upon infection with pathogens (Mehdy et al. 1996). In this study, in according with Lara-Nunez et al. 2006, we determined if the plasma membrane NADPH oxidase could be involved in the production of ROS during allelochemical stress. The decreasing activity of NADPH oxidase observed in WHWW treated plants along the experiment, could be associated with membrane damage caused by both the generation of ROS at 24 h and by a direct interaction of allelochemical compounds to the membrane.

ROS are a major source of nuclear, mitochondrial, and chloroplastic DNA damage (Sharma et al. 2012). Oxidative attack on sugar and the base moieties in DNA results in degradation, single strand breakage, and cross-linking to protein. Here we applied SRAP-assay to reveal the genotoxic potential of WHWW, already assessed through a multimethod approach including other molecular markers as RAPD-assay (Petriccione et al. 2013). SRAP assay has been used for several purposes, such as gene tagging (Zhang et al. 2009; Chen et al. 2012), genetic map construction (Yu et al. 2007; Gulsen et al. 2010), map-based

cloning (Zhang et al. 2010) and genetic diversity analysis (Gulsen et al. 2009; Xie et al. 2009; Dai et al. 2012). In this study SRAP-assay has been chosen to detect DNA damage as previously demonstrated by Deng et al. (2013). In our study, the main changes in SRAP patterns generated from the DNA of WHWW-treated maize roots comprised a loss and/or gain of bands compared with the patterns in untreated roots. The polymorphisms detected through SRAP could be ascribable to the changes in oligonucleotide priming-sites and/or interactions of DNA polymerase with damaged DNA. The present studies indicate a decrease in GTS in maize root following exposure to WHWW in time-dependent manner, thus confirming SRAP-assay as a useful tool to reveal genomic alterations in eco-toxicology studies.

ROS have been proposed as key modulators of PCD in plant, but the complex ROS signaling networks, during PCD, is poorly characterized. Furthermore PCD induction needs a particular timing and intensities of oxidative stress (De Pinto et al. 2013). H_2O_2 is the most stable ROS with essential physiological role (Demidchik 2014) and is considered a signal molecule in PCD (Gechev and Hille 2005). Liu et al. (2007) demonstrated that H_2O_2 is a molecule signal which can activate kinase cascades of the mitogen-activated protein kinase (MAPK), involved in PCD in tobacco. MAPK cascade is the major pathway that transduces extracellular stimuli into intracellular responses in all eukaryotic cells induced by biotic and abiotic stresses (Pitzschke and Hirt 2006; Wu et al. 2011). Several MAPKs were characterized in maize. However Wu el al. (2011) demonstrated that some MAPKs such as ZmSIMK1 were expressed in response to salt stress and under various abiotic stresses and signal molecules (Gu et al. 2010). ZmSIMK1 was highly expressed in maize seedlings by WHWW. Plant metacaspase may play a caspase-like role in the PCD pathway induced by oxidative stresses (Cui et al. 2013) and the number of metacaspase genes in the genomes of different organisms varies considerably (Tsiatsiani et al. 2011). Here, we showed that a maize metacaspase, ZmMCII-1, was induced during WHWW treatment and the transcript accumulation occurred until 6 h of exposure. Our results are in agreement with Ahmad et al. (2012) which showed, for the first time, in maize leaves, the up-regulation of three type II metacaspase genes in response to other abiotic stress. The no expression of ZmSIMK and ZmMCII-1 after 24 h could be due to post-transcriptional regulations, which are known to play an important role in how plants respond to stress. Further investigations are necessary to determine the relationship between ZmSIMK and type II metacaspase activation and development of PCD in response to allelochemical stress.

PCD of the cells was reached when the roots were treated with WHWW, as demonstrated by the formation of

the characteristic ladder pattern of DNA migration. In plant cells, progressive internucleosomal fragmentation was documented during PCD under different biotic and abiotic stress (Koukalova et al. 1997; Fojtova and Kovarik 2000).

The first step of PCDprocess is the cell membrane alteration (Jan et al. 2008), while the nucleus is the last organellum to be degraded during cell death (van Doorn and Woltering 2005; Byczkowska et al. 2012). In our previous study we also observed condensation and degradation of nuclear chromatin with AO/EB staining in WHWW-treated maize roots (Petriccione et al. 2014). Thus, an active process of cell death is induced by WHWW, as suggested by these hallmarks that are ascribable to an autolytic kind of PCD process, as recently defined in plants, in which the term apoptosis has been abandoned (Van Doorn 2011; Van Doorn et al. 2011).

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