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Ozonated water reduces susceptibility in tomato plants to *Meloidogyne incognita* by the modulation of the antioxidant system

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SUMMARY

Few studies have been carried out on the effect of ozonated water (O_3wat) on the oxidative stress of root systems and, in particular, in combination with biotic stress. The aim of this study was to determine whether aqueous ozone is effective in the control of root-knot nematode (RKN) infection and to investigate the concomitant changes in the basal defence system. A tomato cultivar susceptible to *Meloidogyne incognita* was treated with O_3wat as a soil drench. No negative effects were seen following ozone application in comparison with the control under the exposure conditions used. The treatment reduced significantly the nematode infection rate and induced changes in the morphology of nematode feeding sites, some of which were characterized by visible symptoms of senescence. The antioxidant response, as well as parameters of oxidative damage, were examined in untreated and O_3wat-treated galls at 2, 4 and 7 days after inoculation and compared with uninfected roots. High levels of reactive oxygen species (ROS), H_2O_2 and malondialdehyde were generated in galls in response to combined abiotic and biotic stresses. Throughout the experimental period, the activities and relative transcript levels of the antioxidant enzymes catalase, superoxide dismutase and ascorbate peroxidase produced different responses when exposed to ozone treatment and/or infection. The results demonstrate how O_3wat protects tomato against the RKN *M. incognita* through the modulation of basal defence mechanisms.

Keywords: antioxidant systems, defence inducer, feeding sites, *M. incognita*, molecular responses, oxidative stress, ozone.

INTRODUCTION

Several studies have carefully analysed atmospheric ozone (O_3) in the context of plant physiology in terms of its negative impact on the growth and yield of important crop species (Wilkinson *et al.*, 2012). However, ozone is a strong oxidant that has been used as a sanitizing agent for the control of pathogens and many chemical contaminants in a wide range of water and wastewater streams (Graham *et al.*, 2009), as well as for the disinfection and preservation of fresh produce (Tzortzakis *et al.*, 2011; Zhang *et al.*, 2005). As an antimicrobial agent, ozone is active against a broad spectrum of micro-organisms and can be effectively used in both gaseous and/or aqueous states (Perry and Yousef, 2011). Although ozone gas has both well-known and well-characterized phytotoxic effects, these properties are altered when it is applied in aqueous form (Fujiwara and Fujii, 2002). Recently, interest has been shown in the use of sprayed ozonated water (O_3wat) directly onto plants in order to control diseases. For example, O_3wat has been used as an alternative to chemicals for the control of powdery mildew on cucumbers (Fujiwara and Fujii, 2002; Fujiwara *et al.*, 2009) or other airborne diseases, including early blight and leaf mould on tomato (He *et al.*, 2015). Few studies have explored the impact of O_3wat on the control of soil-borne pathogens on tomato (Ciccarese *et al.*, 2006) and little information exists on the use of O_3wat as a new sustainable pest control strategy against nematodes (Sasanelli *et al.*, 2010). Moreover, the mechanisms underlying the effect of O_3wat on the interaction between nematodes and their host plants have not been studied to date. Root-knot nematodes (RKNs) (*Meloidogyne* spp.) represent a major problem for protected crops, especially in Mediterranean countries, where climatic conditions favour their development. *Meloidogyne incognita* is an obligate endoparasite that feeds exclusively on the cytoplasm of living plant cells (Moens and Perry, 2009). Once the infective second-stage juvenile (J2) penetrates inside the root tip, it establishes and maintains a permanent group of cells, known as giant cells (GCs), which serve as the exclusive nutrient source for the developing nematode (Gheysen and Mitchum, 2011).

Plant responses to stresses are highly complex and involve changes at the transcriptome, cellular and physiological levels. Recent evidence has shown that plants respond to multiple stresses differently from how they respond to individual stresses. The occurrence of simultaneous biotic and abiotic stresses presents an added degree of complexity, because the presence of an abiotic stress can have the effect of reducing or enhancing the susceptibility to a biotic

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pest or pathogen, and vice versa (Atkinson and Urwin, 2012; Suzuki *et al.*, 2014). A common plant defence response to stresses, including ozone and pathogen infection, is the production of reactive oxygen species (ROS) (Eastburn *et al.*, 2011; Fujita *et al.*, 2006; Pastori and Foyer, 2002). ROS also play a major role in plant–nematode interactions, where an initial, very rapid, burst occurs in both compatible and incompatible interactions (Melillo *et al.*, 2006). The production of superoxide and hydrogen peroxide contributes to both the killing of the pathogen and/or the activation of further defence reactions (Li *et al.*, 2015; Melillo *et al.*, 2011). Plant responses to elevated ozone resemble defence reactions to pathogens (Blokhina *et al.*, 2003; Mittler, 2006; Singh *et al.*, 2002). In addition, acute ozone induces a hypersensitive response (HR)-like localized cell death in plants similar to the resistance gene-mediated host defence response (Overmyer *et al.*, 2005). High accumulation of ROS in the apoplast leads to oxidative stress in plant cells which, in turn, causes membrane lipid peroxidation and damages macromolecules, including proteins, DNA and lipids. However, recent studies have focused on the role played by ROS as essential integral signalling components in the regulation of numerous biological processes, such as growth, development and responses to biotic and/or abiotic stimuli in plants (Mittler *et al.*, 2011). To utilize ROS signalling molecules, non-toxic levels must be maintained in a delicate and elaborate plant ROS network in which enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), together with non-enzymatic antioxidants, contribute to ROS homeostasis (Gechev *et al.*, 2006; Mittler *et al.*, 2004). The differential regulation of these enzymes may contribute to increases in ROS and the activation of defences following the perception of combined stresses.

Here, we examine the potential downstream benefits of treating a susceptible tomato cultivar with O_3wat as an abiotic elicitor of resistance or tolerance. The specific aims of this study were as follows: (i) to determine whether O_3wat has an impact on tomato growth when applied directly to roots; (ii) to examine the efficacy of O_3wat in containing *M. incognita* infection and its effect on the development of nematode feeding sites; (iii) to investigate the role of O_3wat and nematode infection, alone or in combination, in triggering the plant defence response by evaluating the induction of ROS and H_2O_2; and (iv) to analyse the biochemical and molecular changes in ROS-scavenging enzymes, such as SOD, APX and CAT, and related gene expression.

RESULTS

Effects of O_3wat on tomato growth and rate of pathogenesis

A controlled-environment experiment was carried out in order to examine more closely the effect of O_3wat treatment on tomato. Tomato seedlings treated with O_3wat as a soil drench were analysed 14 days after treatment. As shown in Table 1, there were no significant differences in shoot and root fresh weight in uninfected and infected treated plants, indicating that the direct contact of O_3wat with roots has no phytotoxic effect on the biomass accumulation of tomato. Moreover, no visible morphological differences were observed in O_3wat-treated seedlings relative to the untreated plants (Fig. S1, see Supporting Information).

Table 1 Effect of ozonated water (O_3wat) treatment (10 ppm) on plant growth and *Meloidogyne incognita* infection on *Solanum lycopersicum* cv. Roma.

	Shoot weight (g)	Root weight (g)	Galls/ plant	Giant cell area (μm^2)
Untreated	4.97 ± 0.71a	1.97 ± 0.31a	–	–
Untreated + N	5.13 ± 0.61a	2.00 ± 0.27a	82 ± 3	$21\ 918 \pm 1472$
O_3wat	5.27 ± 0.53a	2.01 ± 0.26a	–	–
O_3wat + N	6.42 ± 0.32a	1.94 ± 0.15a	63 ± 2**	$17\ 212 \pm 1094$*

Data represent fresh shoot and root weights and number of galls per plant from 15 plants at 14 days post-inoculation (dpi) in three independent experiments. Giant cell area was measured on 15 giant cells for each treatment.
Measurements are represented by the mean ± standard error (SE). Values followed by the same letter do not differ at $P \leq 0.05$ (least-significant difference test). Asterisks indicate statistically significant difference (Student's *t*-test: $*P < 0.05$, $**P < 0.01$).
Untreated, untreated and uninfected; Untreated + N, untreated and *Meloidogyne incognita*-infected; O_3wat, O_3wat-treated and uninfected; O_3wat + N, O_3wat-treated and infected.

To establish the effectiveness of O_3wat as an elicitor of plant resistance and/or its direct effect on nematodes, two different trials were set up in the glasshouse. The first experimental trial was performed on tomato plants previously treated for 4 days and then infected with *M. incognita*. The second trial was carried out on plants infected and treated with O_3wat at the same time. Preliminary experiments showed that O_3wat treatment did not affect the viability of J2s directly (data not shown).

Both O_3wat treatments, before or after nematode inoculation, did not influence the dry top weight of tomato plants and suppressed nematode reproduction in comparison with the untreated control (Table 2) ($P \leq 0.01$). The root gall indices observed in treated tomato plants (1.6 and 1.9, respectively) were significantly lower than that observed in the untreated control (3.9). Eggs and juveniles recovered from treated infested roots, the total nematode population density from roots and soil as well as the reproduction rate were also significantly lower than those observed in the untreated control. No significant differences were evident in all nematological parameters between the two trials (Table 2). Therefore, all further analyses were carried out by inoculating nematodes after treatment of the plants with O_3wat.

Nematode feeding site development

As ozone treatment affected *M. incognita* infection by causing a significant reduction in the number of galls in O_3wat-treated roots at 14 days post-inoculation (dpi) relative to untreated roots

Table 2 Effect of ozonated water (O_3wat) treatment (10 ppm) as a soil drench on the *Meloidogyne incognita*–tomato interaction (cv. Roma).

Treatment	Dry top weight (g)	Root gall index (0–10)	Eggs and J2/g root (10^{-3})	Total nematode population (eggs and J2/mL soil) (10^{-3})	Reproduction rate r (P_f/P_i)
O_3wat after nematode inoculation	4.3 ± 0.3 a	1.9 ± 0.2a	1.6 ± 0.1a	30.0 ± 5.4a	120 ± 21.8a
O_3wat before nematode inoculation	4.6 ± 0.3 a	1.6 ± 0.3a	1.5 ± 0.1a	41.7 ± 3.3a	167 ± 13.3a
Untreated control	4.7 ± 0.4 a	3.9 ± 0.3b	4.5 ± 0.6b	127.4 ± 17.7b	509 ± 70.9b

Values are the mean ± standard error (SE) of eight replicates in two independent experiments.
Data flanked in each column by the same letters are not statistically significantly different according to the least-significant difference test ($P \leq 0.01$).

(−23%, $P < 0.01$) (Table 1), we decided to monitor possible cellular alterations in the development of the feeding sites.

A detailed microscopic analysis was performed at 14 dpi in untreated and O_3wat-treated galls. Serial cross-sections (2.5 μm) of 15 untreated and O_3wat-treated galls, cut through their entire length, were examined under a light microscope. Untreated galls contained typical multinucleate GCs occupying the entire vascular cylinder. Within GCs, the central vacuole disintegrated into smaller vacuoles and was replaced with a dense granular cytoplasm containing numerous organelles (Fig. 1a). The observation of more than 250 sections of O_3wat-treated galls (from three different biological replicates) revealed that some GCs showed an appearance comparable with that of untreated galls (Fig. 1b), whereas other GCs had enlarged vacuoles, less dense cytoplasm and dark-stained deposits on their cell walls (Fig. 1c). Others appeared to be entirely collapsed with visible and early symptoms of senescence (Fig. 1d). In addition, GCs were less well developed relative to those in untreated galls. A significant reduction (−17%; $P < 0.01$) in the average area measured on the two GCs showing the greatest expansion was observed in treated galls (Table 1).

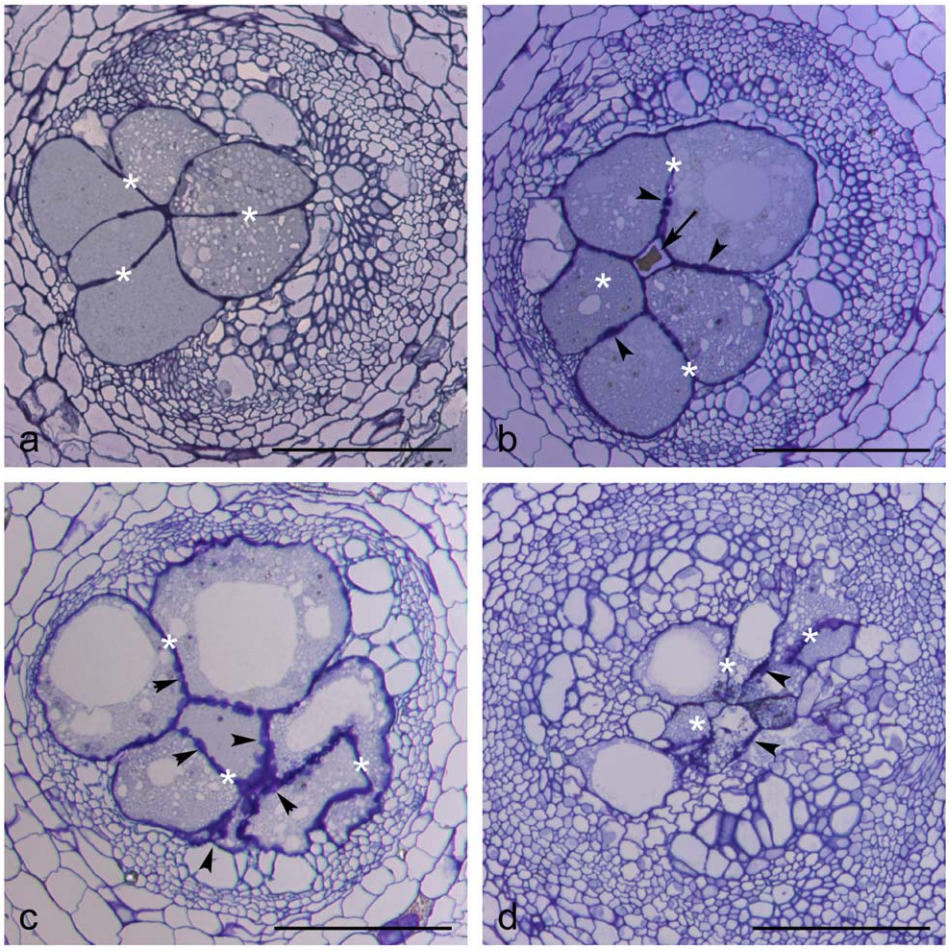

Fig. 1 Microscopic analysis of *Meloidogyne incognita* feeding sites in untreated and ozonated water (O_3wat)-treated tomato galls at 14 days post-inoculation (dpi). Transverse sections were stained with toluidine blue. Untreated galls contained well-developed giant cells (GCs) (a). In O_3wat-treated galls, three different types of GCs were observed, with an appearance similar to untreated galls (b), enlarged vacuoles, less dense cytoplasm and dark-stained deposits on the cell walls (c) and early symptoms of senescence (d). *, GC; arrow, nematode; arrowhead, dark-stained cell wall; scale bar, 100 μm.

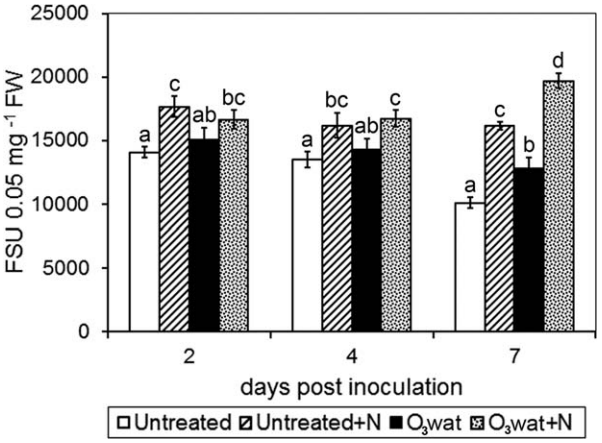

Fig. 2 Reactive oxygen species (ROS) accumulation in untreated and uninfected tomato plants (Untreated), untreated and *Meloidogyne incognita*-infected plants (Untreated + N), ozonated water (O_3wat)-treated and uninfected plants (O_3wat), and O_3wat-treated and infected plants (O_3wat + N) at 2, 4 and 7 days post-inoculation (dpi). Data are the mean ± standard error (SE) of three experiments, each containing a pool of six plants for each treatment and time point. Different letters indicate significant differences between control and treatments (least-significant difference test, $P \leq 0.05$). FSU, fluorescent standard units; FW, fresh weight.

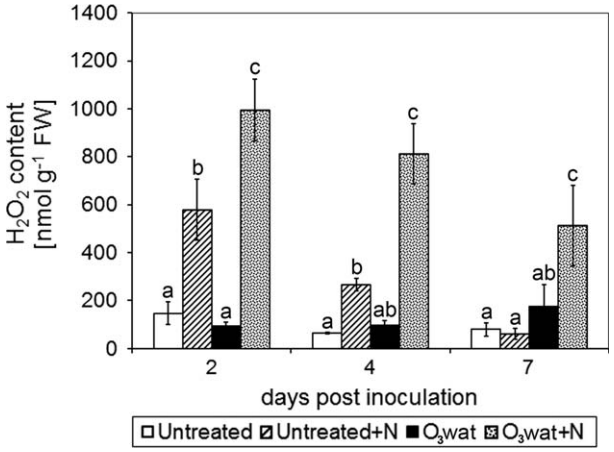

Fig. 3 H_2O_2 content in untreated and uninfected (Untreated), untreated and *Meloidogyne incognita*-infected (Untreated + N), ozonated water (O_3wat)-treated and uninfected (O_3wat), and O_3wat-treated and infected (O_3wat + N) tomato roots. Data are the mean ± standard error (SE) of three experiments, each containing a pool of six plants for each treatment and time point. Different letters indicate significant differences between control and treatments (least-significant difference test, $P \leq 0.05$).

Effects of ozone treatment on ROS and H_2O_2 levels

To test whether O_3wat treatment triggered an oxidative burst, the intracellular accumulation of ROS and H_2O_2 was quantified. ROS content, evaluated by measuring the fluorescence arising from the oxidation of 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), was determined in uninfected and 2-, 4- and 7-dpi untreated and O_3wat-treated roots (Fig. 2). In uninfected roots, O_3wat treatment induced no significant difference in ROS accumulation relative to untreated roots at 2 and 4 dpi. A significant increase ($P < 0.05$) in ROS content was detected at 7 dpi relative to that in untreated uninfected plants. By contrast, nematode infection induced a higher level of ROS at 2, 4 and 7 dpi in both untreated and O_3wat-treated roots (Fig. 2). Interestingly, ROS levels were almost doubled under combined stresses at 7 dpi relative to control plants, and were also significantly higher than in untreated galls (Fig. 2).

Similarly, the H_2O_2 content was determined in uninfected roots and at 2, 4 and 7 dpi in untreated and O_3wat-treated roots (Fig. 3). Ozone treatment alone did not have a significant impact on H_2O_2 production relative to the untreated control, although an increase in H_2O_2 was seen across the time course. The highest H_2O_2 levels were observed in response to nematode infection and, in particular, to combined O_3wat and nematode stresses at the early time point. At 2 dpi, the H_2O_2 content was about 2.9 times higher in infected roots and 5.8 times higher in O_3wat-treated infected roots relative to uninfected controls (Fig. 3). At 4 dpi, the H_2O_2 content in untreated infected roots was still significantly higher than in uninfected roots, whereas, at 7 dpi, it was the same as in the uninfected control. In O_3wat-treated galls, the H_2O_2 levels were significantly higher (11.6 and 5.5 times higher, respectively) than in uninfected, untreated roots at 4 and 7 dpi.

Impact of ozone on transcript levels of CAT, SOD and APX

To understand the effects of ozone stress on the genes encoding antioxidant enzymes, changes in transcript abundance for tomato *CAT1*, cytoplasmic Cu–Zn *SOD* and *APX* genes were analysed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mRNA levels of *CAT*, *SOD* and *APX* were measured at 2, 4 and 7 dpi in both untreated and O_3wat-treated roots, and compared with uninfected and untreated controls at each time point (Fig. 4). At the early stage of infection (2 dpi), *CAT* mRNA levels were similar in all the samples (Fig. 4a). At 4 dpi, the gene was up-regulated, reaching a maximum in infected tissues. It was more highly induced in O_3wat-treated galls (about 34-fold) than in untreated galls (21-fold) relative to the uninfected and untreated roots. Although the expression levels were lower relative to those at 4 dpi, they continued to be up-regulated in both untreated and treated galls at 7 dpi (Fig. 4a). By contrast, O_3wat treatment significantly induced *SOD* gene expression in uninfected roots at all time points measured (Fig. 4b). Neither nematode infection nor the combined stresses affected *SOD* gene expression in both untreated and O_3wat-treated galls during the time course (Fig. 4b). *APX* transcript levels were not influenced by infection and/or ozone treatment at the early time points (2 and 4 dpi). However, they increased significantly in both untreated and O_3wat-treated galls at 7 dpi (Fig. 4c).

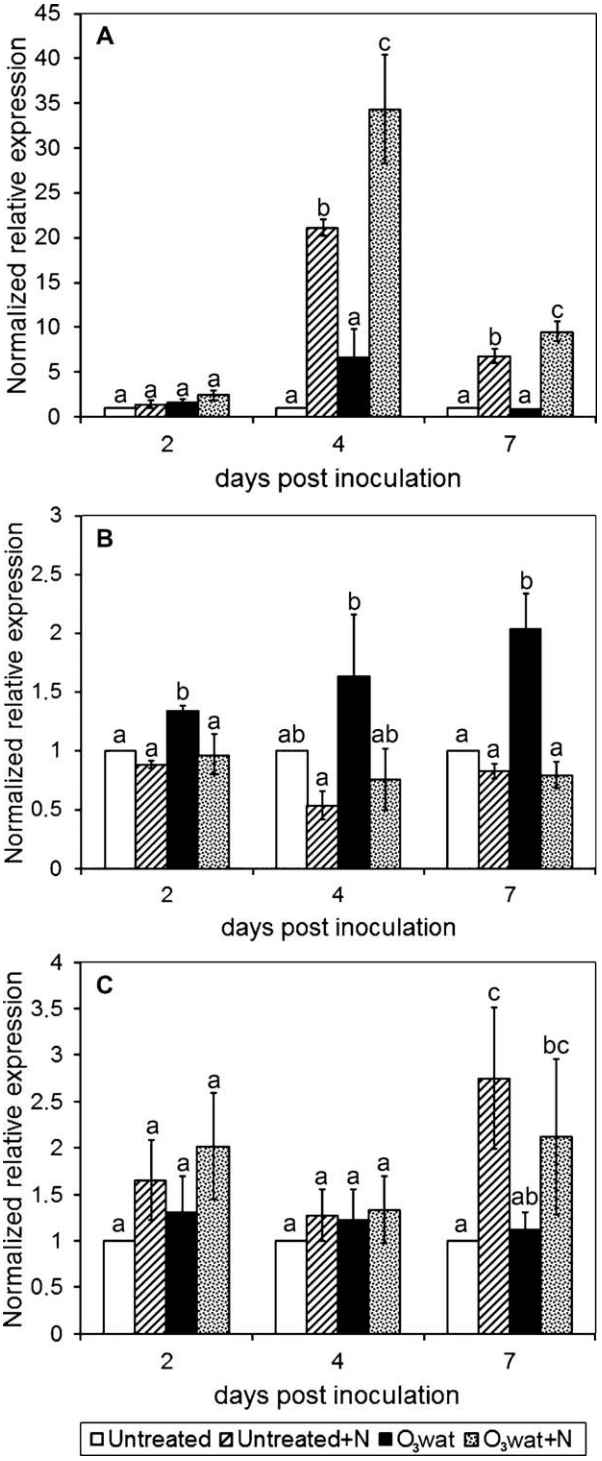

Fig. 4 Relative expression levels of catalase (*CAT*), superoxide dismutase (*SOD*) and ascorbate peroxidase (*APX*) genes in untreated and uninfected (Untreated), untreated and *Meloidogyne incognita*-infected (Untreated + N), ozonated water (O_3wat)-treated and uninfected (O_3wat), and O_3wat-treated and infected (O_3wat + N) tomato roots at 2, 4 and 7 days post-inoculation (dpi) with *M. incognita*: (a) *CAT1*; (b) cytoplasmic Cu–Zn *SOD*; (c) *APX*. Gene expression levels were normalized using the reference gene *actin*. Data are the mean fold change ± standard error (SE) in gene transcript levels relative to control untreated and uninfected tissue (set as unity) at each time point. Each reaction was performed in triplicate and the results represent the mean of three independent biological replicates, each containing a pool of six plants for each treatment and time point. Different letters indicate significant differences between control and treatments at each time point compared with each own control (least-significant difference test, $P < 0.05$).

Effect of ozone on CAT, SOD and APX enzymes

As the expression of the genes encoding the analysed enzymes was not affected at the early time point (2 dpi), changes in the activities of CAT, SOD and APX were examined at 4 and 7 dpi. At 4 dpi, nematode infection alone caused a significant ($P < 0.05$) decrease in CAT activity, whereas ozone alone or combined with nematode infection gave rise to a significant ($P < 0.05$) increase, relative to untreated and uninfected roots (Fig. 5a). At 7 dpi, a different trend was observed. Infection alone induced a strong increase ($P < 0.05$) in CAT activity, whereas a significant ($P < 0.05$) decrease in CAT activity was observed in response to ozone treatment and to combined stresses (Fig. 5a). SOD activity was not significantly affected by nematode infection at both 4 and 7 dpi relative to the untreated, uninfected control (Fig. 5b). O_3wat treatment caused a significant ($P < 0.05$) increase in SOD activity at 4 dpi, both when applied alone and when combined with nematode infection, clearly indicating that this is an effect of O_3wat rather than of nematode infection. By contrast, an opposite trend was detected at 7 dpi, when a significant ($P < 0.05$) reduction in SOD activity was found. The activity of APX, an enzyme known to be important in the removal of H_2O_2 from plant cells, is shown in Fig. 5c. Although no change was observed after nematode infection, treatments with ozone in both uninfected and infected roots caused a significant ($P < 0.05$) decrease in APX activity at 4 dpi. At the latest time point (7 dpi), nematode infection induced a significant ($P < 0.05$) increase in APX activity in both untreated and O_3wat-treated roots. Although ozone treatment caused an increase in APX activity in uninfected roots relative to the untreated control, it did not trigger a further increase in infected roots.

Lipid peroxidation

Oxidative degradation of cell membrane lipids caused by the high accumulation of ROS during nematode infection and O_3wat treatment was measured by the determination of the products of membrane lipid peroxidation. The malondialdehyde (MDA) content in both O_3wat-treated uninfected and infected roots at 4 dpi was significantly higher relative to the untreated control (+21% and +35%, respectively) (Fig. 6). At 7 dpi, lipid peroxidation was significantly higher in *M. incognita*-challenged roots. MDA content increased by around 56% in untreated galls and, in particular (+188%), in response to combined stresses (Fig. 6).

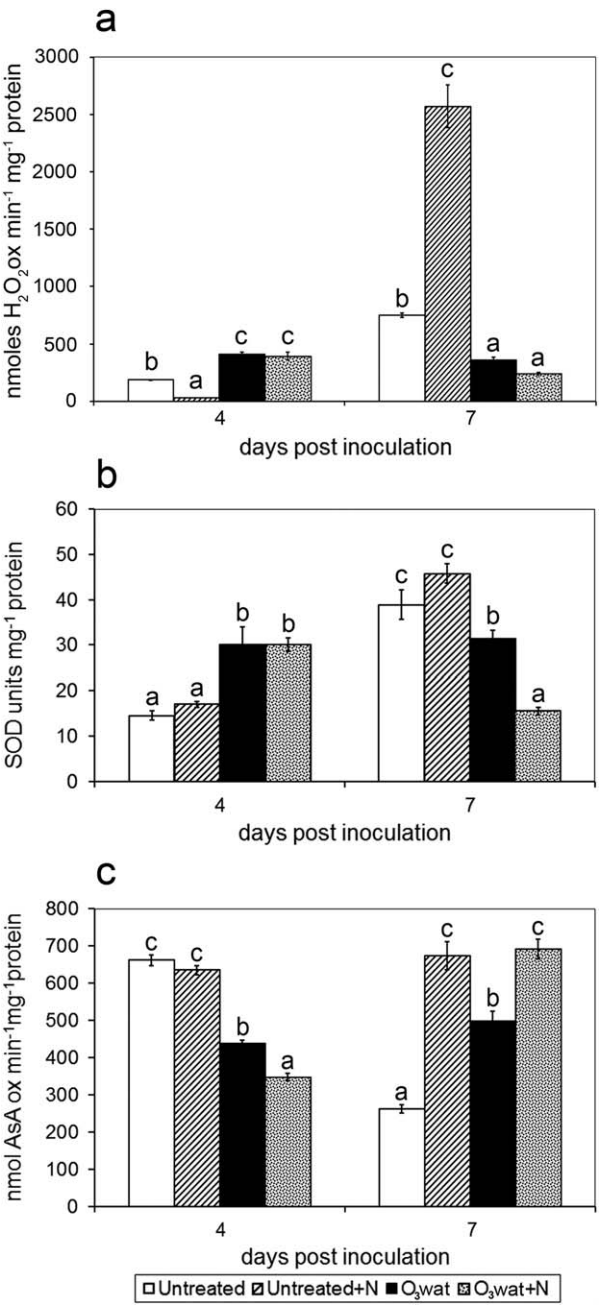

Fig. 5 Catalase (CAT, a), superoxide dismutase (SOD, b) and ascorbate peroxidase (APX, c) activities in untreated and uninfected (Untreated), untreated and *Meloidogyne incognita*-infected (Untreated + N), ozonated water (O_3wat)-treated and uninfected (O_3wat), and O_3wat-treated and infected (O_3wat + N) tomato roots at 4 and 7 days post-inoculation (dpi). Data are the mean ± standard error (SE) of three experiments, each containing a pool of six plants for each treatment and time point. Different letters indicate significant differences between control and treatments at each time point (least-significant difference test, $P \leq 0.05$).

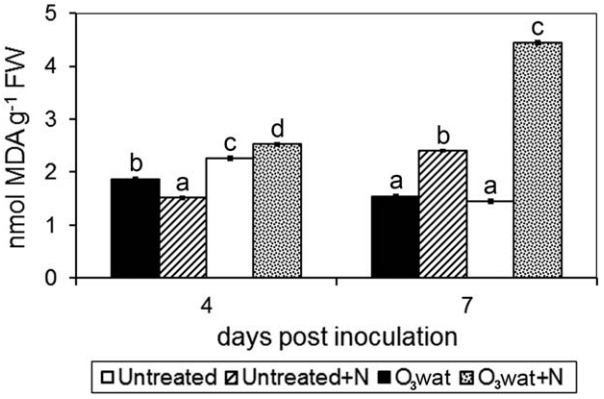

Fig. 6 Malondialdehyde (MDA) content of untreated and uninfected (Untreated), untreated and *Meloidogyne incognita*-infected (Untreated + N), ozonated water (O_3wat)-treated and uninfected (O_3wat), and O_3wat-treated and infected (O_3wat + N) tomato roots at 4 and 7 days post-inoculation (dpi). Data are the mean ± standard error (SE) of three experiments, each containing a pool of six plants for each treatment and time point. Different letters indicate significant differences between control and treatments (least-significant difference test, $P \leq 0.05$).

DISCUSSION

The present study reports, for the first time, the use of O_3, applied as an aqueous solution, to the root system of a susceptible tomato cultivar, as an alternative strategy for RKN management. Furthermore, plant responses to simultaneous biotic and abiotic stresses, in terms of changes in feeding site development, oxidative stress and modulation of enzymes involved in ROS scavenging, were investigated.

As demonstrated previously (Graham *et al.*, 2012; He *et al.*, 2015; Ohashi-Kaneko *et al.*, 2009), ozone application to the root system in aqueous solution has no phytotoxic effect. This is most likely because the half-life of ozone dissolved in water is short (typically less than 20 min) and because the method of application is to the growth substrate, which results in the limited exposure of roots to the gas. In our studies, the use of O_3wat as a soil drench application to tomato roots did not negatively affect the growth parameters (Table 1, Fig. S1). O_3wat was used on susceptible tomato roots to control *M. incognita* infection, and gave rise to a significant decrease in all nematological parameters analysed (Table 2), indicating that O_3wat treatment, both before and after nematode infection, induced a reduction in tomato susceptibility to nematode. The use of ozone may therefore offer an alternative control option for RKNs that might help to answer the growing environmental and public health concerns associated with the use of some nematicides.

During compatible interaction, RKNs induce and maintain a specialized feeding site formed by GCs, which is the only source of nutrients for this root parasite throughout its life (Gheysen and Mitchum, 2011). To determine the effect of O_3wat on nematode feeding sites, a histological analysis was performed at 14 dpi. In

O_3wat-treated galls, ozone had no discernible effect on the ability of nematodes to penetrate, migrate and induce GCs, but feeding site development was compromised. In some plants, O_3wat-treated gall GCs showed an appearance comparable with untreated GCs, whereas, in others, the GCs still contained an enlarged vacuole (occupying almost all the volume of a GC), which is a typical feature of the early stages of feeding site formation (Jones, 1981). In these GCs, the cytoplasm was less dense with a few organelles and dark osmiophilic deposits accumulated on the cell walls. Finally, a number of GCs were characterized by visible symptoms of senescence (Fig. 1d), with the breakdown of cellular material, membrane aggregates and concomitant disorganization of the whole protoplast. Therefore, as for other elicitors effective in producing resistance to RKNs (Sahebani *et al.*, 2011) and in inducing the delayed development of GCs (Melillo *et al.*, 2014), O_3wat application induced a lower susceptibility to *M. incognita* infection, probably as a result of the failure of normal feeding site development. This was confirmed by a decreased area of O_3wat-treated GCs compared with untreated GCs, and this could be the final effect of several responses of the host plant triggered by ozone both alone and/or in combination with the biotic stress.

The phytotoxicity of O_3 is caused by its high oxidative capacity through the induction of ROS in exposed plant tissues (Schraudner *et al.*, 1997). The oxidative burst caused by elevated O_3 elicits defence signalling pathways as seen in pathogen defence responses (Eastburn *et al.*, 2011). It has been reported that increased ROS levels improve resistance to various pathogens (Bilgin *et al.*, 2008). In our experimental conditions, nematode infection induced a ROS burst and the application of O_3wat caused a further increase in ROS levels, particularly at 7 dpi. Indeed, the ozone treatment seemed to act by priming the tissue to subsequent challenge. Moreover, the highest levels of H_2O_2 were observed in O_3wat-treated roots as a response to combined abiotic stress and infection, and these levels may play a direct role as an antimicrobial agent. As seen during the tomato–*M. incognita* incompatible interaction (Melillo *et al.*, 2011), this may be one of the major factors enhancing the resistance of O_3wat-treated tomato roots to RKNs. The H_2O_2 level was coupled with a tendency towards increased MDA concentrations as a secondary end-product of the oxidation of polyunsaturated fatty acids. MDA, which is normally present in cells at a low concentration, is a reliable indicator of the uncontrolled production of free radicals, and hence a consequence of oxidative stress caused by abiotic and biotic stresses (Apel and Hirt, 2004). The increase in MDA content in untreated galls at 7 dpi is likely to have occurred as a result of RKN infection (Korayem *et al.*, 2012). Interestingly, the enhanced response to combined ozone treatment and infection represents the state of membrane lipid peroxidation caused by oxidative stress induced by O_3 (Calatayud *et al.*, 2004; Kumari *et al.*, 2015).

A strict control of the ROS levels is essential in order to prevent unintended damage and to ensure an accurate execution of the signalling function. Therefore, ROS-producing enzymes are used with an elaborate antioxidant system in order to maintain ROS homeostasis in all cellular compartments (Gechev *et al.*, 2006). SOD, CAT and APX are important detoxifying enzymes that play a role in this regard. In the *M. incognita*–tomato compatible interaction, SOD and CAT enzyme activities provide protection against oxidative stress during the development of the nematode feeding site (Zacheo and Bleve-Zacheo, 1995). Changes in these scavenging systems were observed after nematode infection or following O_3wat treatment. The CAT activity level was strongly increased at 7 dpi when the GCs were well developed. This result is correlated with the up-regulation of the *CAT1* gene in untreated galls at 7 dpi. Among the enzymes involved in the removal of excess H_2O_2 (generated either spontaneously or through the dismutation of $O_2^{\cdot-}$), APX plays a key role, together with CAT (Gechev *et al.*, 2006), in the control of the cellular H_2O_2 level. Although similar levels of APX activity were found in untreated galls at 4 and 7 dpi (Fig. 5c), we observed the up-regulation of the APX gene in response to nematode infection at 7 dpi (Fig. 4c), in agreement with data previously reported in both RKN– and cyst nematode–host interactions (Kaur *et al.*, 2013; Simonetti *et al.*, 2010). This may indicate an attempt by the plant cell to reduce the levels of H_2O_2 that otherwise could harm membrane integrity and nematode development. By contrast, SOD was not triggered by nematode infection either in terms of enzymatic activity or transcript abundance.

The scenario was different when following ozone treatment. Increased activities of SOD and CAT, 4 days after the end of O_3wat treatment, in both uninfected and infected tissues, may reflect the response of cells to avoid ROS cellular damage. The increased formation of $O_2^{\cdot-}$, a ROS that is usually the first to be generated (Noctor *et al.*, 2002), may favour greater SOD activity in roots challenged by ozone treatment. The up-regulation of the cytoplasmic Cu–Zn *SOD* gene in response to O_3wat treatment suggests an important role for this enzyme in the protection of the tomato root system against oxidative stress induced by ozone. After 7 days, the decreased SOD and CAT activities show that the SOD–CAT system does not contribute to ROS detoxification. By contrast, the unchanged APX activity and gene expression in O_3wat-treated galls at 7 dpi, relative to those in untreated galls (Fig. 5c), suggest the importance of this enzyme in H_2O_2 removal inside the feeding site. Indeed, as a result of the higher affinity of APX (μM range) than CAT (mM range) for H_2O_2, the APX enzyme can efficiently work in order to eliminate low, but toxic, amounts of H_2O_2. This trend may highlight the presence of a fine cell strategy for the scavenging of small amounts of H_2O_2 in specific cell regions. Thus, the different timing of involvement of CAT and APX depends on the severity of the stresses and how they are perceived by the plant. The data presented here suggest that the

antioxidant system is modulated differently by abiotic and biotic stresses. Interestingly, the significant increases in CAT activity at 7 dpi in untreated galls may be due to the reprogramming of plant cells in response to the detoxification requirements of the nematode feeding site. Conversely, the suppression of the SOD–CAT system at 7 dpi, coupled with the still large amounts of ROS and H_2O_2 in O_3wat-treated galls, may be a way to contain nematode infection, making tomato plants more resistant. This is supported by Kaur *et al.* (2013) and Molinari and Miacola (1997), who noted a decrease in CAT and SOD activity in resistant tomato cultivars after infection.

Changes in transcript abundance were not always linked to the measured activities of the antioxidant enzymes. For example, our results showed that, in O_3wat-treated galls, *CAT1* gene expression and CAT enzyme activity were uncoupled. Enzyme activity is not necessarily correlated with gene transcript abundance. In eukaryotes, protein levels can change independently from the levels of the transcripts that encode them. For example, no correlation was found between peroxidase and CAT gene expression and enzymatic activities in kernels of resistant and susceptible maize genotypes inoculated with various fungi (Lanubile *et al.*, 2015). Moreover, the involvement of unknown effectors and/or post-translational regulation may alter the enzyme kinetic properties (Gibon *et al.*, 2004). However, the trend in gene expression was paralleled by the levels of H_2O_2, confirming this ROS as a signalling molecule for the induction of genes encoding enzymes that protect cells against oxidative damage.

Our results show that plants respond to a specific combination of stresses in a non-additive manner, producing effects that cannot be predicted from the study of each stress applied individually. The positive effect of O_3wat application in the control of nematode infection is probably related in large part to the modulated antioxidant systems, which increase ROS, H_2O_2 and MDA in nematode feeding sites that are morphologically altered as a result. However, further studies are needed to unravel the signalling routes that induce different plant responses to these combinations of stresses.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seedlings of susceptible tomato (*Solanum lycopersicum* L.) cv. Roma were grown (one per pot) in pots containing 50 mL of sterilized sand, in a growth chamber (Sanyo MLR-351), Sanyo Electric Co, Ltd., Morigouchi City, Osaka, Japan at 25 °C, with a photoperiod of 16 h light/8 h darkness at 200 μmol/m^2/s. Plants were watered with Hoagland's solution. Twelve-day-old seedlings were exposed to ozone treatments as a soil drench for 4 days.

Ozone treatments and nematode infection

Filtered tap water was ozonated *in situ* by passing it through an electrolytical O_3wat generator [Ozosafe, Chimia Srl, Muggiò (MI), Italy]. The dissolved ozone concentration in O_3wat was measured by an indigo method using an ozone potentiostatic electrode [SP651 electrode supplied with a CL 125.2 unit (B&C Electronics Srl, Carnate (Mi), Italy)]. Measurements of dissolved ozone were carried out in O_3wat under constant flux until the desired concentration (10 ppm) was reached. Plants were then irrigated daily with 10 mL/pot for 4 days. O_3wat previously added was eliminated by suction with a Pasteur pipette connected to a pump. After the exposure period, plants were irrigated with Hoagland's solution. Control plants were treated with Hoagland's solution only.

After the 4-day treatment, O_3wat-treated and untreated plants were inoculated. An aqueous suspension of 250 freshly hatched J2s of *M. incognita* was pipetted close to the roots of each plant. J2s were collected from egg masses of infested tomato roots that were allowed to hatch in a growth chamber at 25 °C. Uninfected O_3wat-treated and untreated plants were used as controls.

The root systems of O_3wat-treated and untreated plants were examined under a stereomicroscope. The gall formation and position relative to the root tips were carefully considered. Galls of 2-, 4-, 7- and 14-dpi seedlings were hand dissected (Fig. S2, see Supporting Information). The corresponding portions of uninfected roots were collected. A portion of the excised samples was immediately processed, whereas, for biochemical and molecular studies, samples of roots were frozen in liquid nitrogen and stored at −80 °C until use.

Morphometric and morphological analysis

Uninfected and infected seedlings, untreated and O_3wat-treated, were harvested from each pot on the 14th day and the root systems were washed carefully. Fifteen plants were considered for each treatment in three different biological replicates. The biomass of each plant part, shoot (stem and leaves) and root, was determined by direct measurement of its fresh weight. Nematode infection was evaluated by counting the number of galls per plant by examining root systems under a stereomicroscope.

Galls at 2, 4, 7 and 14 dpi from O_3wat-treated and untreated plants were hand dissected and fixed in a mixture of 1.5% glutaraldehyde and 3% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) containing 150 mM NaCl (pH 7.2) for 3 h at room temperature, dehydrated in a graded ethanol series to absolute ethanol and then embedded in acrylic resin LR White (Sigma, St. Louis, MO, USA), which was left to polymerize overnight at 60 °C.

Embedded galls were cut in serial semi-thin cross-sections (2.5 μm) through all their length, and then stained briefly with 1% toluidine blue in 1% borax solution and mounted in Depex. Microscopic observations were performed using bright-field optics on a Leica DM 4500 B light microscope equipped with a Leica DFC 450C camera (Leica Microsystems Srl, Milan, Italy).

In order to determine GC size, semi-thin stained sections were observed in 15 both untreated and O_3wat-treated 14-dpi galls from three different replicates (five galls per replicate). These were cut through their entire length. ImageJ software (http://imagej.nih.gov/ij/) was used to quantify the area occupied by the GCs. Two hundred and fifty sections for each treatment were scored. In each feeding site, the two GCs that showed the greatest expansion were selected to measure their mean area.

Determination of ROS and H_2O_2 contents

ROS contents were determined following the method described by Melillo *et al.* (2006). Root galls from O_3wat-treated and untreated infected roots (2, 4 and 7 dpi) and corresponding portions of uninfected seedlings were excised and pre-incubated for 30 min in potassium phosphate buffer (20 mM, pH 6). Root tissues were homogenized (in a ratio of 1 mL/50 mg of tissue) inside a working solution containing 50 μM DCFH-DA (Sigma) dissolved in potassium phosphate buffer (20 mM, pH 6) with 0.2 g/mL of porcine liver esterase (Sigma), and then incubated for 30 min at 25 °C on a shaker. Fluorescence (excitation, 488 nm; emission, 525 nm) caused by the oxidation of DCFH to DCF was measured by a fluorometer (GloMax-Multi Jr, Promega, Madison, WI, USA). For statistical purposes, fluorometry experiments were performed in triplicate.

H_2O_2 contents were determined by an optimized assay for plant tissue according to Junglee *et al.* (2014). One hundred milligrams of 2-, 4- and 7-dpi O_3wat-treated and untreated galls and the corresponding uninfected frozen tissues were ground in liquid nitrogen, and then directly homogenized with 1 mL of a solution containing 0.25 mL of trichloroacetic acid (TCA) (0.1%, w/v), 0.5 mL KI (1 M) and 0.25 ml of potassium phosphate buffer (10 mM, pH 7) at 4 °C for 10 min. Samples were then centrifuged at 12 000 ***g*** for 15 min at 4 °C and the supernatants were incubated in the dark at 20–22 °C for 20 min. The H_2O_2 content was measured at 350 nm in a Beckmann Coulter DU 800 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). A calibration curve obtained with H_2O_2 standard solutions prepared in 0.1% TCA was used for quantification.

Determination of specific activity of SOD, CAT and APX enzymes

Untreated and treated root tissues, uninfected or infected, were ground with a pestle and mortar at 4 °C in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.8) containing 0.3 mM mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% (w/v) cysteine (buffer A) in a 1 : 3 ratio (w/v). The homogenate was centrifuged at 1000 ***g*** for 5 min. The supernatant was re-centrifuged for 20 min at 25 000 ***g***. The resulting supernatant, assayed as the cytosolic fraction, was desalted by dialysis against 50 mM Tris-HCl (pH 7.8) and used for spectrophotometric analysis. The activities of the enzymes analysed, i.e. CAT (EC 1.11.1.6), SOD (EC 1.15.11) and cytosolic APX (EC 1.11.1.11), were tested according to Paciolla *et al.* (2008). For CAT, 1 U = 1 nmol of H_2O_2 dismutated/min; for SOD, 1 U = the amount of enzyme required to inhibit the reduction rate of nitroblue tetrazolium (NBT) by 50% at 25 °C; for APX, 1 U = 1 nmol of ascorbate oxidized/min. The protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

Lipid peroxidation measurement

For lipid peroxidation, the root tissues were ground with four volumes of 0.1% (w/v) TCA. The homogenate was centrifuged at 10 000 ***g*** for 10 min. One millilitre of the supernatant was diluted with 1 mL of 20% TCA containing 0.5% (w/v) thiobarbituric acid. The level of lipid peroxidation was measured in terms of MDA content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham (1996).

Real-time PCR

Total RNA was extracted from galls of O_3wat-treated and untreated plants and corresponding portions of uninfected tomato roots using an RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized from 0.5 μg of DNase-treated RNA using oligo(dT) primer and a reverse transcription kit (Promega Corporation, Madison, WI, USA). *CAT1*, cytoplasmic Cu–Zn *SOD* and *APX* messages were amplified using the primers CAT-for (5′-TTGTCGTCCTGCTGAGCAGT-3′) and CAT-rev (5′-TGTGAACTTATTCAGGTTCCAACA-3′) (GenBank M93719; 463-bp amplicon), SOD-for (5′-TGCTGGTGATCTTGGTAACATCA-3′) and SOD-rev (5′-AACTGCAGGCACTGTAATCTGCA-3′) (GenBank X14040; 526-bp amplicon), and APX-for (5′-TGAGTGGGGAAAAGGAAGGG-3′) and APX-rev (5′-TATGTCACCACCCTCCCAAC-3′) (GenBank AY974805; 206-bp amplicon), respectively. Real-time PCR assays were performed on a Mx3000P Stratagene system (Agilent Technologies Inc., Santa Clara, CA, USA) in 20-μL reaction mixtures containing 0.4 μM of each gene-specific primer, 10 μL of 2 × GoTaq qPCR Master Mix (Promega) and 1 μL (50 ng) of template cDNA. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 30 s, 58 °C for 1 min and 72 °C for 20 s. The amplification specificity was confirmed by a single peak in a dissociation curve at the end of the PCR and a single band in agarose gel electrophoresis. Expression levels in each sample were normalized to the expression of a 315-bp fragment of the tomato *actin* gene (BT013524) amplified using the primers Act-for (5′-GCTTTGCCGCATGCCATTCT-3′) and Act-rev (5′-GATACCTGCAGCTTCCATACC-3′). The relative gene expression was obtained with the method of Pfaffl (2001); raw fluorescence values, as a function of cycles, were exported into the real-time PCR Miner program (Zhao and Fernald, 2005) in order to calculate the reaction efficiency.

For each stage and treatment, three independent RNA isolations were performed, with two replications for each of the RNA isolations.

Assessment of nematode reproduction

The effect of O_3wat on nematode reproduction was assessed in a glasshouse experiment at 25 ± 2 °C in two different trials with eight replications for each treatment and trial. In the first trial, 12-day-old tomato plants grown in 50-mL clay pots were inoculated with 250 J2s of *M. incognita* per plant and then treated daily with O_3wat (10 mL/pot) for 4 days. In the second trial, 12-day-old plants were first treated daily with O_3wat (10 mL/pot) for 4 days and then infected at the end of the treatment. Untreated infected plants were used as a control. Fourteen days after inoculation, tomato seedlings were transplanted into 1000-mL clay pots filled with steam-sterilized sandy soil (pH 7.2; sand, >99%; silt, <1%; clay, <1%; organic matter, 0.75%). Pots were arranged on benches according to a randomized block design. To avoid a block position effect and the plant position factor within the block, tomato plants were maintained in the glasshouse randomizing the position of the blocks and repositioning each plant within a block every week. Plants received all the necessary maintenance (irrigation, fertilization, etc.). Each trial was repeated twice.

At the end of the experiment, 2 months later, plants were uprooted and dry top and fresh root weights were recorded. The root gall index (RGI) was estimated according to the method described in Bridge and Page (1980).

The final nematode population in each pot was determined by processing 500 mL of soil by the method of Coolen (1979). The numbers of *M. incognita* eggs and J2s in the roots were assessed by cutting up each root system into small pieces and comminuting them in a blender containing 1% aqueous solution of NaOCl for 20 s (Marull and Pinochet, 1991). The water suspensions were then processed according to the procedure described by Sasanelli *et al.* (2002). Nematodes recovered from soil and roots were counted and determined the final nematode population density (P_f) in each pot. The nematode reproduction factor *r* was expressed as the ratio between the final and initial *M. incognita* population density (P_f/P_i).

Statistical analysis

In order to determine significant differences among various treatments, nematological, biochemical, molecular and physiological parameters were subjected to analysis of variance (ANOVA) and the means were compared by the least-significant difference test ($P < 0.05$). Values between treatments and control (untreated and uninfected) were compared at each time point. The number of galls per plant and the GC area measurements at 14 dpi were statistically analysed by Student's *t*-test ($P < 0.01$). Statistical analyses were performed using Plot IT software version 3.20i.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Effect of ozonated water (O_3wat) on the growth of tomato plants. No phytotoxic effects are visible in the shoots and roots of uninfected and infected treated plants relative to untreated plants at 14 days post-inoculation (dpi). (a′), (b′), (c′) and (d′) show enlargements of the root apparatus. Arrowheads in (b′) and (d′) show galls. Bar size, 1 cm.

Fig. S2. Collected material and monitoring of *Meloidogyne incognita* second-stage juvenile (J2) infection. (a, c, e) Hand-dissected galls of untreated tomato roots at 2, 4 and 7 days post-inoculation (dpi). (b, d, f) Galls of ozonated water (O_3wat)-treated roots at 2, 4 and 7 dpi. Histological analysis of nematode feeding sites in untreated and O_3wat-treated galls was performed at 2, 4 and 7 dpi (a′, c′, e′ and b′, d′, f′, respectively). (a′, b′) Cells selected by J2s as feeding sites are evident. (c′, d′) Well-developed giant cells (GCs) clearly occupy the vascular cylinder. (e′, f′) Large and multinucleate GCs acting as a food sink for the growing nematode are present. GCs in O_3wat-treated roots (d′, f′) show less dense cytoplasm and still large vacuoles. *, GCs; arrow, nematode; Bar sizes: 1 mm for galls and 200 μm for sections.