Effects of single or combined water deficit and aphid attack on tomato volatile organic compound (VOC) emission and plant-plant communication

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Abstract

Plants release a broad spectrum of volatile organic compounds (VOCs). The composition of the released VOC blend is dependent on the physiological status and, consequently, is affected by biotic and abiotic stresses. Stress-related VOCs, once released in the atmosphere, can be perceived by different organisms, including natural enemies of herbivores and neighboring plants. Here, the responses of tomato plants (emitters) to single or combined abiotic (water stress) and biotic (aphid attack) stresses, and the effect of VOC released by emitters on neighboring unstressed plants (receivers), have been investigated. A multidisciplinary approach, including the characterization of eco-physiological parameters, VOC release, indirect defense in terms of attractiveness towards an aphid parasitoid and expression of tomato genes known to be involved in VOC synthesis and defense response to biotic and abiotic stress, was used. The emissions of α-pinene and methyl salicylate from plants exposed to single or combined stress, and of camphene from plants exposed to water or combined stress were significantly higher than in unstressed plants. In receivers, only the release of methyl salicylate increased when companion emitters were stressed. The expression of genes related to VOC biosynthesis and plant defense responses was unaffected or declined in water-stressed emitters, and was generally higher in receivers than in emitters. The gene coding for methyl salicylate biosynthesis was particularly active in aphid-attacked emitters and in receivers that were conditioned by the infested emitters. Receivers primed by any combination of stresses were also more attractive towards the aphid parasitoid *Aphidius ervi*. In summary, while interactive biotic and abiotic stresses have an additive impact on the emission of few VOC, they may impact on opposite ways on the expression of genes involved in defensive pathways. VOCs emitted by stressed plants induce VOC emission in unstressed receivers, and this increases attraction of parasitic wasps, which may improve protection against aphid attacks under conditions of reduced water availability.

Keywords: abiotic stress; *Aphidius ervi*; biotic stress; *Macrosiphum euphorbiae*; MeSA; *Solanum lycopersicum*

1. Introduction

Being sessile organisms, plants have to cope with environmental constraints and biotic and abiotic stresses for most of their life (Suzuki et al., 2014). Far from being passive receivers of environmental or biotic stresses, natural selection has allowed evolution of complex physiological, molecular and biochemical mechanisms of defense or communication in plants. One of the most intriguing mechanisms, serving both defense and communication, involve plant volatile organic compounds (VOCs), a large class of secondary metabolites, mainly belonging to the isoprenoid family, and stress hormones (Niinemets et al., 2010; Robert-Seilaniantz et al., 2011).

It has been already documented that single biotic and abiotic stresses change the blend of VOCs emitted by plants, altering the formation of constitutive VOCs and inducing the biosynthesis of new compounds (Holopainen and Gershenzon, 2010; Niinemets, 2010; Rosenkranz and Schnitzler, 2016). In response to abiotic stresses, plants often invest an increasing portion of freshly assimilated carbon into constitutive VOC synthesis (Brilli et al., 2007; Loreto and Schnitzler, 2010; Centritto et al., 2011). Whereas, the blend of VOC profile emitted by plants after herbivory damage largely consists of induced VOCs (Niinemets et al., 2010). In particular, a recent meta-analysis (Rowen and Kaplan, 2016) indicated that: 1) leaf chewing insects, such as caterpillars, induce more volatiles than phloem feeders such as aphids and whiteflies; 2) specialist herbivores induce larger total amounts of volatiles than generalists, albeit this was not true for every class of compounds; 3) stronger volatile responses but less complex VOC blends are induced in domesticated species than in their wild relatives. Once released, VOCs are powerful means of communication (Guerrieri, 2016). Emitted VOCs can act as airborne "warning" signals and infochemicals within the plant, in plant communities, and in plant herbivores/pathogens interactions at multiple trophic level (Shulaev et al., 1997; Engelberth et al., 2004; Heil et al., 2010; Guerrieri, 2016; Coppola et al., 2017).

The salicylic acid (SA) is amongst the main induced metabolites in plant defence signalling (Shulaev et al., 1995; Robert-Seilaniantz et al., 2011). The activation of SA pathway may elicit the defence responses to phloem-sap-feeding insects (Zhu and Park, 2005; Mewis et al., 2012; Ederli et al., 2017; Salerno et al., 2017) by forming distinctive combinations of VOCs, including, among others, the SA methyl ester (i.e., methyl salicylate, MeSA). The release of MeSA seems to be involved in the activation of both direct and indirect plant defence (Ament et al., 2010). In addition, it has been shown that SA may also alleviate the detrimental effects of abiotic stresses (Munne-Bosch and Peñuelas, 2003; Sawada et al., 2006).

Being VOC emission different in response to abiotic and biotic stresses, the combination of two or more stresses may produce unpredictable patterns and levels of emission (Copolovici et al., 2014; Weldegergis et al., 2015; Ponzio et al., 2016; Ederli et al., 2017; Salerno et al., 2017). For example,

Copolovici et al. (2014) found that water stress and herbivore feeding elicited in *Alnus glutinosa* the emission of volatile products of the lipoxygenase (LOX) pathway, a strong and transient emission of methyl salicylate, and emissions of stress marker compounds such as (E)-β-ocimene and the homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene. The induced blend of VOCs efficiently primed plants to control feeding by larvae of green alder sawfly (*Monsoma pulveratum*).

Here we report the response, in terms of VOC emission, of tomato plants exposed to individual stress (either water stress or aphid infestation) or to a combined stress (water stress and aphid infestation). Stressed plants were used as VOC source for unstressed, receiver plants, in order to study, using a multidisciplinary approach, whether the information carried by induced VOCs in stressed plants was able to prime defense mechanisms of unstressed companions.

2. Materials and methods

2.1 Plant material and growth conditions

Tomato (*Solanum lycopersicum cv* 'San Marzano nano') seeds were surface sterilized in sodium hypochlorite for 20 min, washed five times in sterile water, and germinated on wet paper. Seedlings were then grown into 0.4 dm^3 pots filled with sterilized soil and kept in a greenhouse under controlled condition [24 ± 2 °C, 18 h light/6 h dark and relative humidity (RH) = 70 ± 10%]. Plants were watered every other day and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rate (Centritto, 2005). Plants were maintained in well-watered conditions (unstressed control, C), or subjected to either i) water stress (WS); ii) aphid attack (A); or iii) a combination of water stress and aphid infestation (WS+A). Different cabinets were used in order to separate plants subjected to the different treatments.

2.2 Insect rearing

Both the herbivore and its parasitoid were reared at CNR-Institute for Sustainable Plant Protection (Portici) as follows:

- The potato aphid *Macrosiphum euphorbiae* Thomas (*Hemiptera*: Aphididae) was reared on tomato plants *cv* San Marzano nano from material collected in the field (Scafati, SA) on the same plant cultivar in 2001 and periodically refreshed. Rearing conditions were: 20 ± 1 °C, 18 h light/6 h dark, 65 ± 5% RH.

- *Aphidius ervi* Haliday (Hymenoptera: Braconidae) was permanently reared on the pea aphid *Acyrtosiphon pisum* reproduced on broad bean plants (*cv* Aquadulce) from material collected in the

field (Battipaglia, SA) in 2001 on alfalfa, and periodically refreshed. Rearing conditions were: 20 ± 1 °C, 18 h light/6 h dark, 65 ± 5% RH (see Guerrieri et al. 2002 for more details).

2.3 Experimental setup

Water stress was imposed by withholding water for seven days. As indicator of soil water availability, the fraction of transpirable soil water (FTSW; Sinclair and Ludlow, 1986; Brilli et al., 2013) was used. FTSW was estimated as: ($Daily_{potweight}$ - $Final_{potweight}$) / ($Initial_{potweight}$ - $Final_{potweight}$), where $Daily_{potweight}$ is the weight of the water-stressed plants recorded during the water stress cycle, $Initial_{potweight}$ is the weight at pot water capacity, and $Final_{potweight}$ is the FTSW at which FTSW approached ~5% ($FTSW_5$) of the average value of well-watered plants ($FTSW_{100}$).

The aphid attack was realized by transferring twenty mixed-aged adults of *M. euphorbiae* on leaves of well-watered tomato plants. For the double stress, the aphids were added at the onset of water stress imposition ($FTSW_{100}$) and left until the end of water stress ($FTSW_5$).

At the end of the stress treatments, plants were used as "emitters" to prime four-week-old well-watered, uninfested (unstressed) plants ("receivers") in a conditioned cabinet (25 °C, 18 h light /6 h dark, 65 ± 5% RH). The receivers were placed in an aerated cage (Vermadel ®) downwind and 20 cm away from the emitters for three days, keeping the air flow rate toward receivers at 30 cm s^{-1}. Each conditioning treatment was run in a different cabinet and using a different cage to avoid any possible interference by memory effects of VOCs on surfaces.

2.4 Physiological measurements

Gas exchange and fluorescence measurements were made under a photosynthetic photon flux density (PPFD) of 700 µmol m^{-2} s^{-1}, with a leaf temperature of 25 °C and at a RH in the leaf cuvette ranging between 30 and 35%, using the infrared gas analyzer LI-6400 XT (Li-Cor, Lincoln, NE, USA). Steady-state net photosynthesis (*P*n), g_s, quantum yield of PSII in the light (ΦPSII = ΔF/Fm' = Fm' - Fs/ Fm', where F_m' is the maximal fluorescence after saturating pulse and F_s is the level of steady state chlorophyll fluorescence) and photochemical quenching (qP = (F_m' - F_s) / (F_m' - F_o'), where F_o' is the minimal fluorescence] were measured simultaneously with the instrument software on at least seven emitters for each condition.

2.5 Wind-tunnel bioassay

Receivers were tested in a wind-tunnel bioassay for their attractiveness toward the aphid parasitic wasp *A. ervi*. For each experimental condition, a total of ten plants was used and offered individually

daily in a random order to reduce any bias related to the time of the experiments. One hundred parasitoid females were tested singly for each target in no-choice experiments, and observed for a maximum of five min. The percentage of response (oriented flights, landings on the target) to each target plant was calculated. The parameters of the bioassay were set as follows: temperature, 20 ± 1 °C; 65 ± 5% RH; wind speed, 25 ± 5 cm s^{-1}; distance between releasing vial and target, 50 cm; PPFD at releasing point, 700 µmol m^2 s^{-1}.

2.6 VOC collection and analysis

VOCs were collected from ten emitters and ten receivers by four air-tight entrainment systems each consisting of a glass jar (20 dm^3) connected to a circulating pump (closed-loop) whose flow was adjusted to 200 cm^3 m^{-1}. Before re-entering the pump, the air passed through a glass narrow tube filled with a biphasic phase of 30 mg of Tenax and 20 mg of Carboxen (GERSTEL GmbH & Co.KG, Mulheim an der Ruhr, Germany). Clean glass jars and pipelines were used on each measurement, to avoid memory effects. Plants were placed singly inside glass jars and VOCs were collected from the system for 3 h (totaling 3.6 dm^3 of air sampled) under PPFD of 700 µmol m^2s^1, temperature of 25 ± 2 °C and RH of 50 ± 10%. Four complete lines for volatile collection were used to allow simultaneous collection of volatiles from different target plants.

An Agilent 7890 GC-chromatograph coupled with an Agilent 5975C MSD spectrometer was used to analyze VOCs. The following chromatographic conditions were used: column HP-Innovax polyethylene glyco (50 m, 200 µm, ID 0.4 µm DF); splitless mode, oven programme: 40° for 1 min, then a 5 °C min^{-1} ramp to 200 °C, a 10 °C min^{-1} ramp to 220 °C, and a 30 °C min^{-1} ramp to 260 °C, final temperature held for 3.6 min. Mass spectra were acquired within the 29-350 m/z interval operating the spectrometer at 70 eV and at scan speed mode. Three scans s^{-1} were obtained. The identification of VOCs was done on the basis of both matches of the peak spectra with library spectral database, and comparison with pure standards. All standards were purchased by Sigma-Aldrich (Milan, Italy). After identification, each VOC found in the samples was quantified through regression lines built by using a set of serial dilutions of pure standards covering similar spans of VOCs as in sampled leaves. Data were analyzed using Agilent MassHunter Workstation software (Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA).

2.7 RNA extraction, cDNA synthesis and Real-Time RT-PCR

Experiments were carried out on leaves collected for each samples and immediately shock-frozen for molecular analysis. RNA was extracted according to the method of Chang et al (1993). Genomic DNA was removed using the Turbo DNA-free™ reagent (Ambion, Austin, TX, USA) following the

manufacturer's instructions. Absence of genomic DNA was verified by one-step reverse-transcription PCR (RT-PCR), using specific primers for the tomato elongation factor (Table S1). cDNA synthesis was then performed using the SuperScriptII® Reverse Transcriptase (Invitrogen) and 800 ng of total RNA, following the protocol of the supplier (Invitrogen Ltd, Paisley, UK). At the end of the reaction, cDNA was diluted 1:10 for quantitative gene expression analysis (RT-qPCR). Primers for RT-qPCR were designed using Primer3Input, a free software dedicated to design primers from a DNA sequence (http://bioinfo.ut.ee/primer3/). Oligonucleotide sequences are listed in Table S1. Reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems), following the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied Biosystems) as described by Perrone et al. (2012). Thermal cycling conditions were as follows: initial denaturation phase at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression of target transcripts was quantified after normalization to the geometric mean of the endogenous control genes, tomato elongation factor and ubiquitin (*LeEF* and *LeUBI*). Gene expression data were calculated as expression ratios (relative quantity) to controls. All reactions were performed with using three independent biological and three technical replicates. A water stress marker gene coding for a dehydrin (*LeTAS14*), two genes (*LeLOXC* and *LeLOXD*) encoding different lipoxygenase isoforms that participate in the synthesis of jasmonic acid (JA), the germacrene C synthase gene (*LeGCS*), which encodes a protein involved in the biosynthesis of terpenoids (a major class of VOCs in plants), and the hydroperoxide lyase gene (*LeHPL*), producing stress-inducible compounds such as Green Leaf Volatile (*GLV*), and a gene coding for a phenylalanine ammonia lyase (*LePAL5*), which can putatively be involved in salicylic acid (SA) synthesis, were considered. In addition, the expression of a gene coding for a salicylic acid carboxyl methyltransferase (*LeSAMT*), that catalyzes the reaction of salicylic acid (SA) and the methyl donor S-adenosyl-L-methionine (SAM) to methyl salicylate (MeSA; Tiemen et al., 2010) was evaluated.

2.8 Statistical analysis

Differences of physiological parameters and VOCs among treatments were analyzed using a one-way ANOVA with a Tukey post hoc test ($P < 0.05$). A multivariate approach was used to analyse differences in the volatiles emission among plant treatments. Multivariate analysis of variance (MANOVA) was used to compare: emitters *vs* receivers, unstressed *vs* water stressed and uninfested *vs* infested plant. The volatile emission patterns of emitters and receivers plant, measured as peak areas divided by the fresh mass of the plant, were analyzed through multivariate data analysis using partial least squares regression with discriminant analysis (PLS-DA, projection to latent structures discriminant analysis; mixOmics, R package) (Eriksson et al., 2006). The number of parasitoids

responding to each target was compared by a G-test for independence with William's correction. The resulting values of G were compared with the critical values of χ^2 (Sokal and Rohlf, 1995). Gene expression data were subjected to statistical analysis using SYSTAT 10 software, applying the nonparametric Kruskal-Wallis test, and adopting a probability level of $P < 0.05$.

3. Results

3.1 Gas exchange and fluorescence

At the start of the experiment ($FTSW_{100}$), all estimated parameters measured by gas exchange and fluorescence, i.e. *Pn*, g_s, ΦPSII, and qP, did not differ among the plants subjected to the different treatments (Fig. 1). At $FTSW_5$, g_s and *Pn* largely decreased in water-stressed (WS), and in water-stressed and aphids-infested (WS+A) plants (Fig. 1A, B). However, these parameters remained similar to controls (C) in well-watered plants infested with aphids (A). The fluorescence parameters ΦPSII and qP (Fig. 1C, 1D) also significantly dropped in WS and WS+A plants, as compared to C, but the reduction was less evident than that observed for g_s and *Pn*.

3.2 VOC emission

In the emitters, the aphid attack induced a significantly higher release of α-pinene with respect to C, whereas the other isoprenoids were not affected. Methyl salicylate also significantly responded to the aphids, as the emission increased significantly with respect to the tiny amount emitted by C (Table 1). Water stress, alone and in combination with the aphid infestation, further and significantly increased the emission of α-pinene and camphene of emitters, whereas the other volatile isoprenoids remained unaffected with respect to C (Table 1). The release of methyl salicylate also increased in WS and WS+A plants, but the effect of these treatments was similar to that caused by A only (Table 1). Total isoprenoid emission did not differ among treatments, although a trend was clearly noticeable toward higher VOC emissions in stressed plants (Table 1). These differences in volatile emissions as a whole emerged in the PLS-DA analysis that clearly separated control plants from all other ones either single or double stressed (Fig. S1).

In the receivers, there were no significant differences in the emission of isoprenoids, irrespective of the treatment to which the companion emitters were exposed (Table 2). A remarkable (+ 40% on average) but not significant increase of limonene emission was observed in receivers that were conditioned with WS and WS+A emitters. Methyl salicylate emission significantly increased in receivers that were conditioned by biotic or abiotic stresses, with respect to controls (Table 2). This increase was statistically larger than that observed in emitters, and particularly strong in plants

conditioned with WS or WS+A emitters. PLS-DA also separated only plants primed by unstressed control ones from plants primed by single or double stress (Fig. S2) although less clearly than when stressing the emitters. Overall, significant differences were noted when comparing emitters *vs* receivers, unstressed *vs* water stressed and uninfested *vs* infested plants (Table 3).

3.3 Wind-tunnel bioassay (Parasitoid preference)

Receivers primed by emitting plants exposed to any stress treatment (A, WS, and WS+A) showed a significant higher attractiveness towards aphid parasitoid females, both in terms of oriented flight and landings on the target, in comparison to plants that were conditioned by unstressed emitters (Fig 2).

3.4 Gene expression in leaves from stressed and conditioned plants

In the emitters, transcriptional levels of the water stress marker gene *LeTAS14* increased significantly in WS and WS+A and, to a lesser extent, in A plants, compared to C plants (Fig. 3). In the receivers, only plants conditioned with A plants showed significantly higher levels of *LeTAS14* with respect to C (Fig. 3).

In the emitters, the expression of *LeLOXC*, a gene involved in lipoxygenase formation, was up-regulated only by the WS+A treatment, while in the receivers a generally higher expression of *LeLOXC* in comparison to emitters was found, with no significant difference among treatments (Fig. 4A). The expression of *LeLOXD*, the other gene coding for a lipoxygenase isoform, was significantly upregulated only in emitters challenged with A, in comparison to C. In the receivers, as for *LeLOXC*, also *LeLOXD* was more expressed in all conditions with respect to emitters, and especially when plants were conditioned by A and WS+A emitters (Fig. 4B).

In the emitters, the expression of *LeHPL*, the gene involved in production of green leaf volatiles, was significantly downregulated in A compared to C plants (Fig. 4C). The expression of *LeGCS*, a gene of the isoprenoid pathway, was upregulated in WS+A plants, compared to C (Fig. 4D). No relevant differences among the treatments were observed in the expression of these two genes in the receivers, but the expression levels were generally higher in receivers than in emitters.

In the emitters, *LePAL5*, which can putatively be involved in salicylic acid (SA) synthesis, was downregulated by WS and WS+A, and upregulated by A (Fig. 4E). In the receivers, this gene was further upregulated with respect to emitters, in all conditions except than in A plants, where the same level of upregulation was observed. As a consequence, the increase of *LePAL5* expression in receiver plants was particularly significant in the WS and WS+A treatments (Fig. 4E).

In the emitters, *LeSAMT*, a gene involved in the formation of MeSA, was significantly downregulated in WS and WS+A conditions. Whereas in the receivers, this gene was significantly upregulated in plants that were conditioned with A and, to a less extent, with WS+A emitters (Fig. 4F).

4. Discussion

In this study, we have examined the effect of biotic (aphid) and abiotic (water) stresses, alone or in combination, on VOC release of tomato plants and, in turn, the putative priming impact of these VOCs on neighboring unstressed plants. Plants coping with multiple stresses, a rather common situation in nature (Suzuki et al., 2014), need to modulate their response investing energy in different metabolic pathways with frequent cross-talks between them (Bostok, 2005; Ponzio et al., 2016). The outcome of multiple stress factors on plant defence depends on many variables, including the type/species of stressors, the intensity of the stress, the plant species and plant physiological conditions (Atkinson and Urwin, 2012; Tariq et al., 2013; Ramegowda and Senthil-Kumar, 2015). The simultaneous presence of drought and pathogen infection or pest attack can lead to a positive or negative effect of one stress over the other, and to synergic or contrasting plant responses (Atkinson and Urwin, 2012; Ramegowda and Senthil-Kumar, 2015). Here we show that while water stress had a by far more dramatic negative impact on the physiology of tomato leaves (as shown by the large reduction of photosynthesis and stomatal conductance), both water stress and biotic stresses can enhance biosynthesis and emission of selected VOCs in emitting plants directly challenged by the stress, as well as in plants that were not exposed to the stress but received information form stressed emitters (receivers). We also show that the expression of genes putatively involved in VOC synthesis and in defense response was enhanced in receivers, and that changes of VOC profile altered the behavior of a natural antagonist of the aphids, incrementing attractiveness of receivers for the specific parasitoid.

Volatile organic compounds are powerful defensive compounds (Loreto and Schnitzler, 2010), and efficient means of communication between plants and the surrounding environment, including beneficial and harmful organisms as well as neighboring plants (Engelberth et al., 2004; Guerrieri, 2016; Coppola et al., 2017). Three compounds (α-pinene, camphene and methyl salicylate) were released at significant higher rates in emitters that were challenged by single or combined stress than in emitting controls. This indicates a direct impact of stresses on VOC metabolism of tomato plants, and suggests that these compounds effectively act as messengers for companion, unstressed plants. Methyl salicylate is indeed widely reported as an important elicitor of plant-insect interactions (Sasso et al., 2009; Digilio et al., 2012). However, MeSA emission was stimulated in response to aphid attack and water stress, alone or in combination, suggesting a general induction of the salicylic acid

(SA) metabolism, with protective functions (see below). A relationship between the release of MeSA and water stress was also reported for *Alnus glutinosa* (Copolivici et al., 2014). We will first discuss implications for the observed MeSA changes and then focus on volatile isoprenoids.

In tomato, the primary route involved the SA biosynthesis, and consequently in the defense response *via* SA, was reported to be the phenylpropanoid pathway involving phenylalanine ammonia lyase (PAL; Król et al., 2015). In our work, the expressions of a PAL gene (*LePAL5*) and of a gene coding for MeSA transferase (*LeSAMT*), the enzyme involved in the transition from non-volatile SA to MeSA, increased in emitters attacked by aphids, but not in the double stressed plants. These results suggest that a combined abiotic and biotic stress lead to a different expression of defense genes with respect to a single stress condition. Remarkably, Atkinson et al. (2013) have reported that when water deficit and nematode stress were applied to *Arabidopsis* plants in combination, the resulting gene expression profile resembled that of the plant under water deficit alone more closely than under nematode stress alone.

Aphid attack caused a general increase of the expression levels of key genes involved in different plant-response pathways, as already observed (Digilio et al., 2010). In particular, enhanced transcript levels of *LeLOXD* in plants attacked by aphids were observed on tomato plants infested by *M. euphorbiae*. *LeLOXD* codes for a chloroplast-localized lipoxygenase involved in wound-induced jasmonic acid biosynthesis, which leads to an increased expression of wound-responsive genes and, therefore, to an improved plant resistance to insect herbivory attack and necrotrophic pathogen infection (Yan et al., 2013).

We report an absence of effect or even a downregulation of genes involved in defensive pathways in water stressed plants. Downregulation of the impact of aphids on gene expression was also observed in plants exposed to combined WS+A stress. We expected that, being aphids sap feeders, the attack could have an impact on the water balance of the plant, eventually leading to an effect mirroring water stress. However, the expression of *LeTAS14*, a water stress marker gene which encodes a tomato dehydrin (DHN), increased largely when water stress was present (WS, WS+A), while the increase was limited (though significant) in plants attacked by the aphids. We speculate that chemical signals play a more significant role than hydraulic signals in activating defensive pathways of tomato. For example, an important role might be played by chemical compound of aphid saliva that has been reported to alter plant VOC when a threshold is reached (Guerrieri et al., 1999).

In the receivers, MeSA was the sole volatile compound released at a significant higher rate, and the most suitable candidate to alter volatile-mediated relationships between plants and other organisms. Indeed, MeSA stimulation was accompanied by higher *LeSAMT* expression, especially when

receivers were conditioned by aphid-treated plants, suggesting that MeSA priming is particularly effective in response to insect infestation. Specifically, MeSA may have been responsible for the higher attraction of *A. ervi*, as revealed by our behavioral experiment showing increasing flights and landings of the aphid parasitoid on receivers that are exposed to stressed emitters. The rate of emission of MeSA was very low in receivers, when compared to all other compounds of tomato volatiles blend (e.g. Sasso et al., 2007; and Tables 1, 2). It is, therefore, confirmed that tiny releases of MeSA might elicit indirect defense responses, attracting both aphid predators (Zhu and Park, 2005) and parasitoids (Sasso et al., 2007). In fact, MeSA elicits an antennal response by the aphid parisitoid *A. ervi* at a concentration as low as 0.01mg/ml (Sasso et al., 2009). Thus, emitters release MeSA at a rate suitable not only for self-defense, but also to propagate the alarm to neighboring plants. Creation of an "alert" wave propagating quickly among neighboring individuals might be a winning adaptive behavior at plant population level, since aphids are not able to move fast when flying or walking to colonize new plants. Interestingly, genes involved in defensive pathways showed an up-regulation trend in receivers, with the highest expression levels generally observed in plants attacked by aphid, as also observed in emitters. In particular, as *LePAL5* is involved in the response to several stress conditions (Chang et al., 2008) this gene is a good target of the MeSA priming effect in receivers (Arimura et al., 2000). Overall, our results suggest that MeSA is a main component not only as a stress messenger, but also as elicitor (primer) of defensive compound biosynthesis in unstressed tomato plants.

The volatile isoprenoids that were stimulated by stresses might have a direct role as stress defensive compound, either as antioxidants or as stabilizers of cellular membrane (Loreto and Schnitzler, 2010; Velikova et al., 2011). The large reduction of photosynthesis observed in this experiment was attributed to the typical water saving mechanism of tomato adapted to dry Mediterranean conditions, i.e. to a rapid, yet fully reversible, closure of stomata. Volatile isoprenoids might have helped dissipate excess electron flow under those conditions, as often shown (Pollastri et al., 2014). Previous studies showed contrasting results on the effect of multiple stress factors on VOC release, either additive (Vuorinen et al. 2004; Copolivici et al. 2014) or reductive (Himanen et al., 2009). Salerno et al. (2017) have recently reported that in broad bean plants the combination of a water stress with the attack of the green bug *Nezara virudula* reduced VOC emission, although this was not associated to a reduced attraction of an egg parasitoid of the stink bug, despite water deficit promoted both a significantly lower increase in weight and a higher mortality of the stink bug nymphs (Ederli et al., 2017). Here, the release of α-pinene was enhanced in emitter tomato plants subjected to single or combined stresses. However, volatile isoprenoids do not seem to play a role as messenger or priming agent in tomato. VOC-driven elicitation of defensive pathways in neighboring (not attacked) plants

was demonstrated in different plant species interacting with insects, but only when a single stress (elicitor) was considered. For example, corn plants exposed to green leaf volatiles (GLV) released by emitter plants after the attack of a caterpillar started releasing a similar blend of VOC (Engelberth et al., 2004). Coming to volatile isoprenoids, the exposure to β-ocimene, a common stress-related compound, induced the release of defensive VOC in receiving tomato plants, resulting in enhanced attraction of mite predators (Shimoda et al., 2012) and aphid parasitoids (Cascone et al., 2015). β-ocimene was also released at a higher rate by tomato plants exposed to either plants treated with systemin and expressing a systemin precursor, or chewed by a caterpillar, with respect to plants exposed to untreated tomatoes, resulting in a higher attraction for the parasitoid *A. ervi* (Coppola et al., 2017). We do not know why this same action was not observed in our experiment with tomato. Among the possible reasons are limited stress severity, or the absence of VOC acting as active messengers and priming agents in the VOC blend characterizing the emission of our tomato cultivar, or, most probably, a specificity of plant response to different stresses resulting in specific priming volatiles.

In conclusion, we have shown that interactive biotic and abiotic stresses may have an additive impact on VOC emission but an opposite effect on the expression of genes involved in defensive pathways. VOCs emitted by stressed plants may induce VOC emission in companion plants that do not suffer any stress, and this may increase attraction of parasitic wasps. This novel information could be useful for insect pest management, under a realistic climate change scenario.

Conflicts of interest

The authors do not have any conflicts of interest to declare.

Declaration of contributions

Mauro Centritto, Stefano Catola, Annamaria Ranieri and Emilio Guerrieri conceived and designed the experiment; Stefano Catola performed eco-physiological measurements, VOC collection and drafted the manuscript; Pasquale Cascone performed the wind tunnel experiment and participated to VOC collection; Luca Calamai performed VOC analysis; Raffaella Balestrini performed gene expression analyses; Stefano Catola, Mauro Centritto, Annamaria Ranieri, Francesco Loreto, Raffaella Balestrini and Emilio Guerrieri wrote the manuscript.

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Table 1. VOCs released by emitters that were not stressed (C), aphid-attacked (A), water-stressed (WS) and exposed to both water stress and aphid infestation (WS+A). Values not sharing the same letter are significantly different as shown by pairwise t test **(***P* < 0.05). Data are expressed as mean ± SE (n = 10).

	VOC from emitters (nmol m^{-2})			
	C	**A**	**WS**	**WS+A**
α- pinene	12.59 ± 1.46**a**	20.2 ± 1.84**b**	37.46 ± 4.76**c**	44.73 ± 4.42**c**
camphene	14.82 ± 3.8**a**	16.87 ± 4.42**ab**	21.45 ± 2.14**b**	27.03 ± 2.39**b**
α-terpinene	123.96 ± 26.85	160.49 ± 44.55	156.52 ± 32.06	192.76 ± 42.21
α-phellandrene	10.7 ± 3.71	12.15 ± 3.21	12.3 ± 1.88	14.51 ± 2.6
limonene	114.5 ± 38.94	100.08 ± 30.02	106.89 ± 25.6	137.09 ± 31.21
γ-terpinene	176.58 ± 48.46	188.91 ± 45.32	185.33 ± 37.87	192.49 ± 37.06
p-cymene	40.59 ± 15.06	41.98 ± 14.82	32.58 ± 5.92	42.55 ± 9.4
methylsalicilate	0.27 ± 0.04**a**	1.8 ± 0.24**b**	1.55 ± 0.25**b**	2.15 ± 0.35**b**
Total	**494.01 ± 135.84**	**542.46 ± 131.13**	**554.08 ± 93.4**	**653.31 ± 111.47**

Table 2. VOCs released by receivers. These plants were not stressed but were primed by emitters that were not stressed (C), aphid-attacked (A), water-stressed (WS) and exposed to both water stress and aphid infestation (WS+A). Values not sharing the same letter are significantly different as shown by pairwise t test **(***P* < 0.05). Data are expressed as mean ± SE (n = 10).

	VOC from receivers (nmol m^{-2})			
	C	**A**	**WS**	**WS + A**
α-alpha pinene	22.45 ± 4.1	21.36 ± 3.04	25.06 ± 2.88	26.42 ± 3.52
camphene	17.99 ± 3.1	12.86 ± 1.75	15.37 ± 0.94	16.36 ± 2.12
α-terpinene	122.12 ± 28.17	192.96 ± 48.81	125.84 ± 19.87	145.14 ± 26.96
α-phellandrene	9.1 ± 2.19	6.72 ± 1.04	6.69 ± 0.72	8.17 ± 0.92
limonene	60.25 ± 25.75	85.89 ± 20.15	67.73 ± 16.71	100.51 ± 20.96
γ-terpinene	130.89 ± 37.25	116.47 ± 15.02	125.47 ± 26.96	119.96 ± 20.48
p-cymene	32.13 ± 12.39	27.01 ± 5.31	26.87 ± 5.07	33.81 ± 6.84
methylsalicilate	0.51 ± 0.08**a**	3.39 ± 0.87**b**	2.72 ± 0.54**ab**	3.76 ± 1.21**b**
Total	**395.44 ± 98.17**	**466.65 ± 76.29**	**395.74 ± 67.08**	**454.13 ± 69.01**

Table 3. Multivariate analysis of variance (MANOVA) of all volatiles emitted from emitter and receiver tomato plants.

Thesis Tested	Statistic	Value	Num df	Den df	*P*	F
Emitters *vs* Receivers	Pillai	0.23	8	69	0.02	2.51
Well-watered *vs* Water-stressed	Pillai	0.20	8	69	<0.01	6.32
Unifested *vs* Infested	Pillai	0.42	8	69	0.04	2.21

Figure Legends

Fig. 1. Physiological parameters in non-stressed and aphid-attacked plants, in irrigated (C and A, respectively) and water stress (WS, WS+A) conditions. (A) Stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), (B) net photosynthesis (P_n, µmol CO_2 m^{-2} s^{-1}), (C) quantum yield of PSII in the light (ΦPSII), (D) photochemical quenching (pQ). Data are expressed as mean ± SE (measurements were performed at least on seven plants for each condition). Lower case letters denote significant differences between means (Tukey test, $P < 0.05$).

Fig 2. Flight behaviour of the aphid parasitoid *Aphidius ervi* towards "receiver" (named PriC, PriWS, PriA, PriWS) tomato plants primed by C, WS, A, WS+A plants. Values indicate the percentage of female showing oriented flights (A, dark grey columns) and landings on receivers (B, light grey columns). Each assay was conducted using at least 100 females (n = 10). Different letters indicate significant differences between means (G-test, $P < 0.05$).

Fig. 3. Expression changes of a tomato dehydrin gene (*LeTAS14*): i) in leaves of non-stressed (C) plants and of plants attacked by aphid (A), water stressed (WS), and exposed to aphids and water stress (WS+A) (light grey); ii) in leaves of receivers primed by C, WS, A, WS+A emitters (dark grey). Data are expressed as mean ± SE (n = 3). Values of means not sharing the same letter are significantly different as shown by Kruskal-Wallis non-parametric test ($P < 0.05$).

Fig. 4. Expression changes of stress-dependent genes: i) in leaves of non-stressed (C) plants and of plants attacked by aphid (A), water stressed (WS), and exposed to aphids and water stress (WS+A) (light grey); ii) in leaves of receivers primed by C, WS, A, WS+A emitters (dark grey). Data are expressed as mean ± SE (n = 3). Values of means not sharing the same letter are significantly different as shown by Kruskal-Wallis non-parametric test ($P < 0.05$).

Supplementary Information

Table S1. List of the oligonucleotides used for RT-qPCR analyses.

Fig. S1. PLS-DA comparison of the volatile compounds collected from emitters non-stressed and aphid attacked tomato plants, in irrigated (C and A, respectively) and water stress (WS, WS+A) conditions. Score plot of the samples, with the percentages of explained variation in parentheses.

Fig. S2. PLS-DA comparison of the volatile compounds collected from tomato plants primed by C, WS, A, WS+A plants (named PriC, PriWS, PriA, PriWS). Score plot of the samples, with the percentages of explained variation in parentheses.