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Article

Abiotic and Biotic Combined Stress in Tomato: Additive, Synergic and Antagonistic Effects and Within-plant Phenotypic Plasticity

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Simple Summary: This paper focused on the morpho-physiological and metabolic responses to single and combined abiotic and biotic stress evaluating, also, these responses among the differentially vascular-connected leaves of tomato plants. This study aimed to highlight the no-additive effects of the combination of drought stress, N deficiency and herbivory respect to the single ones as intrinsically interesting aspect of the interactions between plants and the heterogeneous and dynamic plant environment. We behave that the results could be useful for plant adaptation to the new environments that will be define by climate change but, above all, could be a useful spur to the ecophysiologists and community and ecosystem ecologists in their studies for understanding the plant-environment interaction.

Abstract: Background: Drought, N deficiency and herbivory are considered the most important stressors caused by climate change in the agro- and eco-systems and varied in space and time shaping a highly dynamic and heterogeneous stressful environments. This study aims to evaluate the tomato morpho-physiological and metabolic responses to combined abiotic and herbivory at different within-plant spatial levels and temporal scales.
Methods: Leaf-level morphological, gas exchange traits and VOC profiles were measured in tomato plants exposed to N deficiency and drought, T. absoluta larvae and their combination. Additive, synergistic or antagonistic effects of the single stress when combined were also evaluated. Morpho-physiological traits and VOC profile were also measured on leaves located at three different positions along the shoot axes.
Results: The combination of the abiotic and biotic stress has been more harmful than single stress with antagonistic and synergistic but non-additive effects for the morpho-physiological and VOC tomato responses, respectively. Combined stress also determined a high within-plant phenotypic plasticity of the morpho-physiological responses.
Conclusions: These results suggests that the combined stress in tomato determined a "new stress state" and a higher within-plant phenotypic plasticity which could permit an efficient use of the growth and defence resources in the heterogeneous and multiple stressful environmental conditions.

Keywords: within-plant phenotypic plasticity; combined stresses; additive, antagonistic and synergic effects; VOC

1. Introduction

Owing to sessile nature, plants are continually exposed to abiotic (mainly drought, heat and salinity) and biotic stresses (pathogens and herbivory) whose intensity and frequency are expected to be increased by climate change. The effects of these stresses and

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how the plants respond to these stressful factors taken individually, have been extensively studied at both the morpho-physiological and molecular scale [1, 2] and plant community level [3, 4]. However, under field condition, these various biotic and abiotic factors are constantly changing during the plant life cycle and, above all, co-occur in nature [5]. Hence, the plants have to make decisions about fine-tuning their responses to allocate resources efficiently for responding to the more serious and different threats at any given point in time. Different studies have uncovered that plants evoke a "unique response" to the abiotic and biotic combined stresses compared to the single stress [5, 6, 7, 8] revealing that the plant responses to combined stress pointed out "a new stress state" with mostly non-additive effects (i.e., synergistic and antagonistic). For example, the insect herbivory antagonized the heat responses in tomato [9], the emission of specific VOCs was synergized by the combination of aphid and drought stress in tomato plants [10] as well in the larvae of green alder sawfly (*Monsoma pulveratum*) and *Alnus glutinosa* interaction [11], some morphological traits of *Pinus sylvestris* were synergized while others antagonized in drought and simulate herbivory combination [12]. In addition to the "new stress state", the plant responses to the stress are strictly dependent on the plant traits, genotypes, species, and type, intensity, frequency and duration of the stress suggesting that more investigation are needed for a better understanding of the abiotic and pest herbivore interaction, the stress combination lesser studied.

The plant responses to the individual abiotic and biotic stress have been showed to observe a modulation (induced and constitutive) with a strong spatio-temporal component (local and systemic, transient and permanent) that determined a high "within-plant variation". For example, the spatial scale of herbivore- induced changes can range from localized at the site of attack [13] to systemic throughout the entire plant or tissue type [14, 15] as well the light and heat gradients determined different responses within the tree canopy [16] or the nutrient deficiency caused different morpho-physiological responses among the root types [17, 18]. The temporal scale of the plant responses can also vary: rapid or long term, ontogenic-modulated [19] and in some cases even trans-generational responses are evoked for the herbivory [20] as well the abiotic stress [21]. The multiple ecological role of the 'within-plant' variation was recently pointed out in the adaptation to individual biotic and biotic gradients [22] and the alteration of plant-antagonist interactions [23] so much so that it was proposed as "functional trait itself" whose influences on ecosystem functioning still neglected [24]. In spite of this important role, the within-plant variation in response to the combined stress has been still no investigated at our knowledge.

Since 2006, the tomato production of the Mediterranean region is under attack by a newly introduced insect, *Tuta absoluta* whose larvae feed on leaves, stems and fruits causing severe damage to the tomato with decreases in production both in the field and greenhouse [25]. Studies revealed that the low nitrogen levels and drought stress inputs to tomato negatively affected the biological traits of the *T. absoluta* [26, 27] but also demonstrated that the N deficiency and drought could be also unfavorable to the tomato plants suggesting to evaluate the trade-off between negative impact on Tuta pests and plant growth. In this respect, experiments were set up to study the spatial and temporal expressions of the morpho- physiological and metabolic responses of the tomato plants to the single and/or combined abiotic (drought+N deficiency) and biotic stress (herbivory by T. absoluta). In particular, the present study investigates the following questions: 1) Are the morpho-physiological responses to individual stresses different from the combined ones in tomato plants? 2) Are additive, synergistic or antagonistic effects in the combined stress? 3) Do the tomato responses to the single and combined stress occurred at between- or within-plant levels?

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2. Materials and Methods

2.1. Experimental procedure and Plant material

This study was constituted by two experimental sets addressing different but consequently related questions.

The first experiment, i.e. the 'synergic, antagonistic and additive effects', aimed to determine the tomato response to the single and combined stress and their temporal evolution and whether the responses to the combined stress were the results of the additive, synergistic or antagonistic effects of the single stress. For this purpose, the effects of the abiotic (drought and N deficiency) (ABIO), biotic (herbivory by T. absoluta) (BIO) and combined stress (abiotic plus biotic stress) (COMB) and the time of exposure (0, 1, 3 and 8 days) on the morphological (leaf fresh and dry weight and water content), physiological (photosynthesis, stomatal conductance, transpiration rate and WUEi) and metabolic (VOC) plant traits were evaluated. The leaf fresh and dry weight are traits directly related to the plant status, while the leaf water content was strictly correlated with the plant drought tolerance [28] but also with the plant palatability [29]. The gas exchange traits (photosynthesis, stomatal conductance, transpiration and water use efficiency) are involved in the plant responses to drought and N deficiency [30, 31] and further the photosynthesis is "…a plant-driven response to the perception of stress rather than a secondary physiological response to tissue damage…" highlighting a strict interactions between photosynthesis, ROS and hormonal signaling pathways for the plant response to insect herbivory [32]. Finally, the VOCs, as direct and indirect defense, are emitted by plants subject to both abiotic and biotic stress [33].

Tomato plants (Solanum lycopersicum L., cultivar nano S. Marzano) (provided by BAVICCHI S.p.a., ITALY) were exposed to the abiotic stress (nitrogen limitation and drought stress simulated by the use of PEG), biotic stress (two first instar larvae placed in a leaf), or their combination and were considered as 'stress condition'. The control plants (CTR) was maintained at optimal N concentration and no drought and herbivory and it was considered as the 'optimal condition'. For the morpho-physiological analysis, we used a randomized block design in which the entire experiment yielded a total of 4 (treatments) x 4 (time of exposure) x 2 (block) x 2 (replications) = 64 samples. The block was introduced because we used two experiments at two different times. A completely randomized design was used for the VOC profiling, in which the entire experiment was constituted by 4 (treatments) x 4 (time of exposure) x 3 (replicates) x 3 (measurements) = 144 samples. The replicates for the VOC were obtained in three different experiments.

The second experiment, i.e. "within-plant phenotypic plasticity", aimed to evaluate the within-plant variation of the tomato morpho-physiological and metabolic traits and how this within-plant phenotypic plasticity changed with each environmental conditions (optimal, abiotic, biotic and combined stress). For this aim, the environmental effects on tomato traits were evaluated on three mature leaves located at three different positions along the shoot axes for each treatment. For each treatment, we used a completely randomized design in which the entire experiments yielded a total of 3 (leaves) x 1 (time of exposure) x 3 (replicates) = 9 samples. For the gas exchanges traits only, we took two measurements for each leaf, hence the experiments provided 3 (leaves) x 1 (time of exposure) x 2 (measurements) x 4 replicates =24 samples.

The Figure S1 reported the experimental protocol schedule of both experiments including the plant growth, treatments and analysis.

2.2. Growth condition

Tomato seeds were surface sterilized for 15 min in 10% (v/v) sodium hypochloride, rinsed with tap water and then were germinated in a Petri dish (diameter 90 mm) on filter paper with 0.1 mM CaSO4. After 7 d of germination (7 DAS), six seedlings of uniform size were transferred to eight hydroponic unit containing 4.5 L of the following aerated nutrient solution at 50% strength and adjusted to pH 6.0 with 0.1 M potassium hydroxide: 5mM KNO3, 1 mM NH4NO3, 1.44 mM MgSO4, 3.99 mM Ca(NO3)2, 0.97 mM KH2PO4, 1

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mM K2SO4, 25 µM H3BO3, 50 µM KCl, 2 µM MnSO4, 4 µM ZnSO4*7H2O, 0.5 µM CuSO4*5H2O, 0.5 µM (NH4)Mo7O24*4 H2O, 20 µM EDTA iron(III) sodium salt.

This nutrient solution (Nutritional recommendation for tomato" downloaded from Haifa website: http://www.haifa-group.com/files/Guides/tomato/Tomato.pdf) was adopted after preliminary experiments that compared different nutrient solutions on tomato growth and SPAD data.

The hydroponic units were placed in a growth chamber at 24°C, 14 h photoperiod; photon flux rate of 300 mmol m-2 s-1; 70% RH.

After 7 days (14 DAS), the nutrient solution was brought to 100% strength and the plants of each pot were reduced at four for the morpho-physiological analysis while they were left to six for the VOC analysis. The nutrient solution was renewed every 2 days.

2.3. Insect Rearing

The tomato leafminer Tuta absoluta (Lepidoptera: Gelechiidae) colony was maintained in climatic chambers (25°C, RH 70%, 16h light). It was kept in cages (Bugdorm® - 60x60x60 cm) and containing tomato plants. Sugar and water were provided ad libitum to adults in rearing cages.

2.4. Abiotic stress and Herbivory treatment

At 28 DAS, six hydroponic units continued to receive the same nutrient solution as previously described in growth condition while in two hydroponic units were added 5% (w/v) Polyethylene glycol 8000 (Sigma PEG8000) and 1 mM nitrogen for simulating the drought stress and nitrogen deficiency, respectively (ABIO group). The final PEG concentration was gradually achieved by the addition of 2.5% (w/v) PEG8000 every two days. The osmotic potential of the solutions, measured by a osmometer (Freezing point osmometer, Osmomat 3000, Gonotec), was -0.55 MPa for 5% PEG and -0.05 MPa for the control solution (0% PEG). To obtain 1 mM N for the nitrogen deficiency, the NH4NO3 was not added and the KNO3 and Ca(NO3)2 were reduced to 1 mM and 0.5 mM, respectively. In order to balance K and Ca, the K2SO4 and CaSO4 were increased to 3 mM and the 3.5 mM, respectively. Preliminary experiments were conducted to individuate the PEG8000 and N concentrations used for simulating the drought stress and nitrogen deficiency, respectively, for the entire experiment without the appearance of dead plants.

At 42 DAS, the hydroponic units were treated as following for obtaining the whole set of traetments (Figure S1):

1) two hydroponic units were renewed the optimal nutrient solution (CTR group);

2) two hydroponic units were renewed the nutrient solution with N deficiency and PEG (ABIO group);

3) two hydroponic units received the optimal nutrient solution but the plants were infested with Tuta larvae to induce the biotic stress (BIO group);

4) two hydroponic units maintained the same nutrient solution with N deficiency and PEG and In addition the plants were infested by Tuta larvae (COMB group).

The plant infestation was obtained by placing two first instar larvae of Tuta in the 1st fully-developed leaf (with 5 leaflets) from the bottom and to avoid larvae escaping, each infested leaf was then bagged with a nylon mesh of 4.7 cm diameter (Figure S2). We added herbivorous insects to plants after 7 days of abiotic stress treatment in order to simulate the effects of a pest outbreak which are predicted to become more frequent with climate change [34].

2.5. First experimental: synergic, antagonistic and additive effects.

2.5.1. Measurements and samplings

At 0 (42 DAS), 1 (43 DAS), 3 (45 DAS), and 8 days from the treatments (50 DAS), the measurements/samplings were realized in order to simulate the short-, middle- and long-time responses, respectively. The measurements for the gas exchange traits were carried out on terminal leaflet of 1st fully-developed leaf (in presence of larvae, we used lateral

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leaflets) while the whole plants was used for the morphological analysis. Three consecutively leaves for each treatments and time of exposure were sampled for the VOC.

2.5.2. Gas exchange measurements

A calibrated portable photosynthesis system (LI-6400; LI-COR, Inc.; Lincoln, NE) was used to measure net CO2 assimilation rate (A, μmol (CO2) m−2 s−1), stomatal conductance (gs, mol H2O m-2 s-1), and transpiration rate (T, mmol H2O m-2 s-1). These gas exchange parameters were measured at 500 cm3 min−1 flow rate, 26 °C leaf temperature, CO2 concentration 400 μmol(CO2) mol(air)−1 (controlled by CO2 cylinder), and 1200 μmol m-2s-1 of photosynthetically active radiation supplied by the LED light source in the leaf chamber. Each measurement was made with a minimum and maximum wait time of 120 and 200 s, respectively, and matching the infrared gas analyzers for 50 μmol (CO2) mol(air)−1 difference in the CO2 concentration between the sample and the reference before every change of plants. The leaf to-air vapor pressure difference (VPD) was set to 1.5 kPa, and continuously monitored around the leaf during measurements and maintained at a constant level by manipulating the humidity of incoming air as needed. All measurements were performed in growth chamber.

Finally, the water use efficiency intrinsic (WUEi) was calculated as the rate of photosynthesis (A) divided by the rate of stomatal conductance to water (gs) [35].

2.5.3. Morphological measurements

All the leaves of the plants were harvested, immediately weighted to obtain the leaf fresh weight (LFW, g) and placed in an oven at 70°C for 2 days to determine the leaf dry weight (LDW, g).

By the above measurements, the leaf water content (LFW, %) was calculated as the following as reported in Jin et al. [36]:

Leaf Water content (%)=(LFW−LDW)/LFW∗100 (1)

2.5.4. VOC analysis

Volatile organic compounds (VOCs) from three leaves per treatments and time of exposure were profiled by HS/SPME method. One leaf was sealed in a 20 ml hermetic vial with butyl lid and allowed to incubate for 20 minutes at room temperature. The fiber (50/30 μm DVB/CAR/PDMS) (Supelco®, Bellefonte, PA, USA) was conditioned according to the supplier's instructions prior to its use and then was inserted into the headspace of the vial containing the sample for 20 minutes for the adsorption of a suitable and representative number of volatiles. The volatiles were desorbed by placing the fiber for 6 min into the injection port of the GC-MS system. All the SPME sampling and desorption conditions were identical for all the samples. Blanks were performed before first SPME extraction and randomly repeated during each series.

GC-MS analyses were performed with a Thermo Fisher TRACE 1300 gas chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness = 0.25 μm, with 10 m of pre-column) and a Thermo Fisher ISQ LT ion trap mass detector (emission current: 10 microamps; count threshold: 1 count; multiplier offset: 0 volts; scan time: 1.00 second; prescan ionization time: 100 microseconds; scan mass range: 30–300 m/z; ionization mode: EI).

GC–MS data were obtained under the following analytical conditions: carrier gas Helium (He 99.99%); flow rate 1 ml/min; spiltless. The initial oven temperature was 60°C for 3 min, after which it was raised to 240°C at 6 °C/min, where it was held for 3 min. The injection port, transfer line, and source temperatures were 250°C, 250°C, and 260°C, respectively.

Qualitative identification was performed using GC–MS reference libraries (NIST x.0). Linear retention indices (LRI) were determined from the retention times of a series of n-alkane mixture (C8-C20, Sigma Aldrich, Milan, Italy) analysed under identical conditions [37]. Percentage of the studied compounds were calculated from the peak areas in the total

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ion chromatograms. The relative abundance of volatile compounds was relative to the total amount or released volatiles, after subtracting eventual contaminants.

2.5.5. Statistical analysis

Morpho-physiological data

By SPSS Inc., V. 10.0, 2002 (SPSS Inc., Evanston, IL, USA), all the morpho-physiological parameters were analyzed by two-way ANOVA with the Treatment (Tr) (CTR, ABIO, BIO and COMB), Time of exposure (Ti) and Block (Bl) as main factors and the TrxTi as interaction. Then, Tukey's test was used to compare the means of all the parameters of each Tr and Ti. All data were tested for normality (Kolmogorov–Smirnoff test) and homogeneity of variance (Levene median test) and, where required, the data were transformed.

VOCs data

The VOC dataset was elaborate using R statistical software 3.5 [38].

Differences among treatments, time of exposure and TrxTi interaction were inferred through PERMANOVA multivariate analysis (999 permutations) using the package vegan. Pairwise comparisons were calculated using a custom script and correcting P values using the False Discovery Rate (FDR) method.

In order to identify VOC key predictors that could constitute a molecular signature identification among the treatments within each time of exposure, we used a preliminary unsupervised (Principal Component Analysis, PCA) and then supervised analysis (Sparse Projection to Latent Structure-Discriminant Analysis, sPLS-DA) using the package mixOmics [39]. Statistical algorithms are detailed in Rohart et al. [39] and they account for multiple comparisons inherent in biomarker datasets, where multiple classification features are considered for a relatively small number of specimens ($p >> n$). In particular, the sPLS-DA procedure constructs artificial latent components of the predicted dataset (VOCs Table denoted $X(N \times P)$) and the response variable (denoted Y with categorical information of samples, e.g. CTR, ABIO, BIO and COMB). To predict the number of latent components (associated loading vectors) and the number of discriminants, for sPLS-DA, we used the perf.plsda() and tune.splsda() functions, respectively. We fine-tuned the model using 5-fold cross-validation repeated 10 times to estimate the classification error rates employing two metrics, overall error rates and balanced error rates (BER), between the predicted latent variables with the centroid of the class labels (categories considered in this study) and specifying the max.dist (which gave the minimal classification rate in this study).

Calculation of additive, synergistic or antagonistic effects in combined stress

To determine if abiotic stress and herbivory treatments exerted additive, synergistic or antagonistic impacts on tomato traits, we used the Bansal et al. method [12] and, specifically, we compared the observed effects (Ob) to expected additive effects (Ex) for the plants exposed to the abiotic stress and herbivory combination (COMB) at 3 and 8 days of treatments, only. The Ob effect sizes were calculated as the absolute value of:

$$Ob = (ob- \bar{x}CTR)/\bar{x}CTR \quad (2)$$

where ob is the measured trait value for each plants and treatment and $\bar{x}CTR$ is the mean trait value for the CTR plants.

The Ex additive effect sizes for the treatment COMB were define in two steps by first determining and then summing the independent effects (In) of each treatment. The In effect sizes were calculated as the absolute value of:

$$Ind = (\bar{x}stress- \bar{x}CTR)/ \bar{x}CTR \quad (3)$$

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where $\bar{x}$stress is the mean trait values from a single stress, and $\bar{x}$CTR is the mean trait value for the CTR plants. Then, the Ex additive effect size for the COMB treatment were calculated using a multiplicative risk model as suggested by Darling et al. [40], that is the sum of two In effects minus their product. Finally, the Ex additive values for COMB plants were compared to the actual Ob additive effects. In particular, we calculated a mean difference (± 95% confidence interval) between the effect sizes of Ob and Ex was for COMB plants. When Ob-Ex > 0 and the lower 95% confidence limit was greater than zero, then the impact from the combination of both stressor was classified as synergistic. Antagonistic effects were defined when the Ob-Ex < 0 and the upper 95% confidence limit was less than zero. Finally, we classified additive effects when the 95% confidence interval crossed the zero line.

2.6. Second experiment: within-plant phenotypic plasticity

2.6.1. Measurements and samplings

The measurements and samplings were carried out at 8 days from the treatments (50 DAS) in the leaves located at three different positions along the shoot axes: basal (B), intermediate (I) and apical leaf (A) placed at first, second and third node, respectively. Preliminary experiments using phloem dying as reported in Orians et al. [41] resulted that the apical leaf, but not the intermediate, is linked to the basal one by vasculature connection (Figure S3). In this respect, we also considered the basal, intermediate and apical leaves as the local (L), no-orthostichous (nO) and orthostichous leaf (O), respectively. The basal/local leaf was used for placing the first instar larvae

Measurements for the gas exchange traits were carried out on two opposite leaflets of the basal/local (B/L), intermediate/noOrthostic (I/noO) and apical/orthostic leaf (A/O) placed at first, second and third node, respectively, and the same leaves were subsequently collected for the morphological analysis.

2.6.2. Morpho-physiological analysis

All the morpho-physiological analysis were carried out as in the first experiment.

2.6.3. Statistics

Within-plant variance of the morpho-physiological traits

The within-plant variance of the morpho-physiological traits was evaluated as in Zywiec et al. [42].

In order to estimate the partitioning of total variation of the morpho-physiological traits among- and within-treatments, we conducted Linear Mixed Models with treatments and plant nested within-treatments as random effects using the whole-plant data. The variance partitions among- and within-treatments and tests on the statistical significance of variance components were conducted using restricted maximum likelihood (REML).

In order to verify the effects of each treatments on morpho-physiological traits of different leaves within the plants, we analyzed the within-plant variation by applying a hierarchical partition to divide total variance into two levels of variation: among plants and among the leaves in the same plants (leaf nested within plant). All levels were considered as random effects, as required for variance partitioning. Analyses were conducted with the mixed procedure of SPSS. The replicate obtained for each leaflets sample allowed us to estimate measurement error and thus assess the variance component and statistical significance (Wald Z and p values) of this component between- and within-individual plants.

Morpho-physiological data

By SPSS Inc., V. 10.0, 2002 (SPSS Inc., Evanston, IL, USA), all the morpho-physiological parameters were analyzed by one way ANOVA with Tukey's test as post-hoc test ($p<0.05$).

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3. Results and Discussions

3.1. Are the morpho-physiological responses to individual stresses different from the combined ones in tomato plants? Are additive, synergistic or antagonistic effects in the combined stress?

The morpho-physiological results clearly indicated an opposite pattern of the tomato plant responses to the single stresses with the ABIO treatments showing more negative impact than the BIO one respect to the CTR plants (Figure 1 and 2).

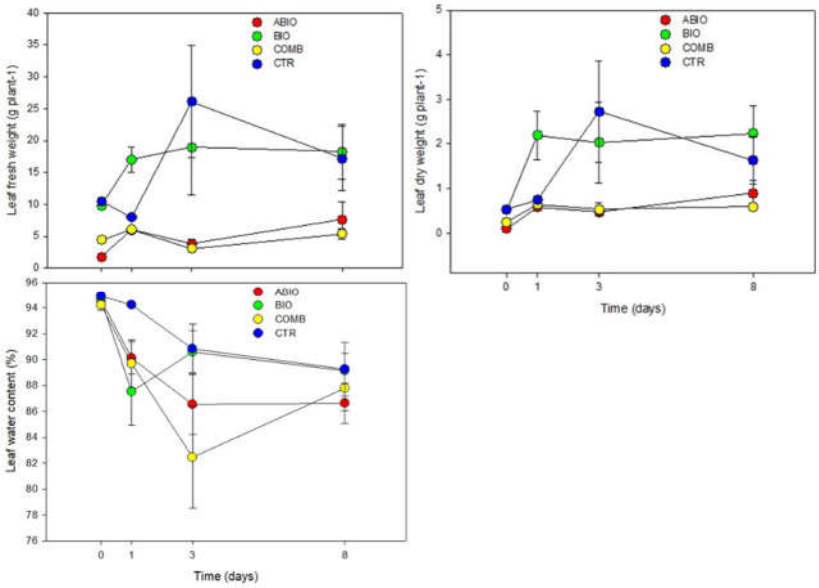

Figure 1. – Morphological traits. Leaf fresh (g), dry (g) and water content (%) of tomato plants treated with different stress (ABIO, BIO, COMB) or not stress(CTR) for different time of exposure (0, 1, 3 and 8 days). The data and error bars indicated the mean and the error standard, respectively (N=4).

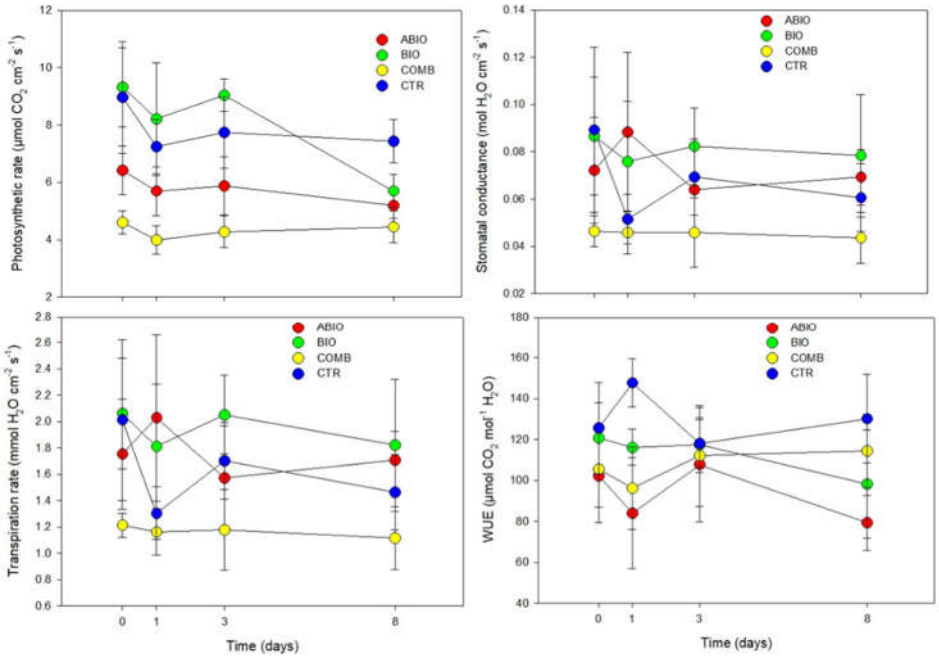

Figure 2. – Gas exchange parameters. Photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), Stomatal conductance (mol H_2O m^{-2} s^{-1}), Transpiration rate (mmol H_2O m^{-2} s^{-1}) and WUEi of tomato plants treated with different stress (ABIO, BIO, COMB) or not stressed (CTR) for different time of exposure (0, 1, 3 and 8 days). The data and error bars indicated the mean and the error standard, respectively (N=4).

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In particular, the leaf fresh and dry weight, leaf water content, photosynthetic rate and WUE were significantly reduced in ABIO plants respect to the control while no significant differences were observed in presence of herbivory except than leaf water content and WUE, only (Figures 1 and 2; Tables 1 and 2).

Table 1. - Results of two-way ANOVA [Treatment (Tr), Time (Ti), Block (Bl), TrxTi interaction (TrxTi)]. Statistics: F- and p-values. Within each root traits and time of exposure, the different letters indicated statistical differences among the means of the treatments ($p<0.05$, test of Tukey). .

Parameters	Statistics		Time (Ti)			
		Treatments (Tr)	t0	t1	t3	t8
Leaf fresh weight	Tr 10.92***	ABIO	a	a	b	b
	Ti 1.40^{NS}	BIO	a	a	a	a
	Bl 6.08*	COMB	a	a	b	b
	TrxTi 1.06^{NS}	CTR	a	a	a	a
Leaf dry weight	Tr 8.87***	ABIO	a	a	b	b
	Ti 2.91*	BIO	a	a	a	a
	BI 17.17***	COMB	a	a	b	b
	TrxTi 1.26^{NS}	CTR	a	a	a	ab
Leaf water content	Tr 3.16*	ABIO	a	ab	ab	a
	Ti 12.01***	BIO	a	b	a	a
	BI 52.15***	COMB	a	b	b	a
	TrxTi 1.50^{NS}	CTR	a	a	a	a

Table 2. - Results of two-way ANOVA [Treatment (Tr), Time (Ti), Block (Bl) TrxTi interaction (TrxTi)]. Statistics: F- and p-values. Within each root traits and time of exposure, the different letters indicated statistical differences among the mean of the treatments ($p<0.05$, test of Tukey). .

Parameters	Statistics		Time (Ti)			
		Treatments (Tr)	t0	t1	t3	t8
Photosynthetic rate	Tr 17.60***	ABIO	a	b	bc	ab
	Ti 2.73^{NS}	BIO	a	a	a	ab
	BI 20.74***	COMB	a	b	c	b
	TrxTi 0.76^{NS}	CTR	a	ab	ab	a
Stomatal conductance	Tr 5.38**	ABIO	a	a	ab	a
	Ti 0.60^{NS}	BIO	a	a	a	a
	BI 61.82***	COMB	a	b	b	a
	TrxTi 0.57^{NS}	CTR	a	ab	ab	a
Transpiration rate	Tr 6.94**	ABIO	a	a	ab	ab
	Ti 0.79^{NS}	BIO	a	a	a	a
	BI 55.73***	COMB	a	a	b	b
	TrxTi 1.13^{NS}	CTR	a	a	ab	ab
WUE	Tr 6.23**	ABIO	a	b	a	b
	Ti 0.47^{NS}	BIO	a	b	a	b
	BI 136.54***	COMB	a	b	a	ab
	TrxTi 1.52^{NS}	CTR	a	a	a	a

It is known that the drought stress alone [43] and together with N deficiency [44] reduced the photosynthesis rate, the stomatal conductance and leaf water content with negatively consequence to the leaf growth of tomato plants through very clear molecular mechanisms [45]. As observed, the BIO treatment did not produce modification of the morpho-physiological traits in comparison to the control (Figures 1 and 2; Tables 1 and 2) and this no response to the herbivory falls in the highly variable effects observed in different plant-insect combination. For example, the leaf dry to fresh mass ratio was not changed by *Monsoma pulveratum* feeding on *Alnus glutinosa* [11] but a weakly negative effects in the soybean-natural herbivory interactions was observed [46]. Further, the high variability in the plant response to the herbivory was observed for the photosynthesis that

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was sharply reduced [47], increased [48] or not modified [49]. Probably, in this study, the Tuta absoluta could have caused 'indirect effects' in leaf tomato such as increase of the photosynthesis and water losses by transpiration rate with reduced WUE and leaf water content as also observed in soybean-japanese beetles and -corn earworm caterpillars interactions [49]. However, in a specific study of the Tuta-tomato interactions, the reduction leaflet growth was pointed out [50]

Although the plant responses to the abiotic and biotic as individual stress are well understood, no information on their impact in the tomato plants as combined stress are detected. The combination of the abiotic stress (N deficiency and drought) with herbivory by Tuta determined the highest reduction of the tomato morpho-physiological traits respect to the control plants (Figures 1 and 2; Tables 1 and 2). This overstate effect of the combined stress could be due to the interactive responses determined by cross-talk hormonal signal and regulation of defence-related genes. Indeed, in the interaction between Solanum dulcamara and the herbivory by specialist *Leptinotarsa decemlineata*, the antagonism of the specific herbivory-induced salycilic acid on the jasmonic acid (JA) prevailed on the synergism of the specific drought-induced ABA with consequent reduction of the defence responses observed at transcriptional levels (increase in the cell wall components and secondary metabolism) [51]. Moreover, the tomato plants subjected to both drought and herbivory by *Spodoptera exigua* stresses pointed out an adaptive response with an increase of the genes related to the photosynthetic machinery and chlorophyll biosynthesis and, consequently, reduction of the secondary metabolite production [51].

The temporal evolution of the plant responses to the environmental stresses results fundamental for the success of the plant adaptation although has been an aspect less studied. In the present work, differently to the single stress, the COMB treatment reduced the leaf fresh and dry weight at 3 days from the stress treatments, while the leaf water content and the physiological traits such as the photosynthetic rate, stomatal conductance and WUE respond faster by a reduction already at 1 day of treatment (Figures 1 and 2; Tables 1 and 2). Probably, the combined stress in tomato plants rapidly activated the stomatal closure to reduce the water losses caused by drought component with consequently reduction of the photosynthetic process which diminished the defence-related metabolites and all this subsequently translates into a lower leaf growth. However, this morpho-physiological pattern could be the final result of the signaling and molecular network which is instead activated in a very rapid responses (within second and minute) as observed in different abiotic- and biotic-stressed plants [21].

To observe also that abiotic stress and herbivory by Tuta negatively affected more the physiological traits than morphological ones (Figures 1 and 2; Tables 1 and 2). The plant physiological plasticity is more related to an enhanced ability to exploit the transient environmental resources, such as water and nutrient patches, or to produce the defence responses (secondary metabolites) to the herbivory attack at low cost by short-term adjustments [52, 53, 54]. Conversely, the plant morphological plasticity is more expensive and functional for the plant adaptation at the long-term [52, 54].

The VOC emission is an important plant defence process in response to the herbivory attack [55] and the abiotic stress also [56]. HS/SPME GC-MS analysis revealed forty-five volatile compounds emitted by the tomato leaves exposed to the single (abiotic and biotic) and combined stress (Table S1). In particular, the volatile profile was mostly characterized by mono- (24% of the total) and sesquiterpenes (44%) but hydrocarbons (11%), ester (9%), alcohol (5%), ether (5%) and aldehyde (2%) were also present (Table S1). Volatile terpenoid metabolites have been recognized as having a range of specific roles in plant/environment and plant/plant interactions [57] and, in particular, in the direct and indirect defence of tomato against the herbivory [58]. Table S2 reported the volatiles from each treatment and time of exposure on the basis of % area of each peak over the total area of the chromatogram. In order to test the influence of the treatments and time of exposure on the volatile profiling, we run a multivariate approach that included, first, the Permanova and, then, the PCA and sPLSDA that permitted to visualize the difference among the groups and to select informative and relevant volatiles. Permanova analysis indicated that

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the treatments, time of exposure and their interaction determined a significant difference in the volatilome of tomato plants (Table S3). Pairwise comparison among the treatments within each time of exposure revealed that the COMB treatment pointed out a volatile profile different to the control at 3 and 8 days of exposure, while the BIO at 8 days of exposure only (padjusted<0.05; Table S4). Since these two times of exposure pointed out differences among the treatments, we only used the volatiles dataset from the four treatments at 3 and 8 days of exposure for running the PCA. Figure S4 showed the results of PCA where it is apparent that this multivariate analysis was not able to separate treatment groups owing to the high variability among samples. Therefore, data were further analysed using sPLS-DA at each time of exposure (3 and 8 days). At 3 days of exposure, the performance step of the sPLSA-DA for the selection of the number of components suggested that 3 components were enough to sharply reduce the balanced error rate around 0.23 (Figure 3A). Further, the final model obtained by tuning process pointed out that the Component 1, 2 and 3 were constituted by 25, 19 and 31 volatiles, respectively (Figure 3B), but with a scarce discrimination among the treatments as highlighted by the sample plots on the first three components (Figures 3C and 3D).

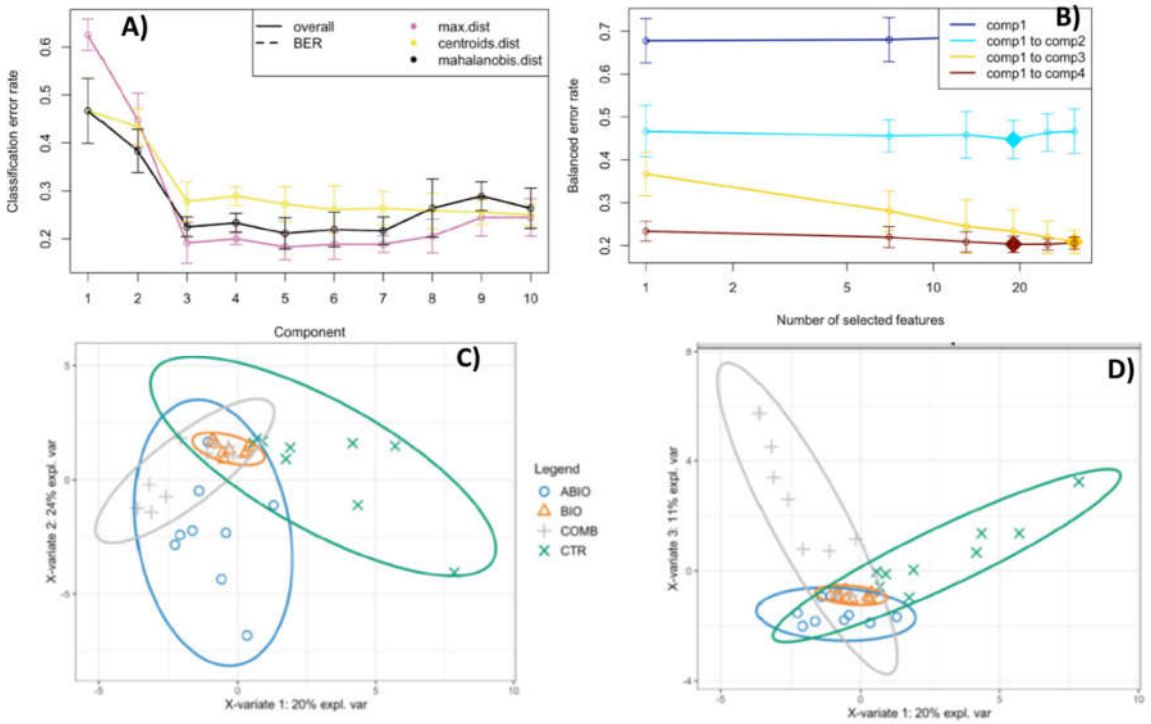

Figure 3. – sPLS-DA of volatile profiles obtained in tomato plants exposed to different stress (ABIO, BIO, COMB) or not stress (CTR) for 3 days of exposure. Choosing the number of components in sPLS-DA by performance test (A). Mean classification by overall and balanced error rate (5 cross-validation averaged 50 times) for each sPLS-DA component. Choosing the number of volatiles for each sPLS-DA components by tuning test (B). Estimated classification balanced error rates for volatile dataset (5 cross-validation averaged 50 times) with respect to the number of selected volatiles for the sparse exploratory approaches. sPLS-DA sample plot for the different components using 95% confidence ellipses (C and D). Component 1 vs. Component 2 (C), Component 1 vs Component 3 (D).

At 8 days of exposure, three components were selected with a balanced error rate around 0.28 and with a molecular signature composed of 16, 16 and 31 VOCs selected on the first three components, respectively (Figures 4A and 4B). The sample plots on the three components permitted to visualize a discrimination among the treatments with 60% of total explained variability split up by 32%, 15% and 13% for the first, second and third components, respectively (Figures 4C and 4D).

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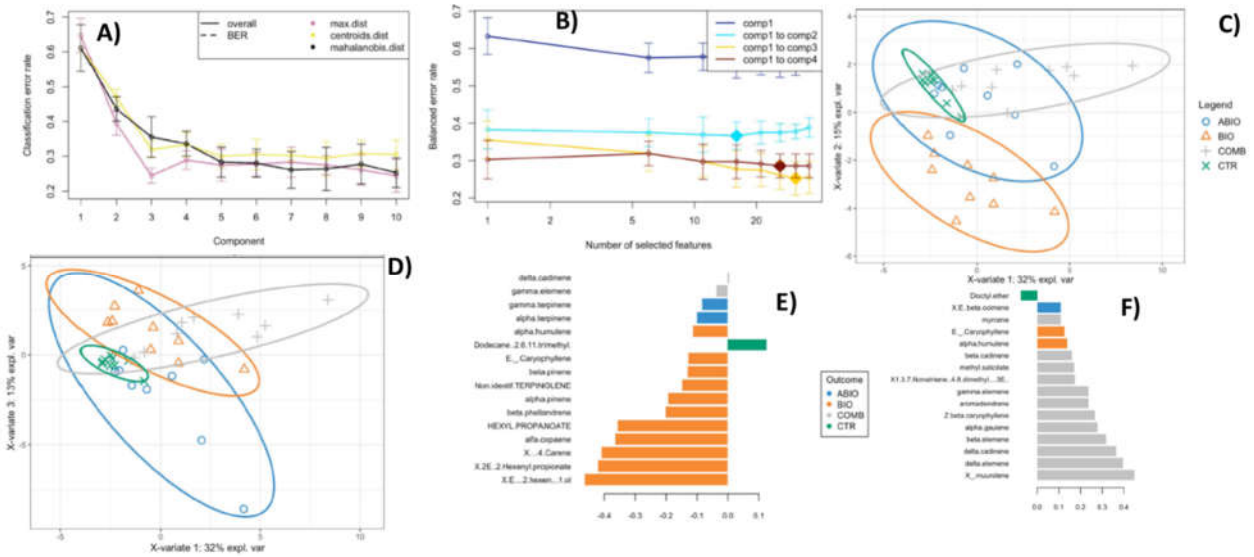

Figure 4. – sPLS-DA of volatile profiles obtained in tomato plants exposed to different stress (ABIO, BIO, COMB) or not stress (CTR) for 8 days of exposure. Choosing the number of components in sPLS-DA by performance test (A). Mean classification by overall and balanced error rate (5 cross-validation averaged 50 times) for each sPLS-DA component. Choosing the number of volatiles for each sPLS-DA components by tuning test (B). Estimated classification balanced error rates for volatile dataset (5 cross-validation averaged 50 times) with respect to the number of selected volatiles for the sparse exploratory approaches. sPLS-DA sample plot for the different components using 95% confidence ellipses (C and D). Component 1 vs. Component 2 (C), Component 1 vs Component 3 (D). Contribution plots by loading weights of the volatiles selected for the Component 2 (E) and Component 1 (F) of the sPLS-DA. The colour indicated the treatments for which the selected volatile has a maximal mean loading weight value.

In particular, plotting the first two components, the BIO was sharply separated from the control and COMB by the second component (Figure 4C). Conversely, the first and third components point out scarce discrimination among the treatments (Figures 4C and 4D). The 16 VOCs selected on the second component all had negative weight in the linear combination, and the followings were highly expressed in BIO: (E)-2 hexen-1 ol, (2E)-2-Hexenyl propionate, (+)-4-carene, α-copaene, Hexyl propionate, β-phellandrene, α-pinene, terpinolene, β-pinene, E β-caryophyllene (Figure 4E). However, the dodecane, 2,6,11-trimethyl was expressed in CTR while the α-humulene and α-terpinene and the γ-elemene and δ-cadinene falled in the ABIO and COMB, respectively (Figure 4E). The (E)-2 hexen-1 ol [59], (2E)-2-Hexenyl propionate [60] and Hexyl propionate [61] are green leaf volatiles which are released in response to different stress conditions to aid in plant defence against herbivory and bacterial and fungal pathogens [62]. The (+)-4-carene, α-copaene, β-phellandrene, α-pinene, terpinolene, β-pinene and E β-caryophyllene are terpenes, organic class mostly involved in the plant defence. For example, the (+)-4-carene, α-copaene and β-phellandrene are the most abundant VOCs emitted by Solanum species in presence of Bactericera cockerelli herbivory [63] and terpinolene and E β-caryophyllene were mainly produced by tomato leaves infested with Trialeurodes vaporario-rum [64]. Further, the α-humulene was responsible of tomato repellence against Bemisia tabaci [65]. The dodecane, 2,6,11-trimethyl that was highly expressed in CTR in this study (Figure 4E), is confirmed as VOC emitted by healthy plants such as olive [66]. Interesting is that the α-terpinene and δ-terpinene were mainly discriminant of ABIO treatments (Figure 4E) confirming the results obtained in tomato plants (cv. Gan Liang Mao Fen 802 F1) fertilized with lower levels of N [65] and in drought-stressed Thymus vulgaris plants [67]. Finally, the present study revealed the VOCs emitted in multi-stressed tomato plants. In particular, the γ-muurolene, δ-elemene, δ-cadinene, β-elemene, α-gauiene, z-β-caryophyllene, aromadendrene, γ-elemene, 1,3,7 Nonatriene 4,8 dimethyl (3E)-, methyl salicylate, β-cadinene and myrcene were the compounds constituting the VOC blend emitted by tomato

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plants when exposed to combined N and drought stress with infestation by *Tuta absoluta* (Figures 4E and 4F). To note that the γ-elemene and δ-cadinene were present in both components while the other volatiles were only observed in the Component 1 that is the lesser discriminant (Figures 4E and 4F). In *Gossypium arboretum*, the δ-cadinene is a precursor of the cyclic secondary sesquiterpene aldehydes, including gossypol, that are insecticide [68]. Conversely to the δ-cadinene, the γ-elemene was poor modified in the tomato leaves by stresses such as, for example, the pest [64]. Besides the δ-cadinene and γ-elemene, the volatiles pointed out in the component 1 were also interesting in plant responses to the abiotic and biotic stress. The methyl salicylate was observed to increase in double drought-stressed and aphid-infested tomato plants [10] but also in the drought-herbivory combination together with 1,3,7-Nonatriene, 4,8-dimethyl-, (3E)-, the two stress-specific VOCs [11]. The other volatiles were present in the VOC profiles of different plant species stressed with the combination of two or more stresses [11, 69, 70].

3.2. Are additive, synergistic or antagonistic effects in the combined stress?

Analyzing the tomato morpho-physiological and metabolic responses to the combined stress is interesting to understand the additive (equal to the sum of the single-stress effects), synergistic (higher than expected) or antagonistic (lower than expected) effects of the single stress. In relation to the results of these effects, the signaling pathways and molecular mechanisms underlying the plant strategy in presence of simultaneous stress could be hypothized. In this respect, the additive, synergistic and antagonistic effects of abiotic (drought and N deficiency) and biotic stress in tomato plants were evaluated by the Bansal et al. method [12]. In general, the physiological (photosynthesis, stomatal conductance and transpiration) and the metabolic (VOC) traits pointed out more synergistic effects than morphological ones especially at early time of exposure, i.e. at 1 and 3 days (Figure 5).

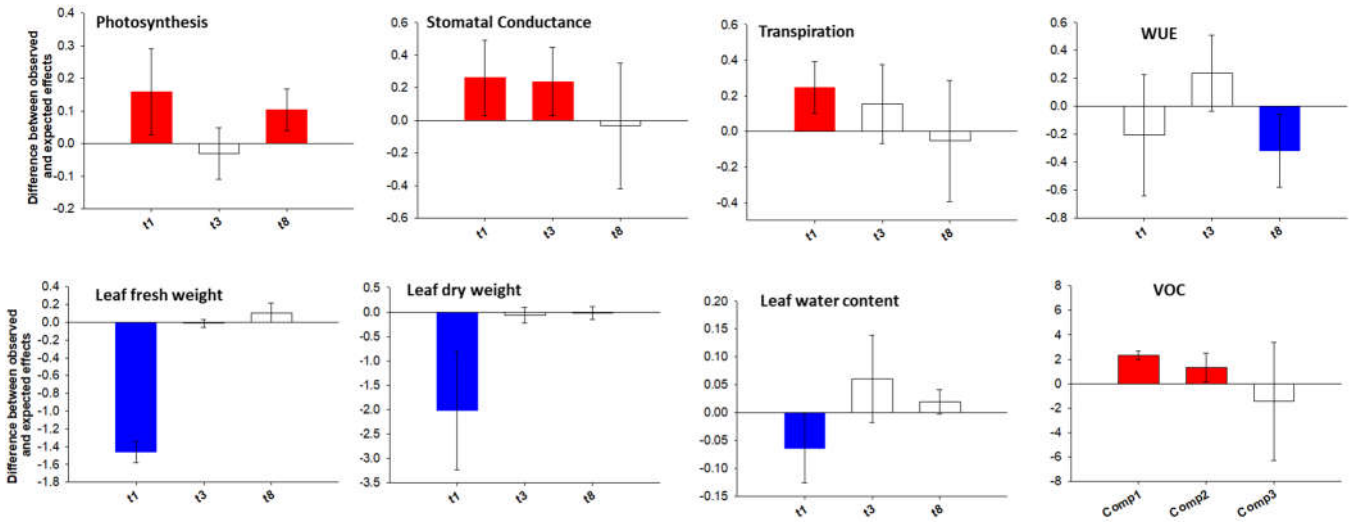

Figure 5. - The combined impacts from abiotic (drought and N deficiency) and biotic stress (Infestation of *Tuta absoluta*) on morpho-physiological traits of tomato plants at 1, 3 and 8 days of treatment and VOC emission at 8 days of treatment. The combined impact of single stressors was estimated as synergist (red colour), additive (white colour) or antagonistic (blue colour) (greater than, equal to or less than expected effects, respectively, based on single stressor effect sizes). The vertical and error bars represent, respectively, the mean and the 95% confidence interval of the overall effect size difference between the observed and expected additive effects from combined abiotic and biotic stress on morpho-physiological and metabolic traits of tomato plants. The zero line represents the expected additive effects from combined stressors. When the means (and their 95% confidence limits) were higher than or less than the zero line, they were considered synergistic or antagonistic, respectively.

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In particular, a synergic effect (Figure 5) was observed for the reduction of the physiological traits (photosynthesis, stomatal conductance and transpiration) (Figure 2) and for the increase of the VOC emission (Table S2). The closure of the stomata is the first plant response to the water scarcity mediated by ABA that orchestrate a network of stress-responsive metabolites and gene expression [71]. The ABA signaling pathways interact also with that of the JA one, the phytohormone that activate the signaling cascades for regulating downstream transcriptional responses to the herbivory [72, 73] and, furthermore, it was observed that MeJA signaling is overlapped with ABA signaling in guard cells [74]. This interaction between the ABA and JA signaling pathway could have caused the synergic effect of abiotic and biotic stress for the reduction of the physiological traits observed in combined stress plants (Figure 5). However, besides the hormonal interactions, unique and novel molecular mechanisms were also found during the stress combination in several studies. For example, the transcriptome analysis revealed that a unique set of transcripts was altered in response to the combination of drought and nematode infection [75], drought, heat stress and virus [76], infection by *Botrytis cinerea*, herbivory by chewing larvae and drought stress [77]. The synergic effect on the reduction of the photosynthesis and transpiration could be determined by the stomatal closure in addition to the herbivory-induced resource reallocation to chemical defence [78] that determined more intense dark respiration [79].

The synergic effect (Figure 5) was also involved in the increase of the metabolic traits such as the VOC emission in COMB-treated tomato plants (Table S2). This synergic effect could be due to diverse reasons. First, the improvement of the formation reactive oxygen species (ROS) by drought stress [80] and nutrient deficiency [81] that could sensitizes the VOC response. Indeed, it is know that the VOCs are emitted by early signaling events involved the ROS during the herbivory [82]. Second, the abiotic stress and herbivory by Tuta could have improved the biosynthesis of VOCs by both hormone cross-talking and higher resource reallocation to chemical defence. For example, the ABA and JA, the phytohormones involved in the plant response to the drought and herbivory, pointed out cross-talking interactions [72, 73, 74] and the reallocation of plant resources to defense by modification of the gene expression profiles after herbivory was also observed [83]. Third, the improved VOC biosynthesis in presence of both stresses could increase their accumulation inside the leaf with consequent formation of a deeply partial pressure gradient between the atmosphere and substomatal cavities along which the VOCs could be highly emitted.

Differently to the physiologic and metabolic traits, the morphological ones pointed out an early antagonistic effect (at 1 days of treatment) that successively transform in additive effect in combined stressed plants (Figure 5). This result could be due to the early prioritization of the plant responses to the herbivory in presence of both stress. Indeed, the tomato plants aimed to reduce the leaf water content more in presence of Tuta rather than in abiotic stress compared to the control through a sharp increase of the leaf dry weight (Figure 1). Why? The water and the dry weight are strictly and negatively linked to the plant palatability towards the pest [84]; hence, the tomato plant in presence of the combined stress redirect the resource allocation towards the formation of carbon-based secondary compounds such as lignin, fibre and silica contents which contribute to leaf toughness and reduce palatability [85].

3.3. Do the tomato responses to the single and combined stress occurred at between- or within-plant levels?

Recent studies pointed out the importance of the within-individual variation of the plant responses to the abiotic and biotic stress rather than that between-individual for the ecology at individual, population, and community levels [86, 87]. For example, a higher within-plant variation of the morpho-physiological responses permitted an improvement of the exploitation of the heterogeneously-distributed resources such as light, CO_2, nutri-

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ent [88, 24], an optimization of the cost-expensive defenses against herbivory and pathogens [89], and an alteration of plant-antagonist interactions [23]. In this respect, we assessed, first, if the among-treatments variance of the morpho-physiological traits and VOC profiles of the tomato plants is more important than within-treatment ones and, then, we evaluated the between- and within-plant variance for each treatment. To calculate the among- and within-treatments variance, we used the morpho-physiological traits and VOC observed at 8 days that is the time with wider and higher modifications of these traits (Tables 1 and 2; Figures 1, 2 and 4) and their measurements have been carried out in three mature leaves located at three different positions along the shoot axes. The contribution of the among- and within-treatment level to the total variance in mean of the morpho-physiological traits and VOC responses to the treatments considered was estimated by Linear Mixed Models and statistically tested by restricted maximum likelihood (REML) (Figure 6).

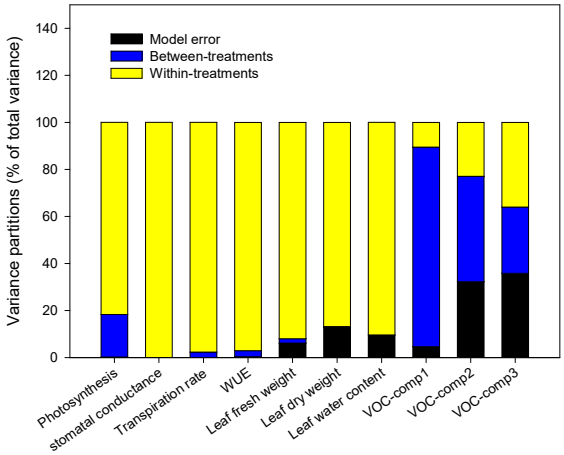

Figure 6. - Dissection of total variance components of the morpho-physiological traits and VOC responses at 8 days of treatments (control, abiotic stress, biotic stress, combined stress). Considering all treatments pooled, the contributions of treatment (blue color) and within-treatment (yellow color) level to the total variance in mean of the morpho-physiological traits and VOC responses in the four treatments considered were estimated by Linear Mixed Models .

The results indicated that most variance occurred at within-treatments, especially for the morpho-physiological traits but not for the VOCs (Figure 6). Hence, differently to the morpho-physiological traits, the emission of the VOCs was more dependent on the stress treatments rather than the individual plants. For this reason, only the morpho-physiological traits were used in the analysis of within-plant variation that was conducted applying a hierarchical partition to divide total variance into two levels of variation: among plants and among the leaves within the same plant. This analysis was performed for each single treatment in order to verify its effects to within-plant phenotypic plasticity. The variance partitions varied substantially among the different treatments and traits considered (Figure 7).

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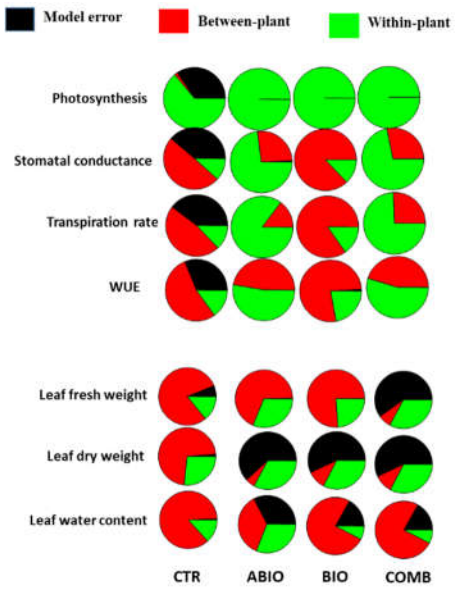

Figure 7. – Nested within-treatment variance partitions (% of the total) in the morpho-physiological responses of tomato plants to different abiotic and biotic stress. The between-plant variance comprise plant within treatment (red color) and the within-plant variance involved the leaves within plant within treatment (green color). The black color indicated the model error.

In general, the physiological traits pointed out a higher within-plant variance (average 54%) while the morphological ones showed more between-plant variance (average 50%) (Figure 7). Why do the individual tomato plants modified more the leaf physiological than morphological traits within their shoot? Probably, the physiological traits being lesser expensive, could be faster modified in response to the abiotic and biotic stress; for example, rapid local and systemic responses through specific signaling pathways have been observed in presence of the light stress [90], the heat tolerance [16] and the herbivory [91]. Among the treatments, the stresses induced a higher within-plant variance than CTR: ABIO (average 58%), COMB (average 53%), BIO (average 31%) and CTR (average 22%) (Figure 7). The within-plant variation in stressful condition could permitted the improvement to exploit the stress-induced transient environmental and soil resources [92, 93] and the optimization of the defences against herbivory [94].

Considering that each treatment pointed out an important within-plant variation for the morpho-physiological traits, we asked if a well-defined spatial pattern of these responses among the leaves of the tomato shoot could be revealed. In this respect, by one-way ANOVA, we compared the effects of three mature leaves located at three different positions [basal (B), intermediate (I) and apical leaf (A) placed at first, second and third node, respectively] along the shoot axes on the morpho-physiological traits for each treatments. Further, the B, I and A leaf can be also considered as local (L), no-orthostichous (nO) and orthostichous leaf (O), respectively, because the apical leaf, but not the intermediate, is linked to the basal one by vasculature connection (Figure S3). Hence, this leaf selection permitted us to evaluate which of the vascular (L vs O vs nO) or architectural patterns (B vs I vs A) caused the within-plant phenotypic variability. For example, the Figure S5 depicted the vascular or architectural pattern or no pattern of the tomato responses to each treatments. The Figures 8-14 showed the results of physiological (photosynthesis, stomatal conductance, transpiration rate and WUEi) and morphological traits (leaf fresh and dry weight, leaf water content) for each treatment.

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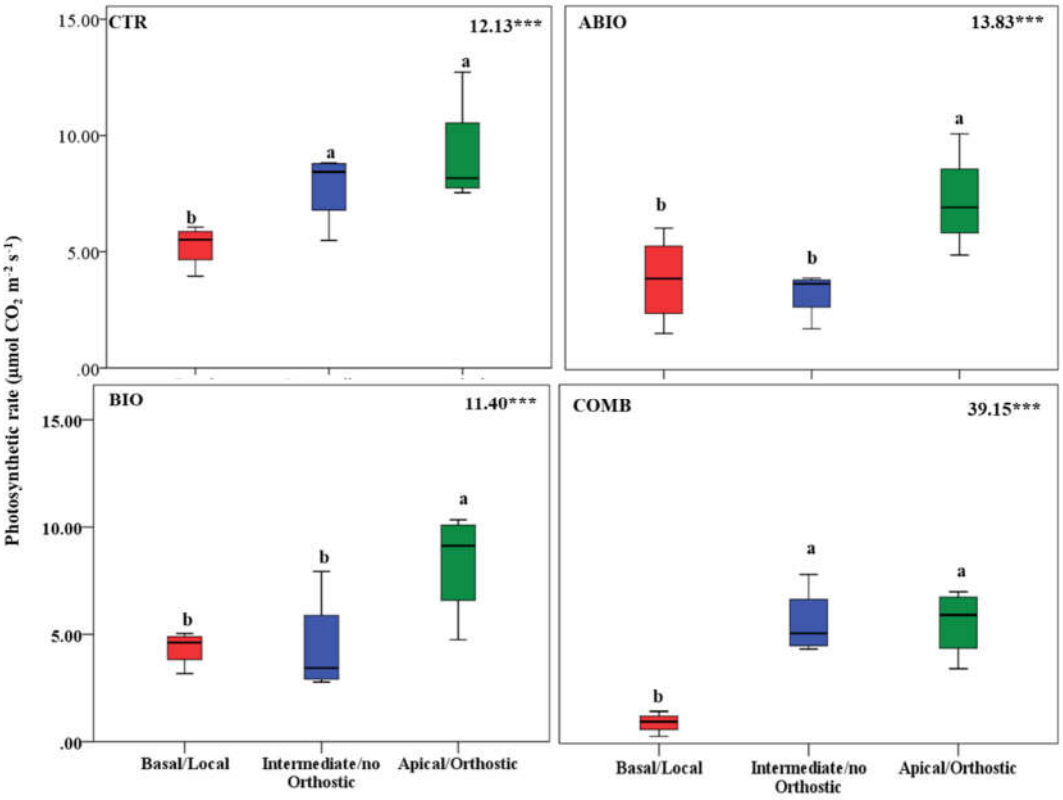

Figure 8. – Photosynthetic rate of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (*** $p<0.001$) derived from one-way ANOVA.

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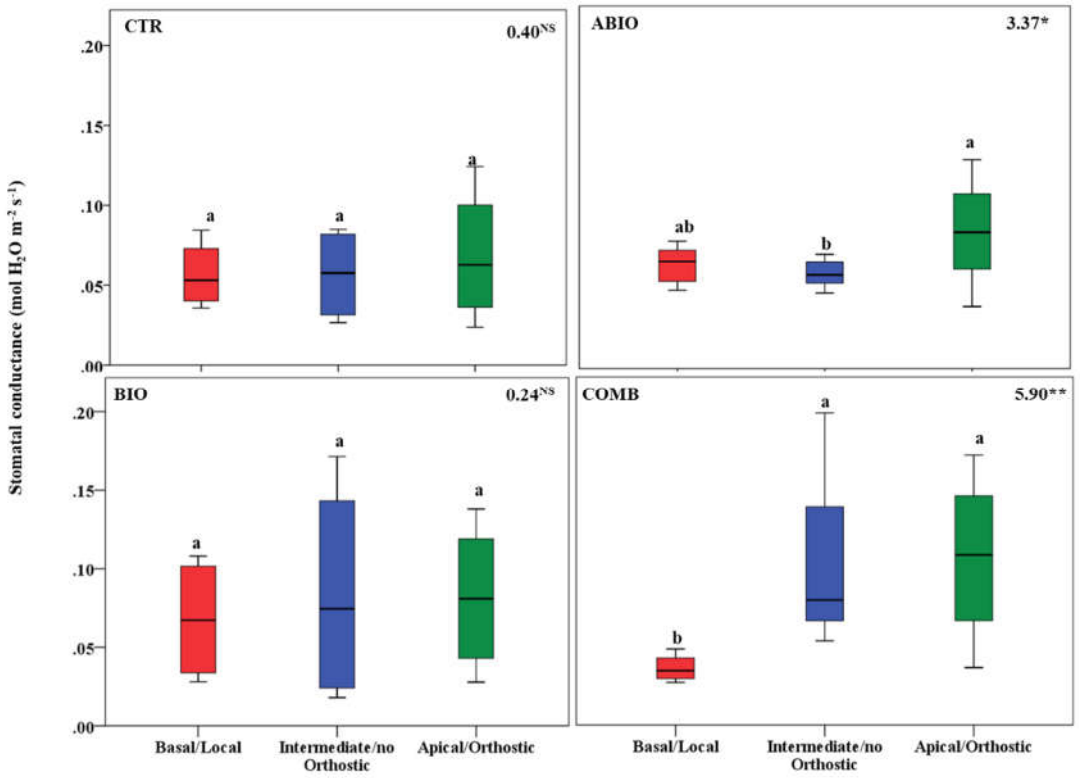

Figure 9. – Stomatal conductance of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (* $0.05<p<0.01$; $0.01<p<0.001$; ns not significant) derived from one-way ANOVA.

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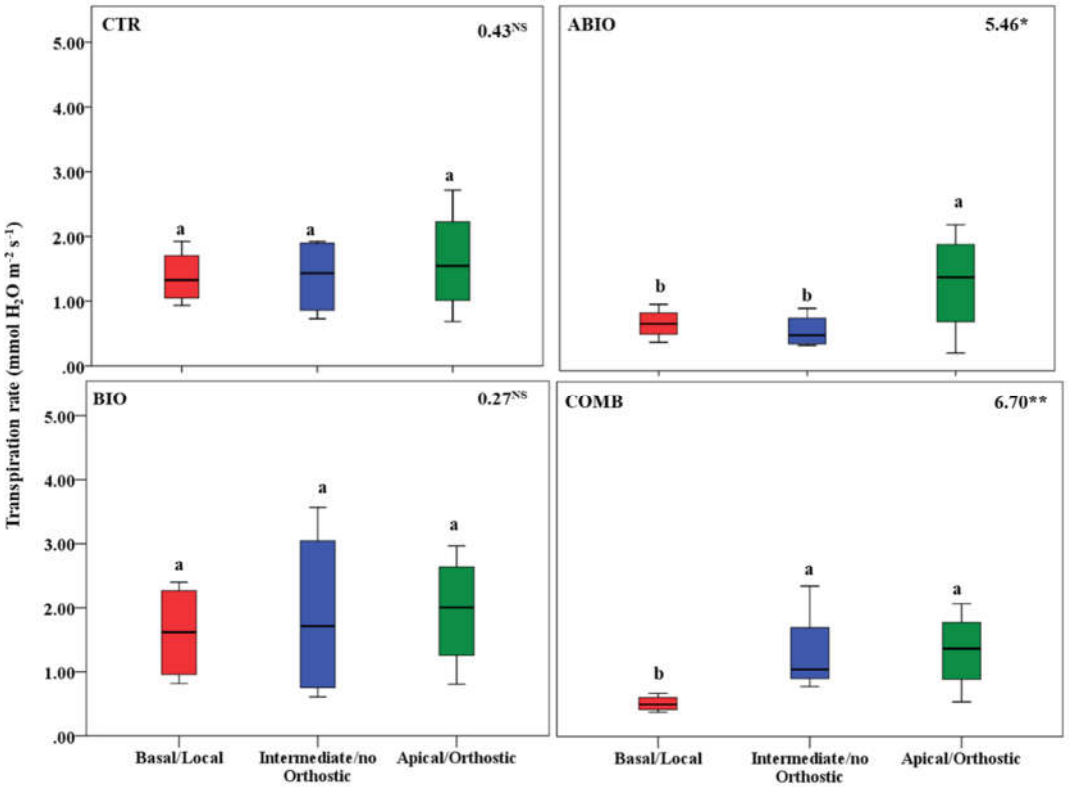

Figure 10. – Transpiration rate of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (* $0.05<p<0.01$; $0.01<p<0.001$; ns not significant) derived from one-way ANOVA.

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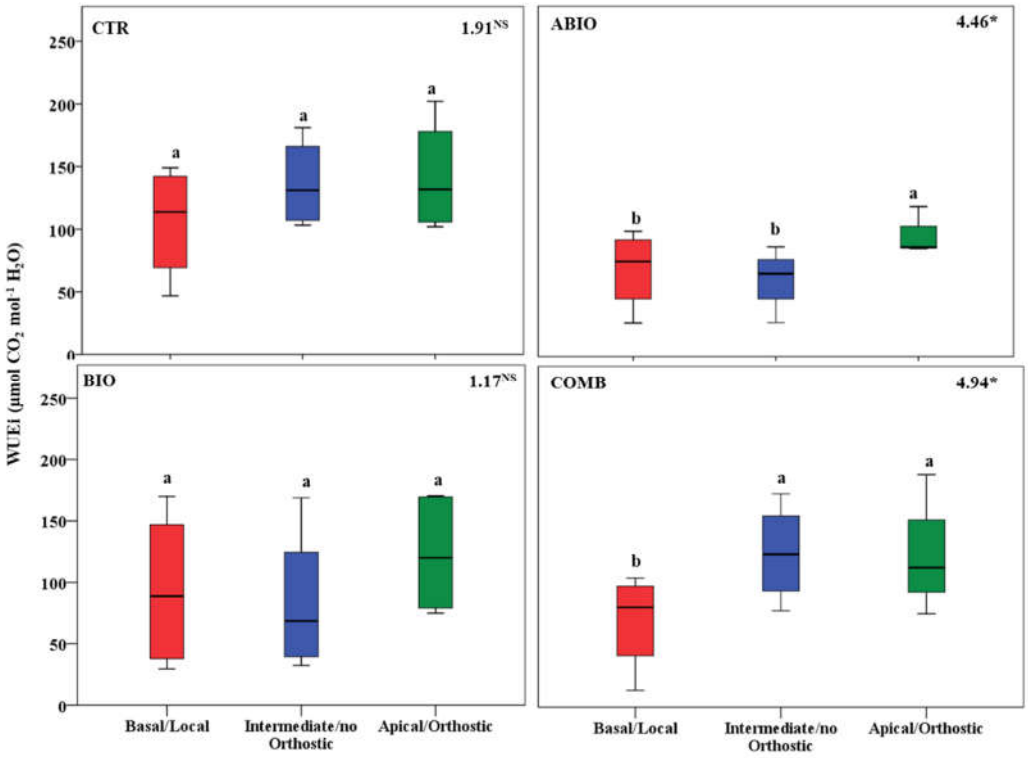

Figure 11. – WUEi of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (* $0.05<p<0.01$; ns not significant) derived from one-way ANOVA.

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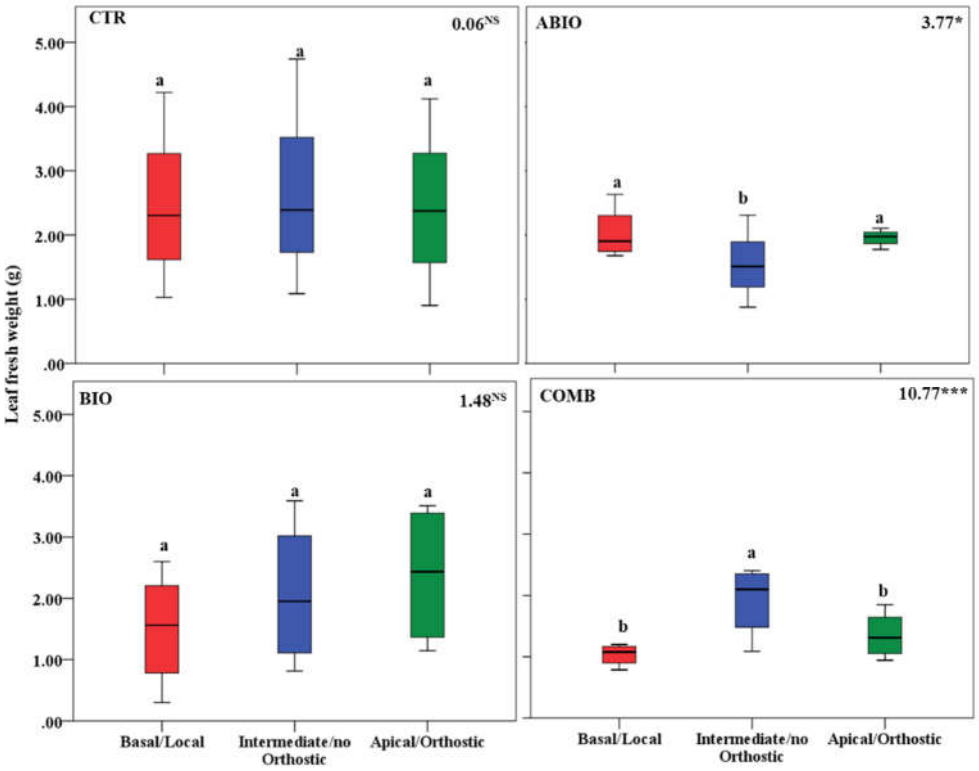

Figure 12. – Leaf fresh weight of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (* $0.05<p<0.01$; *** $p<0.001$; ns not significant) derived from one-way ANOVA.

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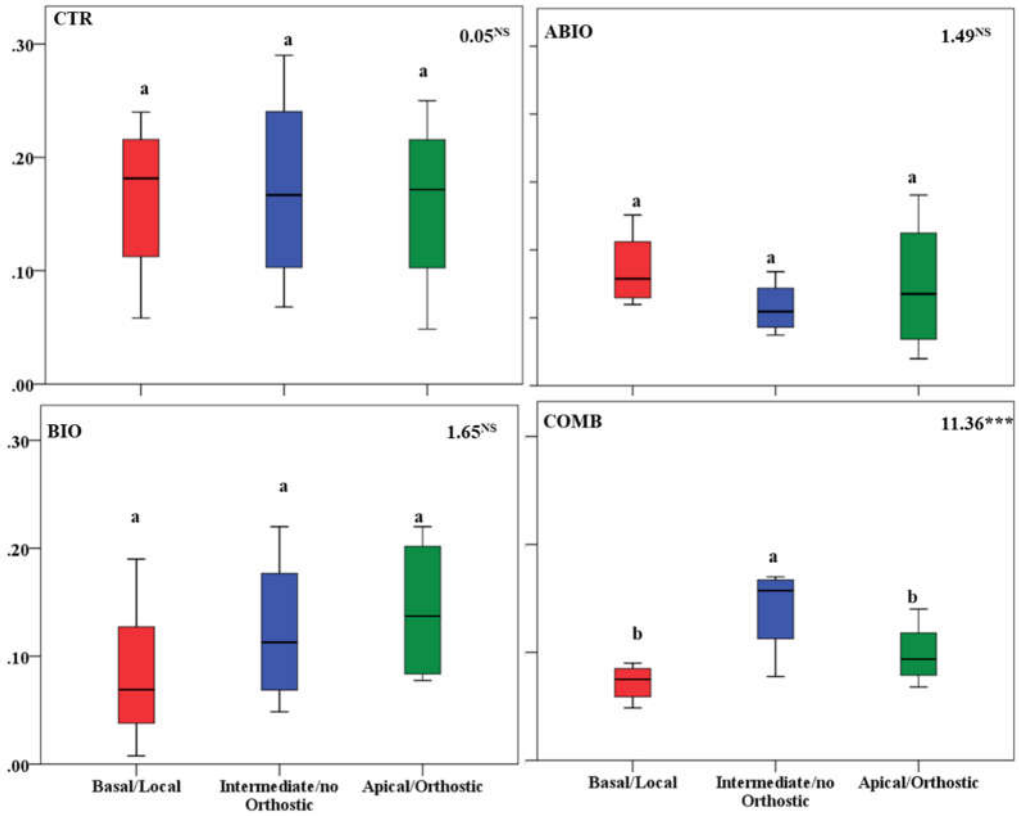

Figure 13. – Leaf dry weight of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (* $0.05<p<0.01$; ***$p<0.001$; ns not significant) derived from one-way ANOVA.

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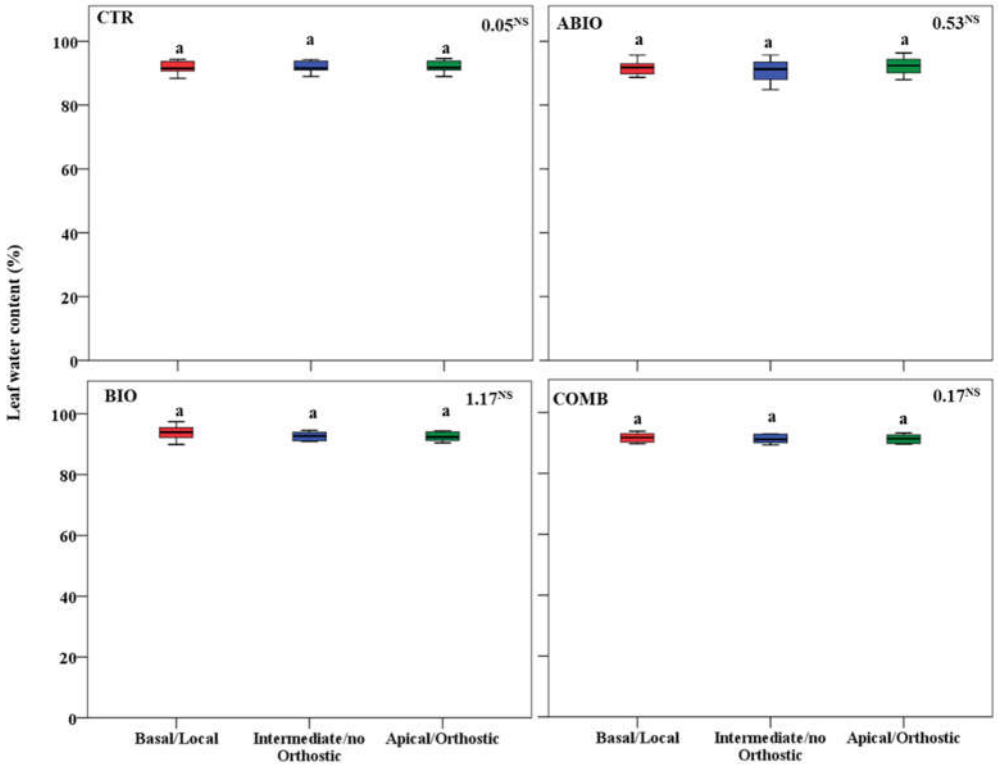

Figure 14. – Leaf water content of three different leaves of tomato plants exposed for 8 days at diverse stresses [abiotic (ABIO); biotic (BIO); combined (COMB)]. No stresses (CTR). The box plot indicated the minimum, first quartile, median, third quartile, and maximum value. Different letters indicated significant difference among the mean groups (N=8; $p<0.05$ test of Tukey). The values within the figure indicated the F statistic with the p values (ns not significant) derived from one-way ANOVA.

An overall result indicated that differently to the CTR and BIO, the ABIO and the COMB treatments significantly modified all the morpho-physiological traits among the three tomato leaves, except the leaf water content and photosynthetic rate (Figures 8-14). Further, comparing with the spatial patterns of plant response (Figure S5), the physiological responses (photosynthetic and transpiration rate, stomatal conductance and WUE) to the ABIO and COMB treatments resembled an architectural pattern while the morphological ones pointed out a vascular pattern (Figures 8-14). The abiotic stress are known to strongly influence the plant photosynthetic traits in relation to the leaf position/age, that is architecture pattern, in order to preserve the highly valuable tissues, such as the young leaf [95]. The vascular pattern of the leaf fresh and dry weight in response to the COMB treatment could be due to the ABA-JA cross-talking signaling pathways [72, 73, 74] which could be observed between the two vascular-connected leaves (local and orthostic). We could hypothized that the Tuta larvae in the local leaf of tomato plants could triggered by vascular connection these hormonal signaling pathways which redirect the photosynthetic resource towards the defence compounds rather growth ones. This kind of hormone cross-talk signaling interaction among the different vascular-connected leaves within the plant was already observed. For example, the abiotic stresses antagonized the immune responses by ABA-SA hormonal interaction in older leaves of Arabidopsis but this effect was suppressed in the younger leaf through a signaling component of the SA pathway [96].

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5. Conclusions

The present study has been addressed to answer questions related to the tomato responses in presence of combined abiotic stress (drought and N deficiency) and herbivore infestation raised from previsions of burst herbivory and increased of drought stress and nutrient deficiency caused by climate change. First result was that the combination of drought, N deficiency and Tuta infestation caused a stronger negative impact on the tomato morpho-physiological traits and induced a specific VOC blend emission than single stress. Probably, hormone cross-talking regulating the signaling and metabolic systems of the plant responses could be evoked. Interestingly, differently to the single stress, the VOC blend emitted by tomato plants exposed to the combined stress was characterized, besides to other, by the homoterpene 4,8-Dimethyl-1,3,7-nonatriene, a rare and fundamental plant alarm volatiles, and by the methyl salicylate, a well-known herbivore-induced plant volatile which attract natural enemies and affect herbivore behavior.

Second result pointed out was the relatively rapid responses of the tomato plants to the COMB treatment and the synergistic effects for the physiological and VOC responses but antagonistics for the morphological ones. In this respect, no-additive effects of the single stress in tomato response to the combined stress are highlighted. This result is important because suggests that a "new stress state" characterized by specific signaling pathways and gene expression, probably orchestrated by hormone interactions, could be evoked in tomato plants stressed by the combination of drought, N deficiency and Tuta infestation.

Finally, except for the VOC emission, the stressful conditions induced a higher within-plant variance in tomato with the abiotic and combined stress as the most influential. The increase of the variability of the morpho-physiological responses within the tomato plants is very interesting considering the higher defense against the herbivore infestation and the maximization of the exploitation efficiency of the scarce soil resources observed in plant with high within-plant variance.

Overall, these results pointed out a "phenotypic picture" of the tomato plants subjected to the single and combined stress that is interesting and worthy to investigate at signaling pathways and gene expression levels for further support our findings on the morpho-physiological mechanisms.

Supplementary Materials: Figure S1: Protocol schedule including tomato growth and treatments and plant sampling events. Figure S2 - Trap for Tuta absoluta's larva. Figure S3 - Evaluation of the degree of vascular connectivity among leaves located in different position along the shoot. Figure S4 - Principal component analysis applied to volatiles emission. Figure S5 – Tomato responses to the experimental conditions resembling the vascular pattern or architectural pattern or no pattern (blue line). Table S1 - Volatile organic compounds identified using gas chromatography/mass spectrometry (GC/MS). Table S2 - Volatile organic compounds (Area %) from the leaves of tomato plants. Table S3 - Permanova results of the VOC emission. Table S4 - PERMANOVA pairwise comparison between treatments.

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Data Availability Statement: The authors declare that the data supporting the findings of this study are available within the article and Supplemental Materials as well as from the corresponding author upon reasonable request.

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