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Tomato-Aphid Interactions in Plants Grown on Soil with Biochar

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Abstract

Plants are affected by various types of stress. The resistance or the susceptibility of plants to stress depends on the mutual characteristics of the plant and the stress. The plant can counteract the stress through the expression of specific genes, through changes in metabolism or through quantitative and qualitative variations of gene expression. Biotic stress is due to the action of viruses, bacteria and small insects and it is the cause of most of the reduction in crop yield. Biochar is a fine-grained vegetable carbon that is obtained from the pyrolysis of different types of plant biomass, and, if added to the soil, it can improve soil characteristics and at the same time it can reduce carbon emissions. Biochar also appears to have an unclear role in the activation of systemic resistance responses to pathogens. Biochar has a carbon content of about 90%; its high porosity increases the retention of water and nutrients by reducing the need for water and fertilizers and increasing agricultural yield. Aphids are one of the major sources of biotic stress for the tomato (*Solanum lycopersicum*), a crop of significant agro-food and socio-economic importance, especially in the Mediterranean area and in southern Italy. In this study, we first evaluated, through a proteomic analysis, the differential protein expression of tomato leaves infected by aphid and grown on control soils and on biochar-modified soil. The results of the proteomic analysis showed a differential expression mainly in the proteins involved in stress and defense, so we decided to deepen this aspect through a molecular analysis. A Real-time PCR of some fundamental genes involved in the Jasmonic acid pathway was made because, although it is clear that aphid infection activates the salicylic acid pathway, we have less data in literature about the resulting tissue damage involves Jasmonic Acid (JA). The regulation of jasmonic acid after phytophagous insects attack is particularly important for the plant's ability to initiate promptly to the defense responses.

Keywords

2-DE, Aphid, Biotic Stress, Biochar, Proteomics, *Solanum lycopersicum*

1. Introduction

Plant stress can be defined as an external condition that adversely affects growth, development or productivity. The plant responds to stress through the expression of specific genes, changes in metabolism or through quantitative and qualitative variations of gene expression [1] (Ghini *et al.*, 2012). Biotic stress is due to the action of exogenous biotic agents such as bacteria, viruses and small insects. During a biotic stress, the plant generally loses energy because the parasite modifies the plant metabolism to its advantage or, in other cases, the loss of energy is determined by the plant itself, which responds to the attack by implementing systems of defence with energy expense. In some cases, plant-pathogen interaction can be strongly limiting for both survival and reproduction, leading to plant death [2]. Biotic stress is the main cause of agricultural crops reduction, globally estimated above 30%. Currently, about 500.000 species of phytophagous insects have been identified and are commonly divided into two categories: phytophagous and phytomites. Phytophages have a chewing jug apparatus able to shred vegetable tissues, whereas phytomish insects have mandibles and jaws fused to form a thin flexible case containing two channels. The alimentary canal is used to ingest vegetable fluids, while the salivary one is used to release saliva inside the stylet and at the feeding site [3]. Insects of the Emittera order such as aphids belong to the second category. Though phytomites produce very limited damage to plant tissues; however, phytophagous are able to remove large amounts of lymph from the plant attached, depriving it of all the compounds necessary for growth, development and reproduction. In addition to the direct damage, aphids also cause indirect damage, such as the transmission of plant diseases due to viruses and mycoplasmas. The plants can activate inducible defence mechanisms against aphids, systemically expressed or locally confined to infested plant parts. The plant under aphids attack, can move resources such as carbon, nitrogen and sulphur to structures unavailable to insects, such as roots, or can direct defence through the production of toxic or anti-nutritive compounds [4] [5]. One of the most harmful insects for tomato crops (*Solanum lycopersicum*) is the aphid *Myzus perspicae*, which is one of the major sources of biotic stress for tomatoes, a crop of great agro-food and socio-economic importance in the Mediterranean area. According to the Koppen climate classification, in the Mediterranean climate the total rainfall in the hot term is less than 30 mm per month. This obviously involves the need to irrigate the crops abundantly, especially those of tomato that require large water availability. A possible solution to the problem of the availability of water for agriculture in dry areas could be the use of biochar, a vegetable carbon produced by pyrolysis, and therefore by combustion in the absence of oxygen, of different starting biomasses. Some studies on the effects of biochar on plants indicated that biochar addition can enhance water holding capacity of soil and improve crop productivity by retaining more water from rainfall in arid regions reducing the frequency or amount of irrigation needed [6]. However, although there are several works confirming the

effects of biochar in improving agricultural yield [7] [8], the data concerning the effects of the addition of this vegetable carbon in the soil on the ability of plants to respond to abiotic stresses are still few and contrasting [9] [10]. Tomato transcriptional changes in response to aphids and molecular mechanisms associated with the development of symptoms are currently largely unexplored, for this reason in this work we wanted to evaluate, through the analysis of protein expression, and of some defence genes activated by phytopathogenic insects, if the addition of biochar in the soil (50 $g \cdot kg^{-1}$) has some effect on the tomato plant attacked by aphids.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted in April-July 2017 in a greenhouse near Benevento, Italy. At fifth leaf stage, tomato (*Solanum lycopersicum*, "San Marzano") seedlings were transplanted into plastic pots (25 cm diameter and 30 height) containing 20 kg of soil for control (CNT) and 20 kg of soil + 1 kg of biochar for Biochar treatment (BIO). The soil used had an average value of 23.0% carbon and 0.9% of nitrogen, and the biochar (Verforfood), was produced from wood with an average temperature of 550 degrees and containing 81.1% of carbon and 1.19% of nitrogen. To study the plant's defense responses to phyto-insects, infestation tests were carried out using eight weeks old tomato plants as hosts, and the aphid *Myzus persicae* as a bug. The infestation protocol adopted was used for the production of plant material used for the evaluation of transcriptional and proteomics variations induced in the plant following the aphid attack. Control plants were grown in the same experimental conditions but in insect-proof cages [3]. Three days after the deposition of the aphid bugs on the leaves, the leaves showing necrotic spots were taken and immediately put in liquid nitrogen.

2.2. Protein Extraction

Protein extraction was performed based on the phenolic extraction method [11]. The leaves were finely pulverized in liquid nitrogen. The powders thus obtained were aliquoted (2.5 g) and treated with 7.5 ml of extraction buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, 2% β-mercaptoethanol, 1 mM PMSF) and an equal volume of phenol for 20 minutes. The samples were centrifuged for 10 min at 11,000 rpm at 4. The phenolic phase was withdrawn and the proteins precipitated with 4 volumes of 0.1 M ammonium acetate in methanol overnight at −20˚C. The proteins were centrifuged for 10 min at 10,000. The pellet obtained was subjected to 3 successive washes, two of which with the precipitation solution and the last one with cold acetone. The protein pellet was dissolved in solubilization solution (9 M urea, CHAPS 4%, Triton X-100 0.5%, DTT 20 mM and ampholite pH 3-10 at 1.2%). The protein concentration was determined spectrophotometrically according to the Bradford assay (Biorad).

2.3. Electrophoretic Analysis of Protein Extracts

IPG strips 17 cm, pH 4-7 (BioRad ReadyStrip, BioRad) were rehydrated overnight with 300 µl of IEF buffer containing 350 µg of total proteins. Proteins were focussed using a Protean IEF Cell (BioRad) at 12˚C, applying 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 53 KVh. After focusing, proteins were reduced by incubating the IPG strips with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of Bromophenol Blue, for 15 min. Electrophoresis in the second dimension was carried out using a Protean apparatus (BioRad) and 12% polyacrylamide gels in 25 mM Tris (pH 8.3), 1.92 M glycine and 1% w/v SDS, with 120 V applied for 12 h. Each sample was run in triplicate. Protein spots were annotated only if detectable in all gels.

2.4. Image Acquisition and Analysis

2-DE gels were stained with colloidal Coomassie G-250 and scanned using a GS-800 calibrated densitometer (BioRad). Image analysis was performed using the PDQuest software (BioRad). Spot detection and matching between gels were performed automatically, followed by manual verification. After normalization of the spot densities against the whole gel densities, the percentage volume of each spot was averaged for six different (three replicates of two samples) gels and Student's t-test analysis ($p < 0.01$) was performed to find out statistically significant protein fold changes associated to biochar presence in soil.

2.5. Protein Digestion and MALDI-TOF-Mass Spectrometry Analysis (MS)

Spots from 2-DE were excised and digested with trypsin, desalted using mZip-TipC18 tips (Millipore) before MALDI-TOF-MS analysis and/or directly analysed by mLCESI-IT-MS/MS. Peptide mixtures were loaded on the MALDI target with CHCA as matrix, using the dried droplet technique. Samples were examined with a Voyager-DE PRO spectrometer (Applera, USA). Peptide mass spectra for PMF experiments were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applera). Peptide mixtures were also analysed by using a LCQ Deca Xp Plus mass spectrometer (Thermo Finnigan, USA) equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan). Peptide mixtures were separated on a capillary Hypersil-Keystone Aquasil C18 Kappa column (100 mm, 0.32 mm, 5 mm) using a linear gradient from 10% to 60% of ACN in 0.1% formic acid, over 60 min, at a flow rate of 5 $mL \cdot min^{-1}$. Spectra were acquired in the range 200 - 2000 m/z. Data were elaborated using the software BioWorks 3.1 provided by the manufacturer.

2.6. Protein Identification

Software ProFound was used to identify spots from NCBI nonredundant data-

base by PMF experiments. Candidates with ProFound's Est'd Z scores >2 were further evaluated by comparison with Mr and pI experimental values obtained from 2-DE. SEQUEST software was used to identify proteins with data derived from mLC-ESI-ITMS/MS experiments. Candidates from NCBI nonredundant or tomato EST databases (ftp://ftp.sgn.cornell.edu/proteins/estscan_predictions/tomato) with more than three identified CID spectra of peptides belonging to the same protein and SEQUEST Xcorr values > 2.5 were further evaluated by comparison with experimental Mr and pl values obtained from 2-DE. Protein functional classification was done according to literature data.

2.7. RNA Extraction, cDNAs Synthesis and Quantitative Reverse Transcription PCR (RT-qPCR)

The "mirPremier microRNA isolation Kit" (Sigma) was used to extract RNA from tomato leaf samples according to manufacturer's instructions. To degrade DNA genomic and obtaining an eluate of pure RNA, the RNeasy/QIamp columns, RNase-Free DNase set (QUIAGEN) was used. The extracted RNA was promptly retrotranscribed to cDNA. For the retrotranscription the ImProm-II Reverse Transcription System Kit (Promega) and the Mj mini thermal cycler (BioRad) were used. The primers (Table 1) for the expression of genes involved in jasmonic acid pathway were designed using the NCBI Primer Blast tool. For the RT-PCR, the EvaGreen 2X qPCR MasterMix-R (Applied Biological Materials) kit was used. The thermal cycler 7300 Real-Time PCR System was set to perform an initial denaturation at 95˚C for 1 min, an annealing phase of 10 min at 95˚C and 40 successive cycles of denaturation (95˚C for 30 s), annealing (60˚C for 30 s), extension (72˚C for 30 s). The experiments were carried out in triplicate for each sample. The relative quantification in gene expression was determined using the 2-ΔΔCt method [12].

3. Results and Discussion

The differences in protein expression in the four different samples (CNT, BIO, CNTA and BIOA) of Solanum lycopersicum leaves, were detected by two-dimensional electrophoresis (2-DE) performed in triplicate and mass spectrometry. Spot acquisition and matching have been performed automatically by

Table 1. List of primers used for real time PCR.

TUB f	GCCTATTTTCTCGTGTAGTTGGT
TUB r	TTAGTGGGGCAAATCCCACC
AOS f	TTGAATCCCACGACGCATCA
AOS r	GCGTTTTCAGTTTCCGACCC
PDF1.2a f	GCTGCTTTCGGTGAGTAATAATG
PDF1.2a r	CCATGTCCCACTTGGCTTCT
PDF1.2b f	GCAGCTTTTGGTTAGTAATGCTCT
PDF1.2b r	AGTACCACTTGGCTTCTCGC

the PDQuest program and then manually. A total number of 405 spots were identified in CNT gels, 360 spots in BIO gels, 260 spots in CNTA gels and 425 spots in BIOA. The PDQuest program made it possible to compare the colour intensity of the spots due to the different concentrations of the overlapping proteins in the four groups of duplicated gels. The statistical analysis (Student's t-test) allowed identifying spots that differed from each other for optical density in the different samples taken with a level of significance equal to 0.01. 27 protein spots with statistical variations were excised from the gel, crushed, reduced, alkylated, digested with trypsin and analysed by MALDI-TOF mass spectrometry (Matrix-Assisted Laser Desorption Ionization—Time of Flight—Mass Spectrometry), or LC-ESI-IT-MS-MS (Liquid Cromatography—ElectroSpray Ionization—Ion Trap Mass Spectrometry/Mass Spectrometry). **Figure 1** shows the master gel with the 27 spots differentially expressed identified.

Identified proteins were divided in groups, based on their biochemical function. The first group includes proteins involved in the deface against stress; the most abundant group contains proteins associated with carbon metabolism and energy production (**Table 2**). This is in agreement with what reported in other papers where changes in metabolism and in the photosynthetic process after insect attack are reported [13] (Duceppe *et al.*, 2012).

The differentially expressed proteins involved in stress and defence are: Catalase 2 (spot 7), Chitinase family (spot11), Heat Shock Protein 70 (spot 13), 1-aminocyclopropane-1-carboxylate (spot 18), Peptidyl-Prolyl cis-trans Isomerase (spot 19), Galactinol synthase 1 (spot 24), Remorin 1 20 kDa (spot 25), Chaperonin (spot 26). Heat shock proteins 70 (HSP70) are induced by a wide range of stresses, recently was discovered their possible involvement in defense responses against pathogens [14] (Argueso *et al.*, 2012). The protein peptidyl-prolyl

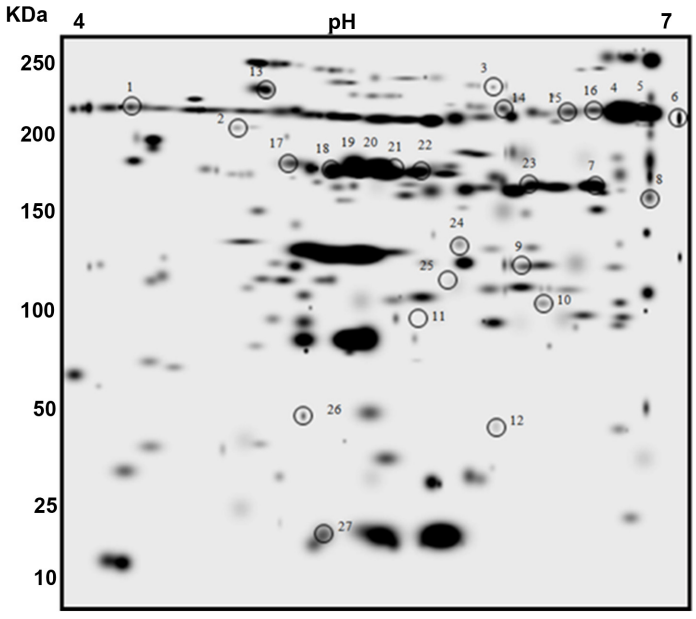

Figure 1. Master gel with the 27 identified spots by mass spectrometry.

Table 2. Proteins with differential expression.

Spot	Proteins	SWISS PROT entry	NCBI entry	CNT	BIO	CNTA	BIOA
	Stress and defense						
7	Catalase 2	CATA2_SOLLC	332661063				
11	Chitinase family	Q7Y0S1_SOLLC	19189				
13	Heat shock protein 70	A8W7B5_SOLLC	929524253				
18	1-Aminocyclopropane-1-carboxylate oxidase	ACCO1_SOLLC	922960051				
19	Peptidyl-prolyl cis-trans isomerase	CYPH_SOLLC	350539643				
24	Galactinol synthase 1	GOLS1_SOLLC	1234486				
25	Remorin 1	Q9XEX8_SOLLC	350536137				
26	20 kDa chaperonin	A0A077DBL2_TOBAC	1027858547				
	Energy and metabolism						
1	Elongation factor G	K4AZ10_SOLLC	940288036				
2	Disulfide isomerase like 1-1	K4BRS2_SOLLC	723699792				
3	Ribulose bisphosphate carboxylase/oxygenase activase	RCA_SOLPN	460380255				
4	Glycine hydroxymethyltransferase	K4CLC9_SOLLC	1109346125				
5	Glycine hydroxymethyltransferase	K4CLC9_SOLLC	1109346125				
6	Glycine hydroxymethyltransferase	K4CLC9_SOLLC	1109346125				
8	Ribulose bisphosphate carboxylase large chain precursor	RBL_SOLLC	92087012				
9	Ribulose bisphosphate carboxylase large chain	RBL_SOLLC	92087012				
10	Triosephosphate isomerase	TPIS_PSESM	28871628				
12	Photosystem I reaction center subunit IV	PSAC_SOLLC	460403271				
14	ATP synthase CF1 beta subunit	A0A0C5CEC7_SOLLC	544163620				
15	Dihydrolipoamide dehydrogenase precursor	Q8GT30_SOLLC	350536667				
16	glycine hydroxymethyltransferase	A0A0Q0HTT0_PSEUB	1109347569				
17	Ribulose bisphosphate carboxylase/oxygenase activase	RCA_SOLPN	460380255				
20	Ribulose bisphosphate carboxylase/oxygenase activase	RCA_SOLPN	460380255				
21	Serine carboxypeptidase-like 21	SCP21_ARATH	1039015599				
22	Ribulose bisphosphate carboxylase/oxygenase activase	RCA_SOLPN	460380255				
23	Putative hydrogenase	D8NVG3_RALSL	299078991				
27	Ribulose bisphosphate carboxylase small chain 1	RBS1_SOLLC	822092500				

Note: Heat maps represent the expression levels in the four treatments (CTR, BIO, CNTA and BIOA).

cis/trans isomerase (PPIase) catalyses the cis-trans isomerization of the peptide bond, and at the level of the thylacoids this enzymatic activity, under reducing conditions, is strongly suppressed [15] (Hanhart *et al.*, 2017). Chitinases (spot 11) are widely distributed in the plant kingdom with several roles in plant growth, development, microsporogenesis, embryogenesis, germination, flowering and abscission, but chitinases are also well-known as pathogenesis-related proteins that are constitutively expressed at low levels in plants, but are dramatically induced when plants respond to infections [16] (Wu and Bradford, 2003).

Some data have indicated that the aphids attack induces in tomato the regulation of genes involved in the biosynthesis and in the perception of ethylene.

Confirming these data, the 1-aminocyclopropane-1-carboxylate oxidase protein, a key enzyme in ethylene biosynthesis, is up-regulated in the CNTA and BIOA samples. Ethylene has a dual role. On the one hand it has a synergistic effect improving the responses induced by stress, on the other it modulates the pathway of jasmonic acid and the responses induced by this phytohormone [17] (Vriezen *et al.*, 1999). Galactinol synthase (spot 24) is involved in the biosynthesis of raffinose family oligosaccharides that function as osmoprotectants. It can promote plant biotic stress tolerance under jasmonate activation [18] (Cho *et al.*, 2010). Remorines are membrane proteins whose functions are not yet fully understood. From some studies of REM levels manipulation in transgenic tomato plants (*Solanum lycopersicum*), it has been shown that the movement of the PVX potato virus from cell to cell is inversely related to the accumulation of these proteins, highlighting the role of this protein in responses to biotic stress. Catalase 2 protects cells from the toxic effects of hydrogen peroxide, this reactive compound acts as an important signal in the defence of plants and in the wound response, determining the induction of defence genes not only locally, but also distal [19] (Miller *et al.*, 2009). Already at the baseline level in the CNT and BIO samples we can observe a down regulation of the proteins involved in stress and defence in the sample grown on soil with biochar; following the attack of the aphids the role of the biochar in inducing a down regulation of the proteins involved in the defence pathways becomes even more evident (**Figure 2**).

Energy and carbon metabolism group contains 19 proteins : elongation factor G (spot 1), disulfide isomerase (spot 2), ribulose bisphosphate carboxylase/oxygenase activase (spots 3, 17, 20, 22), glycine hydroxymethyltransferase (spots 4, 5, 6, 16), ribulose bisphosphate carboxylase large chain precursor (spot 8), ribulose bisphosphate carboxylase large chain (spot 9), triosephosphate isomerase (spot 10), photosystem I reaction centres subunit IV (spot 12), ATP synthase CF1 beta subunit (spot 14), dihydrolipoamide dehydrogenase precursor (spot 15), serine carboxypeptidase-like 21 (spot 21), putative hydrogenase (spot 23) and ribulose bisphosphate carboxylase small chain 1 (spot 27). Many of the differentially expressed proteins identified are involved in the photosynthetic process, such as the Ribulose bisphosphate carboxylase/oxygenase activase (spots 3, 8, 17, 20, 22 and 27), an enzyme involved in the first important phase of carbon fixation, a process by which atmospheric carbon dioxide is converted from plants and other photosynthetic energy organisms—rich molecules. These spots are down regulated in the control sample (CNT) and following the attack of the aphids.

All the identified proteins involved in energy production and carbon metabolism show the maximum expression in the sample BIO, although the aphids attack induces a general down-regulation in these proteins, the comparison between them expression in samples BIOA and CNTA highlights and confirms that the addition of 5% of biochar in soil promotes growth according to the agronomic parameter collected (**Figure 2**, **Figure 3**).

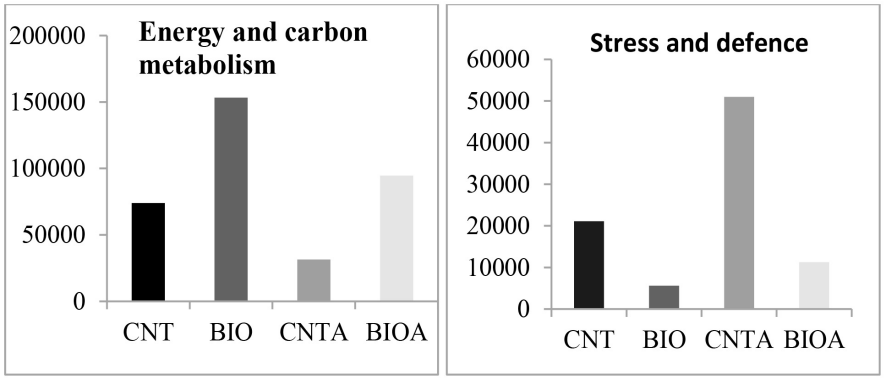

Figure 2. Total expression level of proteins involved in energy and carbon metabolism and in stress and defence in the four samples CNT, BIO, CTRA and BIOA.

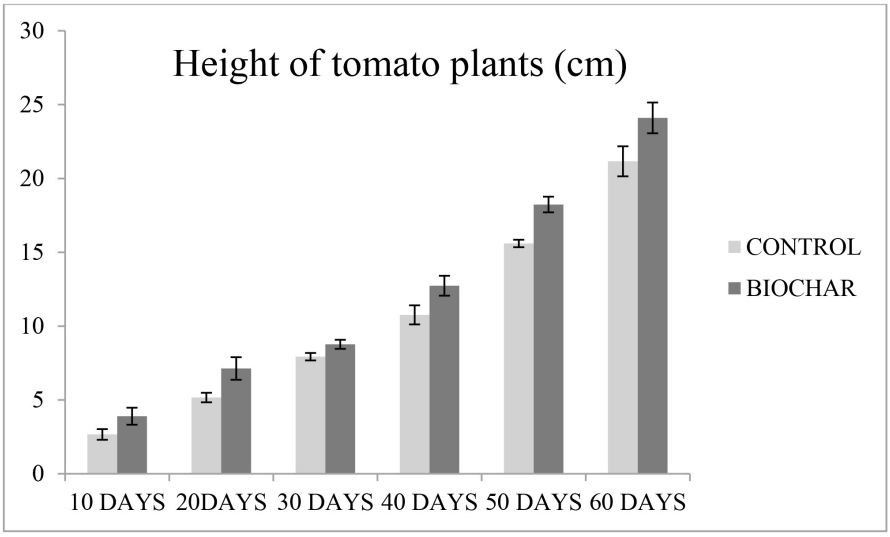

Figure 3. Height of tomato plants collected every 10 days until the aphids infection.

There are many studies in the literature concerning plant-aphid interaction. it is now clear that salicylic acid has a primary role in the activation of the responses of defence against aphids [20] [21] and that the salicylic acid pathway often contributes to the resistance mediated by the genes of resistance, while jasmonic acid and ethylene play a central role in the activation of the wound signal pathways [22]. However the disruption of plant cell wall tissue during aphid feeding has been shown to induce defence responses mediated by the jasmonate in Arabidopsis, Triticum, Sorghum and Nicotiana species [23]. For this reason, we decided to select some genes involved in the jasmonic acid defence pathways already analysed in Viger's *et al.* (2014) work, and make a real-time PCR on them. Using a real-time PCR we evaluated the expression of genes for the allene oxide synthase (AOS) and two genes for the plant defensins (PDF1.2a/b) (**Figure 4**). The expression of AOS determines the defence gene activation, this enzyme catalyses the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids. Jasmonic acid induces the

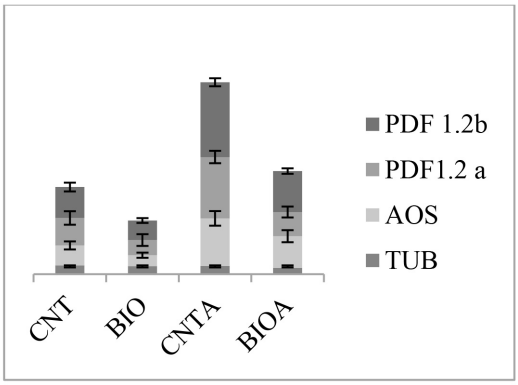

Figure 4. The expression levels of the four genes used for real-time PCR.

production of secondary metabolites such as difensine, thionine and chitinase, which determine the IRS [24].

The results of real-time PCR show that the addition of 5% of biochar induces a down regulation of the three genes involved in the jasmonic acid pathway both at baseline level (BIO) and following the attack of aphids (BIOA) compared to control samples (CNT and CNTA) (**Figure 3**).

4. Conclusions

The aphid *Myzus persicae* is one of the most harmful agents for the tomato (*Solanum lycopersicum*) cultivation. In addition to direct damage, due to aphid nutrition, it can often be a vector of phytopathogenic viruses. To date, there is little information about the proteomic and molecular mechanisms underlying the resistance of the plant to aphids. In order to obtain a better understanding of the changes induced in tomato plants, grown with the addition of a soil improver, a proteomic and molecular analysis was carried out.

The results of these analyses have shown that the presence of 5% of the biochar in the soil shifts the energy of the plant towards the primary metabolism and photosynthesis both at the basal level and following the aphids attack. This, however, goes to the expense of the defence responses that are down-regulated in the samples grown on land financed with biochar compared to controls. The proteomic analysis allowed the identification of some differentially expressed proteins; in those related to the defense, the expression changes significantly in response to the attack of the aphid; in particular in the BIOA treatment these proteins are down regulated respect to the relative control. Moreover, the molecular analysis carried out on three genes related to the wounding and to the jasmonic acid pathway showed an up regulation in the samples attacked by the aphids grown on control grounds (CNTA) respect to the BIOA treatment. On the contrary, the proteins involved in the defense and the genes taken into analysis have a significantly lower level of expression in the samples not exposed to aphids. The present study represents an initial contribution for the subsequent

understanding of specific mechanisms of tomato, grown in soil with biochar, defense against aphids.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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