Full Length Article

Metabolomic Analysis Identifies Synergistic Role of Hormones Biosynthesis and Phenylpropenoid Pathways during Fusarium Wilt Resistance in Tomato Plants

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Abstract

Fungal wilt diseases are among the most destructive pathogens attacking crop plants globally. Host resistance is considered a key for Fusarium wilt management. Some resistant plant varieties have been screened, but the underlying mechanisms are poorly understood. Here detailed metabolomic analysis was performed of resistant and susceptible tomato varieties in response to Fusarium wilt infection. A total of 73 metabolites were identified in the resistant and susceptible tomato plant samples. Functional categorization of these differentially produced metabolites indicated extensive re-modulations in plant physiology. Perception of *F. oxysporum* caused activation of signaling pathways and over production of some precursor molecules in different carbon cycles. These precursor metabolites were mainly channeled into hormones biosynthesis, phenylpropenoid and alkaloid biosynthesis pathways. This all together produced significantly higher quantities of different defense factors such as phenolics, terpenoids and alkaloids in resistant tomato variety upon pathogen attack. This up-regulation of defense-related metabolism contributed to resistance against Fusarium wilt disease in tomato plants. These results improve our understanding about underlying mechanisms and identify metabolites and pathways related to specific resistance mechanisms. Current study suggests that, in contrast to previous knowledge, there exists a synergistic effect between hormone biosynthesis and phenylpropenoid pathway in resistance reaction against Fusarium wilt disease. This knowledge can help to improve the durability of resistance against Fusarium wilt disease. © 2017 Friends Science Publishers

Keywords: Tomato; Fusarium wilt; Metabolomics; Phenylpropenoid pathway; Hormone biosynthesis

Introduction

Plants colonized the land millions years ago. Subsequently, they effectively evolved to survive under stresses. Pathogen attack induce great losses in crop production every year in agriculture fields (Pennisi, 2010). Considerably, plants are thought to be immune to all pathogen races in nature (Pennisi, 2010). Nature has provided plants a range of strategies to protect themselves against an array of pathogens (Chisholm *et al.,* 2006). Plethora of defense responses is initiated by the recognition of specific signaling molecules produced by the pathogens (Boller and He, 2009). Host plants recognize these signaling molecules and initiate their immune responses (Zipfel, 2008; Boller and Felix, 2009). Second defense line in plants is based on gene for gene concept, also termed as effector triggered immunity (Snijders, 1990; Dodds and Rathjen, 2010; Gururani *et al.,* 2012). This immunity works by co-interaction of resistance (R) and avirulance (Avr) genes (Ellis *et al.,* 2000; Bani *et al.,* 2012). Scientists incorporate R genes during plant breeding program by crossing plants with closely related members resistant to that disease (Barilli *et al.,* 2012). These R genes upon expression effectively alter the physiology of plants in response to a specific disease (Jlibene *et al.,* 1992; Montilla-Bascón *et al.,* 2013).

Plant metabolites play a crucial role in the developmental and control of different metabolic pathways by regulating organ formation and development in plants (Croteau, 1988; Gupta and Datta, 2001; DeVos and Jander, 2009). The plant responses to pathogen attack are governed by the changes in small molecules to reach an altered physiological state (Grubb and Abel, 2006). These metabolites are transported throughout the plant body and lead to differentiation of plant organs for adaptation under stressful conditions (Funck *et al.,* 2009). In recent years, metabolomic studies of plants have increased our understandings about plant defense mechanisms and signal transduction pathways. Metabolites synthesis and transport is greatly mediated by the external stimuli (Duan *et al.,* 2013). Many metabolites have been identified and characterized which are involved in plant defense against attacking pathogens. Most of them belong to phenylpropenoid pathway (Duan *et al.,* 2013). This pathway is considered of immense importance regarding plant

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defense responses governing both biochemical and histological mechanisms (Glazebrook, 2005). Ultimately, these responses against pathogen are supported by the reprogramming of metabolomics and histology of plants (Atkinson and Urwin, 2012).

Plant defense system is a composite process that is regulated by interactions at different levels (Boller, 1989; Ballhorn *et al.,* 2009). Therefore, manipulation of a single system is not adequate to study disease resistance process. There is a need to consider complex responses of plants to biotic stresses at a systems biology level. In the past few years, new technology has made it possible to overview the changes that occur at multiple levels inside plant body. The yield losses of the plants under pathogens attack is a common phenomenon in whole world. Therefore, it is necessary to search for new traits to minimize losses caused by pathogen attack. Tomato is primarily cultivated on a large scale by farmers in the whole world due to its importance in food and medicines. Understanding the plant resistance to pathogen has contributed to improve the yield. The wilt disease of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* has been observed in high incidence rates during recent and has caused high losses in some of the cultivated areas (Andolfo *et al.,* 2014). The purpose of this study is to present a comprehensive overview of the changes in the tomato plant's metabolomics under disease stress and to identify important pathways and traits which this plant adapts to effectively cope fusarium wilt attack. For that purpose, tomato plants were interacted with *F. oxysporum* under both compatible and incompatible interactions to understand changes at plants histology and metabolomics re-programing. This information will be useful in breeding for resistance program for number of plants.

Materials and Methods

Plant Growth and Disease Development

Seedlings of two tomato varieties (Pride Burn and Fine Star) with varying susceptibility against Fusarium wilt were raised in sterilized commercial potting media under greenhouse conditions (Akram *et al.,* 2014). A virulent strain of *Fusarium oxysporum* f.sp. *lycopersici* was procured from Fungal biotechnology lab. Institute of Agricultural Science, University of the Punjab. Lahore, Pakistan. After twenty days of emergence, uniform looking seedlings were transformed in sterilized 15 cm plastic pots containing commercial potting mix. After one week of establishment, pathogen was applied in the form of conidial suspension at the rate of 50 mL each of concentration $10x^4$ conidia/mL. Two sets were made of each variety. One was challenged with pathogen, whereas second served as control and provided with 50 mL of distilled water in each pot. Pots were left in greenhouse up to 15 days for disease development. Each set contained ten replicate plants and experiment was repeated twice. After fifteen days of

pathogen application, disease index was assessed by adopting the method as proposed by Cachinero *et al.* (2002).

Metabolomic Analysis

Whole metabolomics analysis of both tomato varieties was performed in accordance with Lisec *et al.* (2006).

Sample Preparation

After ten days of pathogen challenge, leaf samples were collected from third node for each treatment. Leaf samples were crushed in liquid nitrogen and metabolites were extracted over night by suspending one gram of fine powder of leaves in 10 mL of methanol, chloroform and water (80:10:10) solvent system under continuous agitation conditions to ensure maximum recovery of metabolites. Next day, material was filtered and 1 mL of each sample was taken in separate glass tube. Samples were dried completely in liquid nitrogen following derivatization by using MOX and MSTFA reagents according to methodology proposed by Steinfath *et al.* (2008).

GC/MS Analysis

Derivatized plant extract samples were injected in Agilent GC/MS apparatus equipped with capillary column. Helium as a carrier gas was used (flow rate of 1.0 mL/min). Column temperature conditions were as following: 30°C for 5 min, increasing up to 180°C at the rate of 50°C/min.

### Metabolites Identification and DATA Processing

Spectral similarities of detected compounds were compared with the NIST library to identify them. Software package "Mzmine" was used to relatively quantify metabolites. The values of detected compounds were firstly log^{10}-transformed and normalized to the peak size of respective sample (Steinfath *et al.,* 2008). This data was statistically analyzed and graphically represented by using "DSAASTAT" and the "ClustVis" software (Onofri Italy). The principal component analysis was also performed in "ClustVis" software by adopting 'bpca' algorithm. The metabolomic data were summarized as a heatmap with row wise scaling.

Results

Tomato plant histology and metabolomics were studied to understand the mechanism governing the susceptibility and/or resistance of tomato plants upon fusarium wilt disease attack. Two tomato varieties with varying susceptibility against fusarium wilt were used. Successful fusarium infection was confirmed by appearance of wilt symptoms on tomato plants. Here variety Fine Star was found susceptible against fusarium wilt disease with 87.1% disease index (DI) value, whereas tomato variety "Pride Burn" showed 24.3% DI upon pathogen attack (Fig. 1).

Table 1: Functional categorization of metabolites differentially produced in tomato plants of varying susceptibility against Fusarium wilt disease

Metabolic Process	Susceptible		Resistant	
	Down-regulated	Up-regulated	Down-regulated	Up-regulated
Aerobic Respiration	-	3	-	2
ATP Synthesis	1	7	-	5
Cell wall biosynthesis	1	3	2	6
Fatty acid Metabolism	-	3	-	4
Hormones Biosynthesis	2	5	1	9
Lipid Metabolism	5	6	3	5
Organic acids metabolism	5	9	3	8
Pentose-phosphate	2	5	1	7
Phenylpropanoid biosynthesis	3	7	2	9
Photosynthesis	4	6	2	5
Polysaccharide Biosynthesis	-	5	1	6
Signal transduction	-	6	1	5
Sucrose Biosynthesis	3	9	5	8
TCA Cycle	4	6	3	8
Toxin Catabolic Process	-	7	4	3
Transport	2	8	4	3
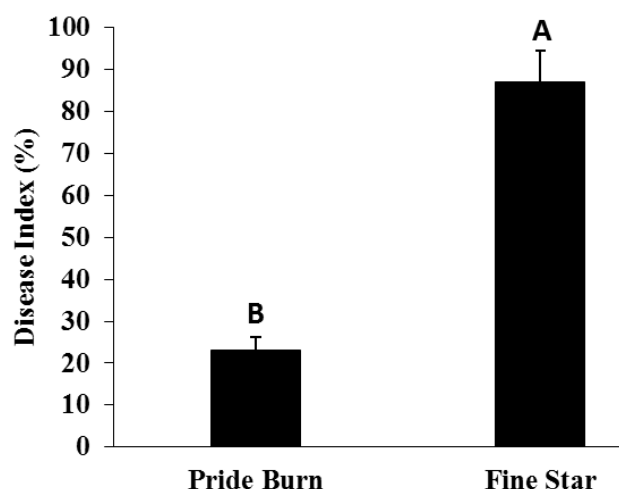
Fig 1: Susceptibility of tomato varieties against Fusarium wilt disease. Vertical bars represent standard error between different replicates of same treatment. Capital letters shows level of significance among different treatments as governed by ANOVA and DNMRT at p=0.05

Subsequent host responses differed markedly following pathogen treatment.

Effect of Fusarium Infection on different Metabolites of Tomato Plants

Both tomato varieties showed obviously different physiological responses. Considerable variations were found in the metabolomics of tomato plants of resistant and susceptible tomato varieties against fusarium wilt disease. In tomato plants, 47 metabolites showed significant differences in their quantities with respect to varying susceptibility against fusarium wilt disease. These included 16 organic acids, 11 amino acids, 13 sugars, 5 sugar alcohols and 2 non-categorized biochemicals that were reproducibly detected in tomato plants receiving *F. oxysporum* inoculum. Based on PCA, resistant and susceptible samples of tomato metabolites showed clear separations by the first components (Fig. 5).

Among amino acids, fusarium infection significantly increased alanine, cerine, glycine, phenylalanine, proline and threonine levels in resistant tomato variety whereas glutamine and cerine were significantly down-regulated in resistant tomato variety. Fusarium treatment significantly increased the quantities of multiple sugars and sugar alcohols (Figs. 2 and 4). Among these sugars and sugar alcohols, the quantities of glucose, fructose, sucrose, melibose, maltose, furanose and glycerol significantly increased in resistant variety (Figs 2 and 4). Fusarium infection significantly increased quantities 11 organic acids, whereas 3 organic acids showed significant down-regulations in resistant variety (Fig. 4).

Re-modulations in Metabolic Process in Tomato Plants under Fusarium Infection

To further investigate the possible re-modulations in primary and secondary metabolism of tomato plants of varying susceptibility against Fusarium wilt disease, these changes were incorporated in different metabolic cycles. The carbon metabolism revealed apparent differences between both tomato varieties receiving fusarium wilt pathogen. Here the fluctuations in resistant tomato variety were relatively higher than that of susceptible variety.

Several secondary metabolic pathways were also differentially regulated in resistant tomato variety viz: amino acid biosynthesis, signal transduction, lignin biosynthesis, phenylpropenoid biosynthesis etc (Table 1). The production of specific signaling molecules like salicyclic acid, was produced in significantly higher quantities in resistant tomato variety as compared to the susceptible one. Regarding other defense related metabolic processes, extensive re-modulations were denoted in phenylpropenoids and lignin biosynthesis. Some precursors of phenylpropenoid pathway (phenylalanine and cinnamic acid) were produced in significantly higher quantities in resistant tomato variety as compared to the susceptible one. When classified to phenylpropenoids, 27 metabolites were successfully quantified. A majority of them were produced in significant higher quantities in resistant tomato plants.

Discussion

Fungal wilt diseases caused by the *F. oxysporum* are serious threat on a range of crop plants, limiting crop productivity (Tsavkelova *et al.,* 2012). Cultivation of disease resistant varieties is the main solution to manage these diseases. Some resistance factors have been identified, but the mechanisms associated are poorly understood.
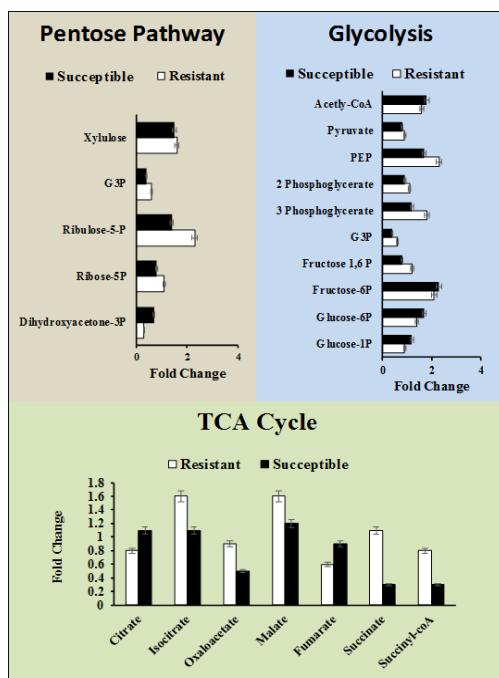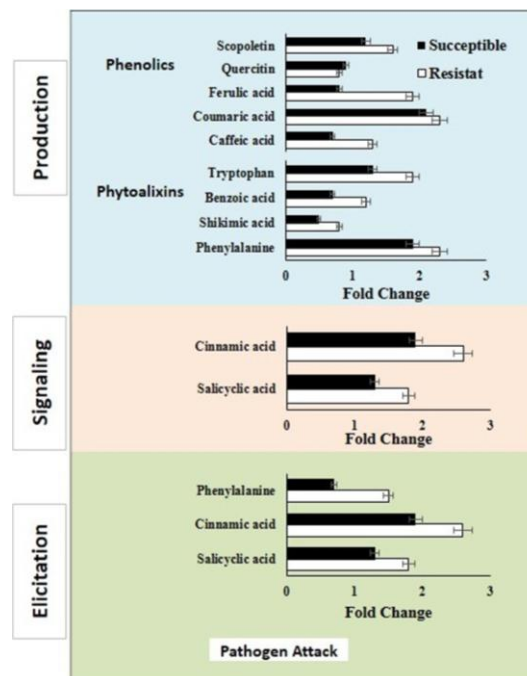
Fig. 2: Change in levels of some metabolites of tomato involved in primary and secondary metabolism of tomato plants of varying susceptibility against Fusarium wilt disease


Fig. 3: Change in levels of some metabolites of tomato involved in defense related pathways of tomato plants of varying susceptibility against Fusarium wilt disease

This research work has provided important clues to interpret resistance mechanisms. The mode of actions associated with re-modulation of metabolomics in resistance tomato variety can be used in breeding for resistance program.

Whole metabolome analysis is an efficient way for describing differences in metabolomics of resistant and susceptible plant varieties. GC/MS identified 69 metabolites and most of these could be related to the plant physiological processes (Table 1). The biological context of these metabolites was further analyzed by channeling them into different physiological processes and ontologies, thus linking specific traits to the resistance process against fusarium wilt disease. In resistant tomato variety, 38 metabolites were significantly up-regulated and 09 were down-regulated as compared to the susceptible one after challenging with the wilt pathogen. These metabolites were belonging to diverse functional groups, including carbon metabolism, signal transduction, amino acid metabolism, phenylpropenoid biosynthesis, alkaloids and flavonoids biosynthesis and hormone biosynthesis etc (Table 1).

In plants, sugars are involved in many important metabolic and signaling pathways. Some sugar molecules act as signaling molecules and contribute to trigger the immune responses against pathogens (Fotopoulos *et al.,* 2003; Azevedo *et al.,* 2006; Rolland *et al.,* 2006). These function as elicitor biochemicals and effectively trigger immunity in plants (Herbers *et al.,* 1996; Roitsch and Gonzalez, 2004). High cellular sugar levels are beneficial

for plants. Sugars also regulate defense mechanisms by controlling gene expression levels and induce pathogenesis-related genes (Roitsch, 1999; Rolland *et al.,* 2006; Bolouri-Moghaddam and Van den Ende, 2012). In resistance tomato variety, some sugars were found in significantly higher quantities as compared to the susceptible one. These higher sugar levels may have contributed in resistance process against *F. oxysporum* in tomato plants.

Plant response to environmental signals is mainly governed by phytohormones. Likewise sugars, plant hormones have important role in governing developmental and signaling processes in responses to a wide range of stresses (Anderson *et al.,* 2004; Achuo *et al.,* 2006; Chisholm *et al.,* 2006). Scientists have suggested the roles of salicylic acid, jasmonates and ethylene in plants during pathogenesis process and plant immunity (He *et al.,* 2007; Flors *et al.,* 2008). Recent studies proposed that different plant hormones are implicated in plant defense signaling pathways (Depuydt *et al.,* 2008; Farrokhi *et al.,* 2008). In current investigation, some plant hormones like gibberellin, were observed in significant higher quantities in resistant tomato variety. Here, these hormones have impaired in modulating plant defense responses against Fusarium wilt pathogen. This investigation also highlights the positive role of some phytohormones as regulators of crosstalk in disease and defense.

Phenylpropanoid compounds mainly function in plant defence ranging from physical and chemical barriers against
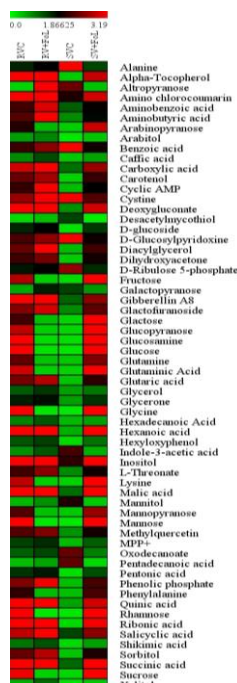
Fig. 4: Heat map showing changes in levels of different metabolites in tomato plants under influence of Fusairum wilt pathogen. SVC= Susceptible variety alone, SV+FoL= Susceptible variety challenged by Fusarium wilt pathogen. RVC= Resistant variety alone, RV+FoL= Resistant variety challenged by Fusarium wilt pathogen
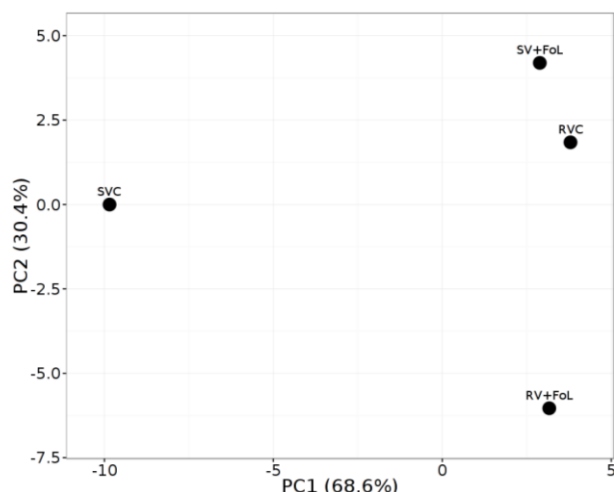
Fig. 5: Principal component analysis of changes in levels of different metabolites in tomato plants under influence of Fusairum wilt pathogen. SVC= Susceptible variety alone, SV+FoL= Susceptible variety challenged by Fusarium wilt pathogen. RVC= Resistant variety alone, RV+FoL= Resistant variety challenged by Fusarium wilt pathogen

infection to pathogen (Dixon *et al.,* 2002; Glazebrook, 2005). GC/MS analysis of both plant varieties clued that Fusarium infection mainly initiated coordinated induction of

phenylpropanoid defense responses in resistant plant variety. It was also observed that some biochemicals that act as precursors molecules were up-regulated in resistant tomato variety. These up-regulated precursor molecules were channeled into the shikimate and phenylpropenoid pathway. Consistent with the carbon metabolomics, the phenylpropenoids production rate in resistant tomato variety was significantly higher as compared to that in susceptible variety receiving fusarium wilt pathogen. Ultimately, the formation rate of phytoalexins such as phenloics, alkaloids and flavonoids increased significantly in resistant variety that hindered further pathogen invasion and establishment inside plant body.

This study concludes that there exists a synergistic effect between hormone biosynthesis and phenylpropenoid pathway in resistance reaction against Fusarium wilt disease. In contrast to previous knowledge, tomato have evolved a different strategy in immune responses. This strategy is based on excessive perturbations in secondary metabolic pathways mainly dealing with hormone biosynthesis and increased phenylpropenoids production. These phenylpropenoids provide a frontline of chemical defense responses against invading Fusarium wilt pathogens.

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