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The plant immunity inducer pipecolic acid accumulates in the xylem sap and leaves of soybean seedlings following *Fusarium virguliforme* infection

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ABSTRACT

The causal agent of the soybean sudden death syndrome (SDS), *Fusarium virguliforme*, remains in infected roots and secretes toxins to cause foliar SDS. In this study we investigated the xylem sap, roots, and leaves of *F. virguliforme*-infected and -uninfected soybean seedlings for any changes in a set of over 3,000 metabolites following pathogen infection by conducting GC/MS and LC/MS/MS, and detected 273 biochemicals. Levels of many intermediates of the TCA cycle were reduced suggesting suppression of this metabolic pathway by the pathogen. There was an increased accumulation of peroxidated lipids in leaves of *F. virguliforme*-infected plants suggesting possible involvement of free radicals and lipoxygenases in foliar SDS development. Levels of both isoflavone conjugates and isoflavonoid phytoalexins were decreased in infected roots suggesting degradation of these metabolites by the pathogen to promote root necrosis. The levels of the plant immunity inducer pipecolic acid (Pip) and the plant hormone salicylic acid (SA) were significantly increased in xylem sap (in case of Pip) and leaves (in case of both Pip and SA) of *F. virguliforme*-infected soybean plants compared to the control plants. This suggests a major signaling role of Pip in inducing host defense responses in above ground parts of the *F. virguliforme*-infected soybean. Increased accumulation of pipecolic acid in foliar tissues was associated with the induction of *GmALD1*, the soybean homolog of Arabidopsis *ALD1*. This metabolomics study generated several novel hypotheses for studying the mechanisms of SDS development in soybean.

1. Introduction

Interactions of pathogens with plants lead to an array of changes in transcriptomes, proteomes, and metabolomes in both hosts and pathogens. Metabolic changes are well known for their roles in defending plants from invading pathogens. For example, phytoalexins and lignins are two long studied secondary metabolite classes that are considered to play major roles in plant defense. Depending on the host cultivar, there can be large qualitative differences in the accumulation of plant metabolites in response to different pathogen infections [1]. For instance, *Fusarium oxysporum*-infected *Brassica rapa* plants have been shown to accumulate more phenylpropanoids, flavonoids, and fumaric acid than those infected with other pathogenic fungi like *Aspergillus niger* and *Leptosphaeria maculans* [1]. Degradation of isoflavanoid phytoalexins by pathogens has been shown to be necessary for disease development [2].

Investigation of the global changes of metabolites can help answer plant biological questions. The utility of metabolomics in dissecting plant-fungal interactions has been reviewed recently [3]. Metabolic profiling was used to study the basal metabolism in *Fusarium* spp. [4]. The roles of small metabolites, such as methyl salicylate, azelaic acid, and pipecolic acid, in the expression of systemic acquired resistance have recently been considered [5]. Xylem

Abbreviations: BLOB, binary large object; BSTFA, bistrimethyl-silyl-triflouroacetamide; ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance; 13-HODE, 13-(S)-hydroxyoctadecadienoic acid; 9-HODE, 9-Hydroxy-10,12-octadecadienoic acid; LAN, local area network; LIMS, laboratory information management system; LIT, linear ion-trap; Pip, pipecolic acid; QC, quality control; SDS, sudden death syndrome.

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sap is known to be involved in long-distance signaling in response to pathogen infection [6]. Changes are observed in xylem sap proteomes and metabolomes in response to abiotic and biotic stresses [6–8]. Pathogen infections can result in increases in phenolic compounds [9] and/or salicylic acid and its derivatives in the xylem sap [10]. Changes in the contents of phenolic and indolic compounds, amino acids, nitrogen compounds, disaccharides, glucosinolates and molecules that lead to an increase in reactive oxygen species have been observed in *Arabidopsis thaliana* infected with *Pseudomonas syringae* pv. tomato [11].

Sudden death syndrome (SDS) is a soybean fungal disease that can cause drastic annual yield losses of nearly 300 million dollars [12]. In North America, this disease is caused solely by *Fusarium virguliforme*, formally known as *F. solani* (Mart.) Sacc. f. sp. *glycines*; whereas, in South America five different *Fusarium* species including *F. virguliforme* are known to cause the disease [13–15]. *F. virguliforme* is a hemi-biotrophic, soil borne, asexually propagated fungus possessing only one mating gene idiomorph, *MAT1-1*; in contrast, *F. tucumaniae*, one of the SDS pathogens in South America, comprises two idiomorphs each having either the *Mat1-1* or *Mat1-2* gene and is therefore able to sexually propagate [13,16]. *F. virguliforme* causes root necrosis and has never been detected in diseased foliar tissues. Host-selective toxins produced by the fungus have been hypothesized to cause foliar SDS symptoms as the pathogen restricted to infected soybean roots [17–19]. Recently, a major *F. virguliforme* toxin FvTox1 has been shown to induce foliar SDS in soybean [20–22]. In addition, five minor candidate toxins have been detected in xylem sap proteomes [7]. Thus, it is becoming apparent that multiple host-selective toxins produced by *F. virguliforme* could be transported to the leaves via the vascular system to cause foliar SDS in soybean [7,20–22].

SDS is an emerging disease with no known genes conferring complete resistance against the SDS pathogen, *F. virguliforme*. Toxin-induced foliar SDS is the highly destructive component of the disease and can lead to total yield losses. To date, there are no fungicides available for controlling this disease. Therefore it is essential to understand the mechanisms of disease development in order to design appropriate SDS management practices. In this study we monitored the accumulation patterns of 273 biochemicals in roots, xylem sap and leaves of *F. virguliforme*-infected and uninfected SDS-susceptible soybean cultivar "Spencer" to determine if there are any biochemicals that are accumulated during SDS development. Elevated levels of the plant immunity inducer pipecolic acid were observed in the xylem sap and leaves of *F. virguliforme*-infected soybean plants. In addition, the levels of peroxidated lipids were increased in leaves of *F. virguliforme*-infected plants, compared to those of uninfected soybean plants. This suggests a possible involvement of free radicals and lipoxygenases in developing foliar SDS symptoms following root infection of soybean with *F. virguliforme*.

2. Materials and methods

2.1. Inoculum preparation, plant material, and xylem sap collection

The cultivar "Spencer", a highly *F. virguliforme*-susceptible soybean cultivar, was used in this study. Mixed inoculum from two *F. virguliforme* isolates, Scott and Clinton, was prepared in sorghum meal as described by Hartman et al. [23]. Mixed inoculum was used to increase the disease pressure. The isolates were grown in 1/3 strength potato dextrose agar (PDA) prior to inoculation of sorghum meal. Detailed descriptions of growth conditions of the plants, inoculum preparation, and xylem sap collection are reported by Abeysekara and Bhattacharyya [7]. In brief, 120 seedlings were grown in soil mixed with either the *F. virguliforme* inocula grown in sorghum as the treatment or with just the sorghum meal as the control [23,24]. Approximately between 14 and 21 days after emergence, xylem sap was collected from the plants showing uniform levels of foliar disease severity. The experiment was repeated four more times. Roots and leaves from each of the infected and uninfected seedlings were collected separately, ground in liquid nitrogen, and stored at −80 °C along with the xylem sap samples until analysis. The experiment was repeated three more times for collecting leaf and root samples to conduct RT-PCR analysis for a few selected genes.

2.2. Sample preparation

Metabolomic analysis of the xylem sap samples was carried out at Metabolon Inc., Durham, NC, USA. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to extraction for quality control (QC) purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and the other one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum prior to reconstitution in another solvent depending on the following analysis (see Sections 2.3 and 2.4).

2.3. Liquid chromatography/mass spectrometry (LC/MS, LC/MS/MS) and accurate mass measurement

The LC/MS was conducted on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and a linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained at least 11 injection standards at fixed concentrations. One aliquot was analyzed using acidic conditions optimized for positive ion detection and the other one using basic conditions optimized for negative ion detection. After injection, each sample aliquot was separated on separate acid/base dedicated 2.1 mm × 100 mm Waters BEH C18 1.7 μm particle columns (Waters, Milford, MA, USA). Extracts reconstituted in acidic conditions were eluted using a gradient of water and methanol containing 0.1% formic acid, while for elution of the basic extracts, the gradient of water and methanol contained 6.5 mM ammonium bicarbonate. For ions with counts greater than 2 million, an accurate mass measurement was performed. Accurate mass measurements could be made on the parent ion as well as on the fragments. The typical mass error was less than 5 ppm. Ions with less than two million counts require a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data dependent manner, but when necessary, targeted MS/MS was also employed, such as in the case of metabolites with lower level signals.

2.4. Gas chromatography/mass spectrometry (GC/MS)

The samples destined for GC/MS analysis were dried by vacuum desiccation for a minimum of 24 h prior to derivatization under nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). A 20 m × 0.18 mm (0.18 mm film phase consisting of 5% phenyldimethyl silicone) GC column (Thermo Finnegan, San Jose, CA, USA) was used and the temperature ramp used was from 40° to 300 °C in a 16 min period. Samples were analyzed on a