**Metabolome profiling to understand the defense response of sugar beet (*Beta vulgaris*) to
Rhizoctonia solani AG 2-2 IIIB.**

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Abstract:

Rhizoctonia crown and root rot (RCRR), caused by *Rhizoctonia solani* Kühn AG 2-2 IIIB, is an important disease of sugar beet. While RCRR can be managed by agronomic practices, plant resistance remains the primary method for control. However, the molecular processes that mediate resistance to *R. solani* are largely unknown. The metabolic changes that occurred during susceptible and resistant *R. solani* interactions were compared and characterized using nontargeted metabolomic profiling. Metabolites from infected and healthy, root and leaf tissue, were taken at 0 and 7 dai and detected using reversed-phase UHPLC-MS and GC-MS. There was a clear distinction in the metabolome between tissue type and genotype, and in response to *R. solani*. 143 compounds were annotated and several metabolites associated with plant defense to fungi were identified in both germplasm.

Keywords: Sugarbeet; Rhizoctonia crown and root rot; host resistance; fungal-host interactions; plant metabolomics.

Abbreviations: RCRR, Rhizoctonia crown and root rot; AG, anastomosis group; dai, days after inoculation.

1.0 Introduction

Sucrose (sugar) is a common, naturally occurring carbohydrate found in many plants and plant parts which is a major component of a vast range of foods, beverages, and pharmaceuticals (Cooke and Scott 1993). Since the mid-1990s, sugar beet (*Beta vulgaris* L.) accounts for a growing percentage of global sucrose production and now constitutes more than 50 percent of United States sucrose supplies (Cooke and Scott 1993;McConnell 2015). Rhizoctonia crown and root rot (RCRR), caused by the fungus *Rhizoctonia solani* Kühn, continues to be a problem in most sugar beet growing areas in the United States, and is an increasing problem worldwide (Büttner et al. 2003;Herr 1996). Rhizoctonia crown and root rot infections often start at the root tip, and progress up the root towards the crown. As the plant matures, the rot increases and can result in partially or completely rotted roots. Sugar beet roots with significant RCRR can cause up to 50% yield reductions (Herr 1996), storage pile deterioration, and reduced juice purity if not removed prior to storage (Hecker and Ruppel 1976).

RCRR is primarily caused by *R. solani* anastomosis group (AG) 2-2 IIIB (Herr 1996), but other AGs can also be isolated from sugar beet although at much lower frequencies (Hanson and McGrath 2011;Herr 1996;Windels and Nabben 1989). RCRR can be managed to a degree by agronomic practices, adequate crop rotation, and chemical measures (Stump et al. 2004).

However, it is difficult to achieve adequate control with these strategies alone, therefore plant resistance is an important component for effective RCRR management.

While significant progress has been made in breeding for resistance to RCRR (Gaskill 1968;Hecker and Ruppel 1975;Hecker and Ruppel 1976;Panella 1999) little is known about the specific genes and mechanisms that condition host resistance to this important pathogen. The resistance to *R. solani* in sugar beet is believed to be polygenic, involving at least two loci, with two or three alleles, and an unknown number of modifying genes that contribute to resistance (Hecker and Ruppel 1975). Lein et al. (2008) found that there are at least three major quantitative trait loci (QTLs) located on sugar beet chromosomes 4, 5, and 7 which corroborates earlier findings that RCRR resistance is due to many minor genes. Other mapping efforts support an even more complex picture of resistance (Janssen et al. 2009). Identification of novel sources of germplasm with broad-spectrum tolerance to the highly diverse *R. solani* populations would greatly benefit the sugar beet community, but screening can be tedious and long within traditional breeding programs.

Quantitative resistance mechanisms generally involve both metabolites and proteins. In order to characterize and better understand these mechanisms, functional genomics including transcriptome, proteome, and metabolome analyses, can reveal the potential biochemical pathways that serve as a basis of resistance (Fiehn et al. 2000;Heuberger et al. 2013). While functional genomics has traditionally been approached as a unidirectional flow of information from gene to transcript to protein, we now know that enzymes can affect metabolic pathways leading to phenotypic changes in the host and that this traditional “linear” thinking is no longer true (Hollywood et al. 2006). In reality these cellular processes are intimately networked and contain many overlapping feedback-loops with protein complexes interacting with many

metabolites in response to pathogen attack. The metabolome is the quantitative complement of all the low-molecular weight molecules present in the plant cell and metabolic studies are believed to be complementary to transcriptomic and proteomic studies, to further the characterization of the defense response (Heuberger et al. 2014;Hollywood et al. 2006).

Until recently, few genomic resources were available in sugar beet to understand the genetic basis for disease resistance; however, the complete genome of sugar beet is now available (Dohm et al. 2014). Proteomics has already been used to characterize protein changes that occur in sugar beet during infection by the soilborne fungi *Fusarium oxysporum* and the fungi-transmitted virus *Beet necrotic yellow vein virus* (BNYVV) (Larson et al. 2008;Larson et al. 2007;Webb et al. 2014;Webb et al. 2015). In those studies it was determined that proteins are temporally regulated and proteins predicted to contribute to resistance in other plant species were also found during the sugar beet defense response (Larson et al. 2008;Larson et al. 2007;Webb et al. 2014;Webb et al. 2015). While many common protein families were up-regulated during infection, there were also a number of proteins unique to each specific pathosystem in question. This indicates that while sugar beet may utilize similar defense pathways for a wide range of pathogens, resistance to a particular pathogen, such as the necrotrophic *R. solani*, should be studied separately to identify novel mechanisms for disease control unique to each pathogen and to understand how each pathogen uniquely interacts with sugar beet to cause disease. While proteomic profiling is beginning to elucidate potential pathways that may be contributing to sugar beet defense against plant pathogens, protein identification can be cumbersome. This is primarily because of the lack of relevant sugar beet proteins in search databases and the lack of significant differences in protein separation and identification methodologies. Therefore other

methodologies to identify the downstream physiological pathways associated with sugar beet interactions with *R. solani* are warranted.

Metabolomics provides an analytical tool which enables the qualitative and quantitative profiling of metabolites in a biological system and serves as a link between genotypic and phenotypic responses (Fiehn et al. 2000;Heuberger et al. 2014). A better understanding of plant metabolism in response to different biotic stresses (including pathogen infections) facilitates a better understanding of plant physiology which is crucial for the development of future applications in plant breeding, biotechnology, and crop protection (Aliferis and Jabaji 2012a;Jung et al. 2015;Stark et al. 2015). Metabolomic profiling has been used previously in sugar beet to complement proteomic studies characterizing the response of carbohydrate metabolism and the TCA cycle to iron deficiency in the plant (Rellan-Alvarez et al. 2010). Likewise, Aliferis and Jabaji (2012b) have described the metabolome of potato in response to infection with *R. solani* AG 3. However, to date there have been no known reported studies on the effects of *R. solani* on the global metabolic network of sugar beet. Additionally, metabolomic approaches have been utilized in characterizing compounds found in *R. solani* that are directly important to the fungus during critical life stages as well as in characterizing the metabolites found in fungal exudates that may contain clues to secreted effectors (Aliferis and Jabaji 2010a;Aliferis and Jabaji 2010b) and for the resolution of *R. solani* isolates into anastomosis groups (Aliferis et al. 2013).

Here, we employed a nontargeted metabolomics approach to profile the sugar beet metabolome in order to characterize the biochemistry that underlies the complex phenotype of RCRR resistance during interactions with *R. solani* AG 2-2 IIIB.

2.0 Materials and Methods

2.1 Plant treatments:

Sugar beet germplasm FC709-2 (resistant; Panella 1999) and FC901 (aka FC901/C817; susceptible; Gaskill et al. 1967) were grown in a greenhouse with temperatures set for 22-26°C/16°C (day/night), 25-50% relative humidity, and an 18 h photoperiod until plants were 10 weeks after sowing. Artificial inoculum using hull-less barley (Bob's Red Mill, Milwaukie, OR) was either colonized with *R. solani* AG 2-2 IIIB isolate R-9 ("inoc") (Hecker and Ruppel 1975) or left uncolonized ("mock") using a modified protocol originally described by Pierson and Gaskill (1961). Ground inoculum or mock infected barley was used to inoculate four pots, each containing 3 plants, of each germplasm by placing ~0.6mL (about 0.50 - 0.55 g) of ground barley in the soil directly next to tap roots (about 1-2 cm deep) for each time point. Plants were arranged in a greenhouse in a split-split plot design (by germplasm, mock vs. infected) and maintained at 27°C/24°C (day/night temperatures), with an 18 h photoperiod. Soil was kept moist, but not sodden by daily watering. Plant tissue was collected from each pot at 0, 1, 3, 5, and 7 days after inoculation (dai) by harvesting the entire plant, washing the roots free of soil then separating the root and leaf at the crown (by removing ~1-2cm that is above the soil line). All root and leaf tissues (respectively) were pooled for all pots for each treatment (4 pots per treatment). Experiments were biologically replicated twice.

2.2 Metabolite extraction

For metabolite extraction, leaf and root tissue were extracted separately at each time point. Leaf tissue was immediately ground in liquid nitrogen (LN₂) and stored at -80°C, while root tissue was washed in distilled water to remove soil, cut into small slices, and then frozen at -80°C. Root tissue was lyophilized for at least 48 h, then ground to a fine powder using a

Cyclone Grinding Mill (Udy Corporation, Fort Collins, CO). Metabolites were extracted by placing 20 mg of ground lyophilized tissue into a 1.5mL microcentrifuge tube followed by 1 mL 80% methanol (v:v) and vortexing (Vortex Genie 2, Scientific Industries, Bohemia, NY) at room temperature for 2 h (Heuberger et al. 2013). After vortexing, samples were placed at -20°C for 20 minutes and then centrifuged (Eppendorf, Hauppauge, NY) at ~16,000 rpm for 20 minutes at 4°C. Metabolites were removed as the supernatant fraction after centrifugation and placed into a new 1.5mL microcentrifuge tube which was kept at -80°C until mass spectrometry could be performed.

2.3 Metabolomic analysis

For GC-MS analysis, 100 μ L of extract was dried using a speedvac, resuspended in 50 μ L of pyridine containing 50 mg/mL of methoxyamine hydrochloride, incubated at 60°C for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Next, 25 μ L of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) was added and samples were incubated at 60 °C for 30 min, centrifuged at 3000xg for 5 min, cooled to room temperature, and 80 μ L of the supernatant was transferred to a 150 μ L glass insert in a GC-MS autosampler vial. Metabolites were detected using a Trace GC Ultra coupled to a Thermo ISQ mass spectrometer (Thermo Scientific). Samples were injected in a 1:10 split ratio twice in discrete randomized blocks. Separation occurred using a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 μ m film thickness) with a 1.2 mL/min helium gas flow rate, and the program consisted of 80 °C for 30 sec, a ramp of 15 °C per min to 330 °C, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec after electron impact ionization. The ionization source was cleaned and retuned and the injection liner replaced between injection replicates.

For UHPLC-MS analysis metabolite extracts were injected onto a Waters Acquity UHPLC system (Waters Corporation, Milford, MA) and separated using a Waters Acquity UPLC T3 column (1.8 μm , 1.0 by 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min and followed by a linear gradient to 95% B in 12 minutes, then held at 95% B for 3 min, before being returned to starting conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 $\mu\text{L min}^{-1}$ flow rate. The column and samples were held at 50°C and 5°C, respectively. Column eluent was infused into a Waters Xevo G2 Q-TOF-MS (Waters Corporation, Milford, MA) with an electrospray source in positive mode, scanning 50–1200 m/z at 0.2 s per scan, alternating between MS (6 V collision energy) and MSE mode (15–30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temp at 150°C and nitrogen desolvation temperature at 350°C with a flow rate of 800 L h^{-1} . Each sample was injected in duplicate ($n=2$ injections) in two discrete blocks, with injection order completely randomized within each block. The signal intensity values were averaged before statistical analysis was performed.

2.4 Statistical analysis

All processing and statistical analyses were performed in R. MS files were converted to netCDF format and processed with XCMS for peak detection, retention time alignment, and feature grouping on both low and high collision energy channels. The feature based XCMS data were then subjected to RAMClustR (Broeckling et al. 2014). Spectra were searched against in-house, NISTv14, Metlin (Smith et al. 2005), and Massbank (Horai et al. 2010) databases for annotations. Metabolite isomers are inferred by highly similar idMS and idMS/MS spectra at multiple retention times, and glycerolipid positional isomers classified as previously described

(Heuberger et al. 2013). Analysis of variance was conducted on each compound using the aov function in R, and p-values were adjusted for false positives using the Bonferroni-Hochberg method in the p.adjust function in R (Benjamini and Hochberg 1995). PCA was conducted on mean-centered and Pareto variance-scaled data using the pcaMethods package in R.

3.0 Results

3.1 Effects of plant genotype on metabolome

Metabolite profiles by UHPLC-MS and GC-MS clearly distinguish the two germplasm tested in both the root and leaf tissue, and the magnitude of these differences was dependent on tissue type using tissue collected on the day of inoculation (0 dai) (Supplemental Table 1, Supplemental Table 2, Figure 1). GC-MS and UHPLC-MS data were analyzed using the unsupervised multivariate method, principle component analysis. The fitted principle components (PCs) were subjected to ANOVA to determine which PCs were responsive to genotype and/or infection status. For GC-MS analysis of root tissue, the best separation between germplasm was revealed using principal components (PC) 3 and 5, which collectively explained 11.5% of the Pareto scaled dataset wide variance. Analysis of the UHPLC-MS data from root tissue revealed germplasm sensitivity in PCs 1 and 2, which collectively explained 40% of the Pareto scaled dataset wide variances (Figure 1). A similar pattern was observed for leaf tissue, in which the PCs separating the two germplasm explained much more variation by UHPLC-MS (52%) than GC-MS (28%). These results indicate that the differences between germplasm were revealed more effectively using the UHPLC-MS platform (largely secondary metabolites) than GC-MS (largely primary metabolites).

Examination of the loadings plots for the PCs which separate the two germplasm at day zero revealed root UHPLC-MS compounds 4, 16, 28, 82, 172, 262, 353, 378, and 527 to

contribute disproportionately to the separation in PC1 (FDR corrected $p < 0.001$, outlier test on loadings values), while several of those same metabolites, compounds 6, 5, 56, 65, and 374, contribute disproportionately to PC2. Compounds 4, 5, and 6 contain spectral signatures consistent with secondary metabolite glycosides, and likely represent terpenoid glycosides of the betavulgaroside family. Compound 16 is consistent with the mass of codonocarpine, a polyamine phenolic conjugate. Compounds 28 and 65 represent tryptophan and tyrosine, respectively. Compound 172 is consistent with betaine, 262 represents a disaccharide signal, and 353 possesses a strong signal at 439.3577 consistent with an oleanolic acid $[M+H-H_2O]^+$ signal. Compound 378 is consistent with glutamine. When treated in a univariate manner, the most significant UHPLC-MS signals at baseline included compounds annotated as caffeic, ferulic, and coumaric acid conjugates, including glycosides, polyamines, and unknown conjugates. In total, 199 compounds were found to significantly differ in the UHPLC-MS root dataset, using either PCA loadings p-values (only from PCs determined to be significantly impacted by the experimental design) or by analysis of variance (Supplemental Table 1).

When analyzed by GC-MS, the signals contributing to the separation of the two germplasm include several amino acids in PC5 (oxo-proline, pyroglutamate, valine, serine, and aspartic acid) in addition to several unannotated signals. In total, 163 compounds were found to significantly differ in the GC-MS root dataset, using PCA loadings p-values or analysis of variance (Supplemental Table 2). Collectively, these results indicate a difference in both primary and secondary metabolism between the two germplasm, with phenolic acids and their conjugates demonstrating a role in distinguishing the two root basal metabolomes.

In leaf tissue, UHPLC-MS data revealed 319 significantly altered compounds, including compounds such as phenolic acids and conjugates, phloroglucinol, tyrosine, 5-

methylthioadenosine, monoacylglycerols, pantothenic acid, betagarin, and terpenoid-like compounds (Supplemental Table 3). These compounds represent ~20% of the leaf UHPLC-MS metabolome, demonstrating a dramatic difference between the two germplasm before challenge with the pathogen. In contrast, only 24 compounds differed between the two germplasm in the leaf GC-MS dataset (Supplemental Table 4). These compounds include several carbohydrates, in addition to phosphate, malate, and lactate, which were found as contributing significantly by PC loadings outlier analysis.

3.2 Genotype-dependent effects of *R. solani* on metabolome (7 dai)

To explore the influence of pathogen challenge on the metabolome, we examined samples from 1, 3, 5, and 7 dai using principal component analysis as well as an ANOVA model containing both germplasm, inoculation status, and the interaction between them. This ANOVA model was applied both to the principal component scores and the individual metabolites. We report metabolites as significantly responsive to inoculation if the ANOVA inoculation term and the interaction term was significant, either for metabolites or PCs. Based on this analysis no significant data were revealed in either germplasm in response to inoculation with *R. solani* at 1, 3, and 5 dai (data not shown).

For samples collected at 7 dai, PCA results (Figure 2) revealed that GC-MS was again less sensitive at detecting the differences in the metabolomes of FC709-2 and FC901 than UHPLC-MS. In fact, using GC-MS, none of the PC's were significant for either the root or leaf tissues nor were any significant for either the inoculation or the inoculation by genotype interactions. However, a clear response of the metabolome to inoculation was detected by UHPLC-MS, particularly for the FC709-2 germplasm from root tissue. In leaf tissue a response was also observed in PC5 ($r^2 = 0.066$) (r^2 is equal to 'variance explained') for FC901, and in PC7

for FC709-2 ($r^2 = 0.031$). These results indicate that the response to inoculation is apparent in UHPLC-MS data in both roots and leaves, that this response is strongest in FC901 roots (visible in PC1, $r^2=0.313$), and that the leaf response to the pathogen at this time point is higher in the *R. solani* susceptible germplasm (FC901), which separates in PC5 ($r^2 = 0.066$) while FC709-2 separates in PC7 ($r^2 = 0.031$).

There were 44 compounds in the UHPLC-MS root dataset which demonstrated statistically significant results by ANOVA or PCA (Table 1). One of these compounds represents a known phytoalexin, betavulgarin (Geigert et al. 1973; Johnson et al. 1976). The response for this metabolite to inoculation was dependent on the germplasm, with FC901 demonstrating a stronger response at 7 dai than FC709-2, however both germplasm responded with increased accumulation of this phytoalexin (Figure 3). The remaining 43 compounds represent lipids, four phenolic polyamine conjugates, terpenoid-like signals, 3-methoxytyramine, and currently unannotated signals. Several annotated compounds were of interest due to their putative roles in plant defense. Four tentatively identified polyamine conjugates, N1-caffeoyl-N10-feruloylspermidine, codonocarpine-like, codonocarpine-like.1, and codonocarpine-like.2 all show similar trends, in which the basal levels are much higher in FC709-2 than FC901 (~10 fold). In the resistant FC709-2 the levels of codonocarpine-like compounds all drop slightly upon infection (not all p-values were significant as they ranged from 0.004-0.063; Figure 4). FC901 has levels of these compounds that approach the limits of detection. Additionally the susceptible genotype (FC901) in this study responded to pathogen challenge with a reduced accumulation of 3-methoxytyramine as compared to the resistant variety (p-value=0.04; Figure 5).

In leaf tissue, contrary to root tissue, both germplasm responded to inoculation, but responded in a qualitatively and quantitatively different manner. The FC901 response is contained largely in PC5, for which 25 metabolites contribute disproportionately (Table 2). These metabolites include a dopamine-like compound, betavulgaroside, betaine, a flavanone conjugate, betagarin, a methyl-dopamine-like compound, docosenamide, and a disaccharide signal. Nine of these compounds also significantly contributed to the separation in PC7, which separates the inoculated vs mock samples for FC709-2 (Table 2). One of the few compounds in this list that responded similarly between germplasm was tentatively identified as betavulgaroside. This compound tended to increase following inoculation, but the variation was high and these results were not statistically significant by ANOVA.

The response to inoculation for the FC709-2 genotype included a capsazepine-like compound, docosenamide, betaine, betavulgaroside, a 3,5-dimethoxy-4-hydroxycinnamic acid, trigonelline, and a digalactosyl diacylglyceride, among several unannotated compounds. Only two of these compounds also demonstrated significant responses to inoculation via ANOVA, C28 and C68. These two spectra contain signals indicative of a glycosidic structure, with apparent neutral losses of 162.05, consistent with the loss of a hexose. The molecular ion for compound 28 is consistent with a diacetylated, di-hexose conjugate of kaempferol. The two molecular ions are separated by 14.01 Da (679.187 for C28, 693.202 for C68), and C68 elutes ~40 seconds later, consistent with an addition of a methylene group for C68 vs C28. These compounds likely represent two compounds of the same class, as indicated by the spectral similarity as well as the conserved response. Each compound is found at reduced levels in the susceptible genotype, and slightly increased levels in the resistant genotype following inoculation.

4.0 Discussion

Using a nontargeted metabolomics approach we have characterized the plant metabolic factors that are associated with plant defense in sugar beet (from both leaf and root tissues) during early susceptible and resistant interactions with *R. solani*. Using this approach we identified and annotated approximately 143 compounds of which there was a clear distinction between tissue type, germplasm, and response to inoculation with the pathogen. UHPLC-MS was more effective than GC-MS in identifying compounds during this study therefore more secondary metabolites, including several phytoalexins, terpenes, and alkaloids, were identified than primary metabolites. UHPLC-MS is an analytical platform that is more compatible with measurement of secondary metabolites, which may explain why we saw stronger responses to infection in UHPLC-MS than GC-MS datasets. In this study, we also saw significant basal differences (at time 0) in the two germplasm that were tested even in the absence of pathogen inoculation, but by 7 dai, there were even further separation of the metabolomes associated with each germplasm in response to *R. solani* infection. Generally, the resistant germplasm had higher basal levels of several metabolites that included codonocarpine, tryptophan, betaine, oleanolic acid, and glutamine whereas the susceptible genotype generally had higher basal levels of betavulgarosides. Since such a clear difference in germplasm was apparent even in the absence of pathogen infection, future work to fully characterize the basal differences in germplasm using lines with more similar genetic backgrounds should be utilized to determine whether differences functionally contribute to resistance. Once functional metabolites associated with resistance (or at least highly reliable metabolic markers) are identified, targeted metabolomics could be used to pre-screen germplasm prior to field testing in breeding programs.

R. solani is considered a necrotrophic root pathogen that has a different ecology, epidemiology, life cycle, and infection process than foliar pathogens (Okubara and Paulitz 2005). While the molecular aspects of the interaction of foliar pathogens with their hosts are fairly well described, much less is understood about how root pathogens, such as *R. solani*, interact with the host or their mechanisms for resistance. Only a small number of defense pathways reported for foliar pathogens have been described in roots (Okubara and Paulitz 2005) indicating a critical need for further research. In sugar beet, it has been shown that different genes are expressed in different plant organs (i.e. root, leaves, and inflorescence) and that some genes are preferentially expressed in roots (Bellin et al. 2002). This suggests that it is crucial to not only assess the plant defense responses at several time points during infection by soil-borne pathogens, but also to determine whether responses are expressed in tissues other than roots and distant from the site of pathogen infection. Our data indicated that sugar beet potentially utilizes specific pathways for defense against *R. solani*, and that these responses were differentially found in root and leaf tissues. More specifically, our data indicated that a metabolic phenotype can be seen in leaf tissue following inoculation by a root pathogen suggesting that a complete understanding of sugar beet responses to a root pathogen should include analysis of the metabolome from multiple tissue types. This also suggests that further exploration of metabolic markers to screen for disease resistance may enable sampling of leaf tissue (which is much easier to sample) to reveal disease status of the root.

During these experiments we focused on early interactions during the sugar beet-*R. solani* pathosystem (0, 1, 3, 5, and 7 dai). We saw little influence of inoculation with *R. solani* at the earliest time points, but did see a slight change of metabolite profiles by the last day of the study (7 dai). This may be due to the reported life cycle during the *R. solani* infection process where

the pathogen must 1) first grow towards the plant, 2) grow over the plant, 3) attach to the plant and 4) produce the necessary infection structures which are then used to 5) penetrate the host and 6) finally colonize host tissues. The time and extent of this process depends on the specific isolate of *R. solani*, as well as the host that it is infecting, and therefore full colonization of the cortex has been reported to take up to 3-4 days after infection was initiated (Sneh et al. 1996).

We found several metabolites that were differentially induced in root tissues to be of interest for more specific analysis in future experiments. N1-caffeoyl-N10-feruloylspermidine and codonocarpine both had higher basal levels in the roots of the resistant germplasm, however these levels dropped after inoculation with *R. solani* by 7 dai. Codonocarpine (Doskotch et al. 1971) and N1-caffeoyl-N10-feruloylspermidine are polyamide alkaloids and have had described roles in plant defense against both abiotic and biotic stresses as well as in plant development (Martin-Tanguy 1985; Onkokesung et al. 2012). These results suggest a role of the polyamide alkaloid biosynthetic pathway in resistance against *R. solani*. However, because polyamines have multiple and complex roles it is difficult to disentangle their individual activities from one another and therefore their specific roles in plants remain largely unknown (Bitrian et al. 2012; Onkokesung et al. 2012). Aliferis and Jabaji (2012b) studied the defense response of potato (*Solanum tuberosum* L.) during interactions with *R. solani* AG 3 where they also found a relationship with the biosynthesis of alkaloids, suggesting that this may be a conserved defense response to *R. solani* or other necrotrophic fungal pathogens. Future studies to better describe the anti-fungal activity of these metabolites and other regulatory factors in the same biosynthetic pathway may better describe how sugar beet responds to invasion to *R. solani*. Two additional compounds that were found in the resistant germplasm and had increasing abundance after infection with *R. solani*, were annotated as “oleanic acid-like” compounds. Oleanic acids are

triterpenoid glycosides that belong to a class of compounds called saponins (Sparg et al. 2004). Saponins (including oleanic acids) have been reported to have a wide range of biological activities which also include antifungal and insecticidal activities (Sparg et al. 2004). There have been reports of direct roles of some triterpenoid glycosides in plant defenses against fungal plant pathogens (Bednarek and Osbourn 2009) and therefore is an interesting compound to follow-up with in future experiments during resistant sugar beet/*R. solani* interactions.

The behavior of other compounds found primarily in the susceptible genotype were also quite interesting. For example, 3-methoxytyramine has been associated with phenolic accumulations at infection sites in association with plant cell wall thickening during interactions with fungal pathogens (McLusky et al. 1999). In our study, the resistant genotype (FC709-2) had generally higher levels of 3-methoxytyramine which would support a potential role in host defense. Phytoalexins are low molecular weight, antimicrobial compounds that are formed in response to biotic agents and appear to be involved in disease resistance (Keen 1986; Paxton 1981). Here, we found one reported phytoalexin, betavulgarin (Geigert et al. 1973; Johnson et al. 1976) that was present in both germplasm but more highly induced in the susceptible germplasm. Betavulgarin is reported to have antifungal activities in sugar beet, primarily to *Cercospora beticola* (Johnson et al. 1976; Takahashi, Sasaki, and Ito 1987). While resistant reactions are often seen in plants that accumulate phytoalexins, there are some instances in which susceptibility has been associated with higher levels of the phytoalexin as seen in our case. For example, while pea is known to accumulate the phytoalexin pisatin, and the fungal pathogen *Aphanomyces euteiches* was sensitive to pisatin, it was reported that the fungus was still able to colonize pea plants despite the presence of pisatin. It was then shown that other compounds (i.e. lipids) decrease the sensitivity of the fungus to pisatin and that this allows some fungi, such as *A.*

euteiches, to escape the effects of some phytoalexins in some way (Keen 1986). It is unknown how betavulgarin accumulation is affected by *R. solani* infection, nor how these responses differ from previously described reactions during sugar beet infection with other fungal pathogens, but this opens an interesting avenue to further explore sugar beet defense responses to improve our understanding of how sugar beet responds to multiple fungal pathogens.

5.0 Conclusions

In conclusion, utilizing a nontargeted metabolomics profiling approach we determined that metabolite abundance in sugar beet is significantly altered based on the germplasm in question and during the presence of *R. solani*. Several biochemical pathways appeared to be involved during susceptible and resistant interactions with *R. solani*, and indicated a complex role of primary and secondary metabolites in sugar beet during fungal interactions. Several important biosynthetic pathways have been identified for future study and we now have a greater understanding of how *R. solani* interacts with sugar beet.

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8.0 References

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Table 1: Significant metabolites found by UHPLC-MS in root dataset at 7dai during *R. solani* interactions with resistant (FC709-2) and susceptible (FC901) sugar beet germplasm.

Compound ^a	Annotation	Var ^b	Inoc ^c	Var:Inoc ^d	PC6 ^e
		ANOVA	ANOVA	ANOVA	
C4	oleanic acid-like	0.0005	0.6595	0.2469	0.0001
C5	oleanic acid-like.1	0.1133	0.2352	0.5253	0.0000
C16	codonocarpine-like	0.0000	0.0635	0.2405	0.0000
C17	lysoPC(18:2)	0.7759	0.7384	0.2599	0.0137
C24	docosenamide	0.8763	0.5716	0.9890	0.0001
C27	MG(18:2).1	0.6615	0.9301	0.3124	0.0000
C29	codonocarpine-like.1	0.0000	0.0017	0.0046	0.9564
C44	peptide.3	0.0000	0.0440	0.0482	0.9564
C54	C54	0.9418	0.0092	0.4459	0.9564
C63	betavulgarin	0.0088	0.0010	0.0597	0.2816
C95	C95	0.0000	0.0199	0.2673	0.9564
C115	MG(18:3).1	0.0137	0.0735	0.0067	0.9564

C122	C122	0.4945	0.0443	0.7813	0.9564
C135	DGDG(34:2)	0.5302	0.8465	0.8543	0.0002
C136	DGDG(32:2)	0.9301	0.7368	0.9015	0.0036
C137	DGDG(34:1)	0.8124	0.8396	0.2028	0.0046
C154	C154	0.0082	0.0213	0.2824	0.9564
C167	C167	0.0000	0.0210	0.0787	0.9564
C182	C182	0.0001	0.1963	0.0182	0.9564
C202	C202	0.8975	0.5716	0.9868	0.0001
C226	3-methoxytyramine	0.1260	0.5724	0.4738	0.0130
	N1-caffeoyl-N10-				
C233	feruloylspermidine	0.0000	0.0010	0.0257	0.9564
C236	codonocarpine-like.2	0.0000	0.0106	0.0144	0.9564
C262	disaccharide	0.7045	0.1005	0.8785	0.0000
C317	linoleoyl ethanolamide	0.0000	0.3057	0.0001	0.9564
C328	C328	0.3620	0.0250	0.7301	0.9564
C333	C333	0.0005	0.0099	0.2265	0.9564
C353	calenduloside E-like	0.9393	0.3477	0.2544	0.0000
C358	MG(16:0).1	0.9319	0.0352	0.4330	0.9564
C374	C374	0.8465	0.4461	0.2382	0.0000
C392	C392	0.9125	0.0486	0.1199	0.9564
C462	C462	0.4154	0.0257	0.3566	0.9564
C470	C470	0.0005	0.0305	0.1889	0.9564
C482	C482	0.0578	0.8396	0.0440	0.9564

C489	C489	0.0000	0.0420	0.0178	0.9564
C510	C510	0.0208	0.0003	0.0282	0.9564
C527	C527	0.0140	0.4975	0.4185	0.0000
C797	C797	0.0735	0.0349	0.9868	0.9564
C816	C816	0.0040	0.0316	0.1923	0.9564
C853	C853	0.0001	0.2180	0.0172	0.9564
C860	C860	0.0000	0.0472	0.2444	0.9564
C897	C897	0.5290	0.0493	0.0520	0.9564
C972	C972	0.0008	0.0349	0.6419	0.9564
C978	C978	0.0426	0.0486	0.1438	0.0000

^ap-values for each metabolite using either analysis of variance (ANOVA) or principal component analysis (PCA) for each metabolite are shown.

^bVar = Sugar beet germplasm; resistant (FC709-2) or susceptible (FC901)

^cInoc=Inoculated treatment; with *Rhizoctonia solani* AG 2-2 IIIB or mock

^dVar:Inoc= Variety by inoculated comparisons for all treatments.

^e p-values based on an outlier test on the loadings value for PC6. Outliers in the loadings distribution can be interpreted as contributing disproportionately to the separation imparted by PC6.

Table 2: Significant metabolites found by UHPLC-MS in leaf dataset at 7 dai during *R. solani* interactions with resistant (FC709-2) and susceptible (FC901) sugar beet germplasm.

Compound ^a	Annotation	Var ^b	Inoc ^c	Var:Inoc ^d	PC5	PC7
		ANOVA	ANOVA	ANOVA		
C10	13-docosenamide, (Z)	0.7791	0.7145	0.8557	0.0158	0.0000
C21	C21	0.5107	0.8260	0.3120	1.0000	0.0000
C22	C22	0.8580	0.3896	0.9860	0.0006	0.0179
C28	C28	0.0000	0.0130	0.0119	1.0000	0.0000
C39	C39	0.1058	0.8813	0.8744	0.0007	0.0071
C44	C44	0.8733	0.5647	0.9832	0.0230	0.0254
C50	DGDG(36:6)	0.9796	0.9302	0.9852	1.0000	0.0452
C52	C52	0.9005	0.6279	0.7620	0.0027	0.9249
C60	C60	0.8638	0.9229	0.9609	0.0000	0.2399
C68	C68	0.0002	0.9023	0.0181	1.0000	0.0000
C78	C78	0.9537	0.6369	0.9537	0.0000	0.9249
C83	C83	0.5437	0.9637	0.9608	0.9154	0.0103
C86	C86	0.8870	0.9234	0.9715	0.0000	0.5255
C126	C126	0.8760	0.4947	0.7803	0.0000	0.9249
C136	dopamine	0.3467	0.9255	0.9243	0.0000	0.9249
C143	C143	0.2578	0.9545	0.9206	1.0000	0.0001
C161	C161	0.7808	0.7608	0.7155	0.0921	0.0045
C162	betaine MS spectrum	0.9302	0.9947	0.8607	0.0000	0.0024

C163	C163	0.9223	0.8549	0.9202	1.0000	0.0414
C170	C170	0.0254	0.8371	0.5791	0.0046	0.0000
C179	betagarin	0.6333	0.7305	0.9913	0.0000	0.5047
C180	C180	0.7608	0.9947	0.8719	1.0000	0.0047
C194	C194	0.9138	0.9913	0.9738	0.0263	0.0485
C230	C230	0.6869	0.7206	0.9637	0.0084	0.9249
C233	C233	0.7620	0.1635	0.3085	1.0000	0.0041
Dihydroxy-6,7-methylenedioxyflavanone						
C237	glucoside	0.5079	0.7145	0.6643	0.0000	0.9249
C274	capsazepine-like	0.8723	0.9008	0.8229	1.0000	0.0000
C305	methyldopa-like	0.2267	0.9023	0.9610	0.0029	0.1055
C308	C308	0.3019	0.5050	0.7925	0.0000	0.9249
3,5-dimethoxy-4-hydroxycinnamic acid.1						
C333	hydroxycinnamic acid.1	0.0314	0.7223	0.7988	1.0000	0.0320
C372	C372	0.5381	0.7145	0.7925	1.0000	0.0014
C410	C410	0.9008	0.5657	0.9435	0.0263	0.9249
C422	C422	0.9917	0.7410	0.9860	1.0000	0.0254
C493	C493	0.1961	0.5791	0.5160	1.0000	0.0010
C552	disaccharide	0.2133	0.9886	0.8580	0.0430	0.5291
C555	C555	0.0196	0.4106	0.8874	0.2497	0.0045
C620	C620	0.8076	0.7321	0.6897	0.0000	0.9249
C679	C679	0.0000	0.0423	0.0487	1.0000	0.9249

C706	C706	0.0019	0.2286	0.0206	1.0000	0.9249
C729	C729	0.5107	0.1093	0.5268	0.3723	0.0000
C828	trigonelline	0.9005	0.8549	0.6256	1.0000	0.0369
C959	C959	0.2352	0.8836	0.8371	1.0000	0.0191
C984	C984	0.7608	0.8836	0.9998	1.0000	0.0222
C1002	C1002	0.4561	0.5657	0.9138	0.0000	0.0537
C1009	C1009	0.9533	0.9320	0.1046	1.0000	0.0127
C1161	C1161	0.7188	0.8472	0.6256	1.0000	0.0000
C1187	C1187	0.6577	0.4631	0.4253	0.0000	0.0369
C1189	C1189	0.9302	0.8928	0.7859	0.4631	0.0000
C1251	C1251	0.7298	0.1126	0.4487	0.0000	0.9249
C1257	C1257	0.0002	0.0314	0.0248	1.0000	0.9249
C1422	C1422	0.2741	0.0477	0.8428	1.0000	0.5255
C1543	C1543	0.0167	0.9842	0.6432	1.0000	0.0078
C1567	betavulgaroside	0.9622	0.6829	0.9202	0.0000	0.0165

^ap-values for each metabolite using either analysis of variance (ANOVA) or principal component analysis (PCA) for each metabolite are shown.

^bVar = Sugar beet germplasm; resistant (FC709-2) or susceptible (FC901)

^cInoc = Inoculated treatment; with *Rhizoctonia solani* AG 2-2 IIIB or mock

^dVar:Inoc = Variety by inoculated comparisons for all treatments.

Figure 1. Principal component analysis (PCA) of the root and leaf datasets at 0 dai by GC-MS and UHPLC-MS of sugar beet germplasm FC709-2 (resistant) and FC901 (susceptible) during interactions with *R. solani* AG 2-2 IIIB. R^2 is equal to ‘variance explained’ for each principal component.

Figure 2. Principal component analysis (PCA) of the root and leaf datasets at 7 dai by GC-MS and UHPLC-MS of sugar beet germplasm FC709-2 (resistant) and FC901 (susceptible) during interactions with *R. solani* AG 2-2 IIIB or mock treated. R^2 is equal to ‘variance explained’ for each principal component.

Figure 3. Box plot data of the annotated metabolite betavulgarin at 7 dai during interactions of sugar beet germplasm FC709-2 (resistant) and FC901 (susceptible) with *R. solani* AG 2-2 IIB (Inoc) or mock treatments. Significance was tested at $p\text{-value} < 0.05$.

Figure 4. Box plot data of three annotated codonocarpine-like metabolites at 7 dai during interactions of sugar beet germplasm FC709-2 (resistant) and FC901 (susceptible) during

interactions with *R. solani* AG 2-2 IIB (Inoc) or mock treatments. Significance was tested at p-value<0.05.

Figure 5. Box plot data of the annotated metabolite 3-methoxytyramine at 7 dai during interactions of sugar beet germplasm FC709-2 (resistant) and FC901 (susceptible) during interactions with *R. solani* AG 2-2 IIB (Inoc) or mock treatments. Significance was tested at p-value<0.05.