University of São Paulo
"Luiz de Queiroz" College of Agriculture

Identification of candidate resistance metabolites to *Leifsonia xyli* subsp. *xyli* in sugarcane through metabolomic profiling

Fernanda Raquel Rezende de Castro Moretti

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

Piracicaba
2017

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PUBLICATIONS

1. Oliveira TS, Dallagnol LJ, Araujo-Filho JV, **Castro-Moretti FR**, Camargo LEA. First report of powdery mildew caused by *Erysiphe platani* on *Platanus* × *acerifolia* in Rio Grande do Sul, Brazil. Plant Disease, vol. 99, p. 157, 2015.
2. Araujo-Filho JV, **Castro-Moretti FR**, Bonfim-Junior MF. *Pratylenchus brachyurus* (Nematoda: Pratylenchidae) in Guariroba in the state of Goiás, Brazil. Helminthologia, vol. 51, p. 352-354, 2014.
3. Dallagnol LJ, Ferreira LV, Araujo-Filho JV, **Castro-Moretti FR**, Camargo LEA. Gray mold caused by *Botryotinia fuckeliana* on edible pods of pea in Brazil. Plant Disease, 2013.
4. Dallagnol LJ, **Castro FR**, Garcia EN, Camargo LEA. First report of powdery mildew caused by *Golovinomyces* sp. on *Plantago australis* in Brazil. Plant Disease, v. 97, p. 421-421, 2013.
5. Dallagnol LJ, **Castro FR**, Frare G, Camargo LEA. First report of powdery mildew on flamboyant tree caused by *Erysiphe quercicola* in Brazil. Plant Disease, v. 96, p. 589, 2012.

FIELDS OF STUDY

Plant Pathology, Molecular Biology

In quoting others, we cite ourselves

Julio Cortázar

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RESUMO

Identificação de metabólitos candidatos em cana-de-açúcar para resistência à *Leifsonia xyli* subsp. x*yli* através da análise de perfil metabólico

O Raquitismo-da-soqueira (RSD) é uma grave doença que afeta todos os paises produtores de cana-de-açúcar. O principal sintoma do RSD é tamanho reduzido das plantas, observado apenas nas plantas-soca, o que pode resultar em perdas de biomassa em até 80%, dependendo das condições climáticas. A doença é causada por *Leifsonia xyli* subsp. *xyli* (Lxx), uma bactéria gram-positiva e fastidiosa, descrita até o presente momento como hospedeira natural apenas da cana-de-açúcar, colonizando principalmente os vasos do xilema. Todavia, a detecção precoce deste patógeno é o principal desafio para prevenção do RSD. O melhoramento genético para resistência ao RSD, apesar de viável, não é uma medida de controle adotada na prática. Como existe diferenças entre as variedades de cana em relação ao grau de colonização por Lxx e as perdas estão diretamente relacionadas ao título bacteriano, uma estratégia de melhoramento promissora é a seleção de genótipos que apresentam resistência à multiplicação bacteriana. Portanto, o conhecimento das respostas da cana-de-açúcar ao RSD em termos “ômicos” é um passo inicial primordial para a identificação de alvos-chave para melhorar variedades resistentes. O objetivo geral deste estudo foi determinar os perfis metabólicos de duas variedade, uma suscetível (CB49-260) e uma resistente (SP80-3280) inoculada ou não com Lxx e comparar os resultados com dados já existentes de proteômica e transcriptômica para definir um núcleo de alvos (proteínas, genes e metabólitos) que possam ser testados como marcadores de resistência em uma coleção de cana-de-açúcar. Os títulos bacterianos foram quantificados por PCR em tempo real (qPCR). Os perfis metabólicos foram elaborados a partir de folhas e fluído xilemático coletados aos 30 e 120 dias após inoculação (DAI). A análise não-direcionada foi realizada por cromatografia gasosa acoplada à espectrometria de massas (GC-MS), usando folhas e extratos coletados aos 120 DAI. Já a análise direcionada foi efetivada via cromatografia líquida acoplada à espectrometria de massas em tandem (LC-MS/MS), em ambos tecidos e tempos de coleta. Para validar os resultados de metabolômica, um grupo de metabólitos destacado nas análises de metabolômica foi escolhido para testes *in vitro* e por fim detectar alterações no crescimento de Lxx. O resultado do qPCR confirmou a suscetibilidade da CB49-260, pois esta continha títulos superiores à SP80-3280. A análise global revelou que ambos variedades e tecidos possuem perfis metabólicos distintos, porém essas diferenças foram mais quantitativas que qualitativas. A análise direcionada identificou mais aminoácidos, açúcares, ácidos organicos e compostos fosforilados no genótipo suscetível não-inoculado, enquanto que o resistente apresentou maior abundância de compostos fenólicos. Também foi demonstrado que a inoculação com Lxx resultou em maior quantidade de aminoácidos, ácidos orgânicos, compostos fosforilados e fenólicos. Ademais, um aminoácido essencial à sobrevivência de Lxx foi relacionado à inoculação de ambas variedades, assim como um composto fenólico relacionado a defesa de plantas. O teste *in vitro* mostrou que, apesar de alguns compostos causarem inibição, é necessário aprimorar a metodologia utilizada para confirmar os resultados obtidos.

Palavras-chave: Bacteriose; Biomarcador; Metabolômica; Defesa de plantas; Raquitismo-da-soqueira

ABSTRACT

Identification of candidate resistance metabolites to *Leifsonia xyli* subsp. *xyli* in sugarcane through metabolomic profiling

Ratoon stunting disease (RSD) is a serious disease that affects all sugarcane producing countries. The major symptom of RSD is plant growth reduction, which is only seen in ratoon plants, causing up to 80% biomass reduction depending on environmental conditions. The disease is due to *Leifsonia xyli* subsp. *xyli* (Lxx), a gram-positive and nutritionally fastidious bacterium that so far has been found to specifically colonize the xylem vessels of sugarcane. However, the successful early detection of this pathogen is currently the main challenge for RSD prevention. Breeding for resistance to RSD, although not in practice, is a viable control measure. Since sugarcane varieties differ in relation to their degree of colonization by Lxx and losses are directly related to population densities of the pathogen in the plant, a promising breeding strategy would be to select for genotypes that are resistant to bacterial multiplication. Thus, knowledge on the responses of sugarcane to RSD at the “omics” level is an essential starting step to identify key metabolic targets for breeding resistant varieties. The overall goal of this study is to determine the metabolic profiles of a susceptible (CB49-260) and resistant (SP80-3280) variety inoculated or not with Lxx and to compare the results with existing proteomic and transcriptomic data to define a core of targets (proteins, genes, and metabolites) that can be tested as markers of resistance in a collection of sugarcane varieties. Bacterial titers were quantified by Real-Time PCR (qPCR). The metabolites were profiled from the leaves and from the xylem saps collected at 30 and 120 days after inoculation (DAI). Untargeted analysis were performed with Gas Chromatography - Mass Spectrometry (GC-MS) and were carried out on leaves and sap from 120 DAI. Targeted analysis was executed with Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) on both tissues at both timepoints. To validate metabolomics results, a set of metabolites was chosen to be tested *in vitro*, in order to detect growth alterations caused to Lxx. qPCR confirmed the susceptibility of CB49-260 as it had higher titers than SP80-3280. Global analysis revealed that both varieties and tissues have different metabolic profiles but that those differences are more quantitative than qualitative. The targeted approach identified more amino acids, sugars, organic acids and phosphorylated compounds in the non-inoculated susceptible genotype, while the resistant one had higher abundance of phenolics. It was also shown that inoculation with Lxx results in more relative abundance of amino acids, organic acids, phosphorylated compounds and phenolics. Furthermore, a key amino acid for Lxx survival was related to inoculation on both varieties, as well as a known phenolic compound related to plant defense. Distinguished phenolics resulting from the targeted analysis were selected to evaluate their effect on Lxx growth *in vitro*. Although some compounds caused inhibition, further optimization of the methodology is needed to confirm these results.

Keywords: Bacterial pathogen; Biomarker; Metabolomics; Plant defense; Ratoon stunting disease

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1. INTRODUCTION

1.1. Sugarcane – food and bioenergy crop

The importance of sugarcane to the Brazilian economy goes back to the 16th century, when Portuguese settlers began to grow sugarcane in Brazil and traded sugar in Europe in partnership with Dutch explorers. Today it represents a major crop as more than 13% of the country's cropped area is allocated to its cultivation. This year alone, the harvested area is estimated to be more than 9.1 million hectares (CONAB 2017). Ethanol started to be used as a biofuel in Brazil in the first decades of the 20th century, and then in the 1970's it was established as an alternative fuel throughout the country (INT 2017). As such, sugarcane is a dual-purpose crop: it produces sugar for human consumption and ethanol as a bioenergy source.

Brazil is responsible for more than 30% and 25% of the world's sugarcane and sugar production, respectively (UNICA 2017). More than 100 countries import Brazilian sugar, which accounts for 2/3 of the national production and represented over 10,436 million US dollars in exports in 2016. When it comes to ethanol, the country´s production exceeded 30 million cubic meters (m^3) in 2016, ranking it as the second world ethanol producer and consumer after the USA (MAPA 2017).

Sugarcane is a semi-perennial crop, vegetatively propagated through stalk cuttings (setts) containing lateral buds. As such, in the first year, a mother cane arises from the sett followed by tillering at a later stage, when lateral shoots emerge, and form multiple stalk around the initial mother one. After growth and maturation, canes are harvested annually and new plants regenerate in the stubble giving rise to ratoons (Figure 1). In this way, a single planting can be harvested up to 8 times before the cane field is replanted.

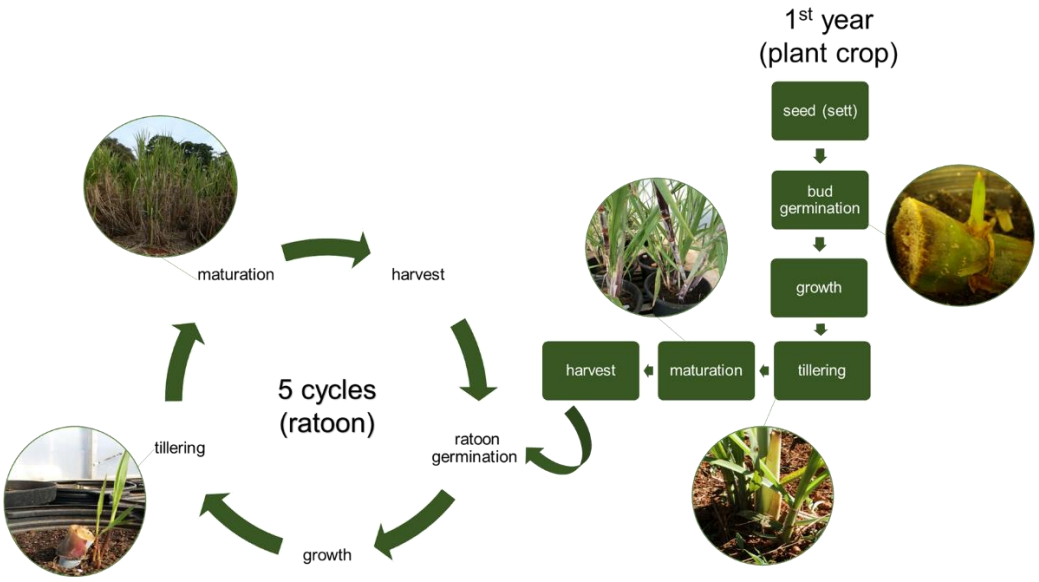


Figure 1. Sugarcane life cycle

The first sugarcane varieties resulting from breeding were introduced by the end of the 19th century, when Dutch growers began crossing wild varieties (Buckeridge et al. 2012). Higher sugar content, biomass increase for ethanol production, drought stress tolerance and resistance against insects and pathogens are desirable traits for modern sugarcane varieties. However, the complex genome of this grass represents a challenge to this and, in practice, breeding programs have focused on improving sugarcane content alone (Dal-Bianco et al. 2012). As such, new approaches must be developed to select for other traits and to speed up the release of new varieties which normally takes up to 12 years to be accomplished (Gazaffi et al. 2014).

Sugarcane pests and diseases cause yield losses and reduce sucrose content and quality. Biotic disorders that affect sugarcane are induced by diverse agents, which can be of fungal (e.g. smut - *Sporisorium scitamineum and* orange rust – *Puccinia Kuehnii;* Marques et al. 2016; Zhao et al. 2011); viral (e.g. sugarcane yellow leaf virus; Debibakas et al. 2014) or bacterial nature (e.g. ratoon stunting disease – *Leifsonia xyli* subsp. *xyli* and sugarcane leaf scald – *Xanthomonas albilineans*; Comstock 2002; Pieretti et al. 2015). To date, the best measure to avoid losses caused by these diseases is to use healthy planting material (Ogden-Brown 2015) obtained by heat treatment or by regenerating plantlets *in vitro* usually through apical meristem culturing. Heat treatment consists of immersing the setts in hot water for 30 min at 52ºC or for 2-3 h at 50ºC (Damann, Jr. 1983). Even though this practice reduces bacterial titers, it

does not eliminate the bacteria. Thus, even a small amount of infected planting material can act as a source of inoculum and spread to healthy plants in the field through the blades of harvesting machines. It is therefore essential to develop fast tools for detecting and diagnosing sugarcane pathogens.

1.2. Ratoon stunting disease and *Leifsonia xyli* subsp*. xyli*

Ratoon stunting disease (RSD) is an important disease affecting all sugarcane producing countries (Li et al. 2014). This disease was firstly described in Australia in 1944. However, its etiology was only established in 1980 (Teakle et al. 1973; Davis et al. 1980). Croft et al. (2002) estimated losses to RSD to be greater than 6 million U.S. dollars; Australia alone is estimated to lose more than 11 million dollars annually due to this disease (Fegan et al. 1998). In China, RSD incidences under natural conditions is estimated to vary between 61 and 85 % (Fu et al. 2016). In the major sugar and ethanol producer state of Brazil (São Paulo) incidences in commercial fields have been reported to be as high as 80% (Urashima and Marchetti 2013), accounting for estimated annual losses greater than US$ 1 million (Urashima et al. 2017). As the methods used to detect Lxx in commercial fields are not reliable, it is highly probable that losses are greatly underestimated even on present days (Young 2016b).

The major symptoms of RSD are shorter internodes and thinner stalks that develop only in ratoon plants due to an increase in bacterial titers over the harvestings, leading to a significant reduction in biomass over the years. Diagnosing RSD based on symptoms is difficult, as these can be easily mistaken for symptoms of many environmental stresses that affect plant growth. For those reasons, diagnosis depends on time-demanding and expensive molecular techniques. Breeding of resistant varieties is an effective control method as it is not feasible to eradicate the bacterium from sugarcane fields. Nonetheless, the only control method of RSD used at a commercial scale is to subject propagative material to heat treatment in an attempt to eliminate the bacterium, but the efficacy of this method is variable and it also may affect bud germination (Young et al. 2012). Because the causal agent is present in sugarcane fluids, it is spread mechanically to healthy plants through harvesting blades and any other harvesting tool (Hoy et al. 1999). This makes the pathogen very difficult to control once it is established in the field as the number of infected plants tend to increase with the consecutive harvests. Genetic control of RSD using resistant varieties is a

promising strategy as sugarcane genotypes differ in relation to their resistance to bacterial growth in their tissues (Comstock et al. 1996; Grisham 1991; Davis et al. 1988a). Since losses due to RSD are related to bacterial densities (Davis et al. 1988b; McFarlane 2002), a breeding strategy would be to select for genotypes that restrict the multiplication of the bacterium. However, phenotypic selection based on inoculation trials is not feasible because quantification of Lxx *in planta* requires time-consuming serological or molecular methods. For instance, screening 250 thousand seedlings would be necessary to develop a single commercial variety just by selecting for higher sugar content alone and not considering other traits (Dal-Bianco et al. 2012). It was hypothesized that varieties more resistant to RSD have different anatomical features in the xylem vessels, with more branching in the nodal region (Davis et al. 1988a; Teakle et al. 1975). This feature results in a reduced Lxx concentration when compared to more susceptible plants that have less branching and thus allow for increased Lxx population. Defense response to pathogens can be constitutive or induced and is mostly performed by secondary metabolism (Bennett and Wallsgrove 1994). Preformed defense is constituted by accumulated metabolites that form natural non-host barriers to prevent or reduce pathogenesis (Lattanzio et al. 2006). In contrast, induced defense mechanisms are enhanced when the host recognizes the pathogen through resistance gene signaling and produces barriers to prevent the disease from spreading (Dixon 2001). Biochemical mechanisms involved in resistance have not yet been described for this pathosystem. Likewise, compounds that promote Lxx growth in sugarcane would result in greater susceptibility. The possibility of the involvement of a plant-derived constitutive bacterial growth factor as a mechanism of resistance is realistic and appealing. Indeed, an unknown xylem sap metabolite of maize, possibly a non-reducing sugar, was found to enhance the growth of the Lxx-closely related organism *Leifsonia xyli* subsp. *cynodontis*, an endophyte of grasses, including sugarcane (Haapalainen et al. 2000). Additional resistance mechanisms to Lxx must be studied to develop fast and diverse tools to select resistant genotypes.

Leifsonia xyli subsp. *xyli* is a gram-positive, coryneform bacterium measuring approximately 0.25 x 1.4 µM (Davis et al. 1984). It has no flagella and colonizes the xylem vessels and meristem cells (Brumbley et al. 2004). Recent studies using Lxx-GFP cells showed that this bacterium also colonizes the mesophyll and the bundle sheath cells surrounding the vascular system (Quecine et al. 2016). *In vitro* cultivation of Lxx is difficult and requires a complex growth medium due to its fastidious nature

(Davis et al. 1980). For these reasons, this pathogen was only described as the causal agent of RSD 40 years after the disease was detected. It was first classified as *Clavibacter xyli* subsp. *xyli*, and then reclassified as *Leifsonia xyli subsp. xyli* due to the biochemical composition of its cell wall, which contains D- and L- 2,4-diaminobutyric acid isomers in its peptidoglycan and MK-11 as the major menaquinone (Evtushenko et al. 2000). It is hypothesized that *Saccharum spontaneum* might be the natural host of this bacterium and that modern sugarcane acquired Lxx when hybridization was done using this species as a parent. Thus, a single clone of Lxx might have been disseminated around the world after exchange of hybrid breeding materials (Young, 2016). This theory explains the genetic uniformity among Lxx strains and its worldwide distribution. For instance, the genome of a Chinese strain is almost identical to the Brazilian reference strain CTCB07, with an average nucleotide identity of 93.61% (Zhang et al. 2016). Studies on the pathogenicity of Lxx began only after its genome sequence became available (Monteiro-Vitorello et al. 2004). Lxx is recognized as a plant pathogen with a low number of genes linked to pathogenicity. Only 105 genes were found to be related to this trait and about 18% of these appear to be pseudogenes. The presence of various ABC transporters suggest that Lxx can use diverse sources of carbon, such as fructose, arabinose, ribose, maltose, trehalose and xylose (Ventura et al. 2007). Even though obstruction of the xylem vessels can been seen in some infected plants, it is unlikely that this blocking matrix is produced by Lxx (Brumbley et al. 2006a) because it lacks of gum producing genes (Monteiro-Vitorello et al. 2004a). Despite not causing evident necrosis in the xylem, the bacterium possesses pectinase-coding genes that could lead to cell-wall degradation (Ventura et al. 2007; Monteiro-Vitorello et al. 2004a). Although the genome sequencing revealed very important information about this bacterium, the mechanisms involved in the interaction with its host are yet poorly understood. Therefore, further studies are necessary to understand how these bacteria cause RSD, why some sugarcane varieties are more susceptible than others and how environmental conditions influence the development of the disease. A systems biology approach combining multi-omic data can help to understand this intricate pathosystem composed by a host with very complex genome and by a pathogen with many peculiarities.

1.3. Application of transcriptomic and proteomic studies to RSD

Even though RSD has been affecting sugarcane fields for more than 77 years, little is known about the interactions between the causal agent and its host (Young 2016a). Two recent studies evaluated sugarcane response to Lxx by measuring physiological parameters and enzymatic defense activity (Zhang et al. 2017a), and by analyzing vascular changes and endogenous hormonal levels (Zhang et al. 2016). Lxx-inoculated plants had smaller heights, exhibited decreased enzymatic activity for phosphoenolpyruvate catalase, and later on time there was also a reduction on photosynthesis and respiration rates (Zhang et al. 2017a). Additionally, it was concluded that Lxx induces its host to produce ABA (abscisic acid) and to reduce IAA (auxin) (Zhang et al. 2016). Because these two hormones are directly involved in plant growth (Osakabe et al. 2013) - ABA inhibits shoot growth whereas IAA induces it (Powell and Lenhard 2012) - Zhang results correlate with RSD most important characteristic: plant stunting. On the other hand, this report fails to relate the bacterial titer among stalks: their experimental design assumes that Lxx concentration is the same among all stalks in the same plant, which does not correspond to previous reports (Root et al. 2002).

Studies developed by our group (Carvalho 2012; Cia 2014) showed that plants inoculated with Lxx presented altered expression of proteins and genes involved in the control of the cell-cycle (e.g.: cyclin dependent kinase and cyclins), in ABA perception (e.g.: calreticulin, protein G, and alcohol dehydrogenase), and in the synthesis of ethylene and methionine. It is noteworthy that the genes of the cell cycle were all down-regulated, which is consistent with the major symptom of RSD. Interestingly, a methionine synthase encoding gene was up-regulated in inoculated plants. The upregulation of the synthesis of methionine in plants infected with Lxx provides a nice example of host manipulation by a pathogen, as the synthesis of this amino acid is possibly impaired in Lxx due to mutations in two essential genes, metE and metF (Monteiro-Vitorello et al. 2004a). Genes related to signal transduction pathways and to transcription factor expression were also activated. Expression profile analyses from susceptible and resistant plants to RSD persuaded that tolerance to Lxx might be associated to extracellular signal perception (Ferro et al. 2007). A system biology approach on RSD is a promising strategy to get a global overview of this pathosystem,

investigating biochemical relationships between Lxx and its host and how they connect with transcriptomics and proteomics studies.

1.4. What metabolomics could bring to the study of RSD

Metabolomics is the study of metabolites of organisms and relies on state-of-the-art techniques to detect molecules at very low concentrations (Núñez et al. 2013). This approach is widely used in pharmaceutics and other bio-analytical procedures and is now becoming an indispensable tool in the study of plant-pathogen interactions. Metabolites perform diverse roles in plant metabolism, such as but not limited to enzyme regulation, cell-to-cell signaling, and defense against pathogen attack (Vinayavekhin and Saghatelian 2010). The approach can be used to detect a series of metabolites related to infection, such as molecules secreted by pathogens during colonization (Tsuge et al. 2013) or amino acids and sugar alcohols that are produced by the host and enhance pathogen growth. *Plasmodiophora brassicae*, for example, takes over the cytokinin production control from its host and induces gall formation in infected members from the Brassicaceae family, causing clubroot disease (Malinowski et al. 2016). Amino acids from potato root exudates stimulate spore germination of the pathogenic fungus *Spongospora subterranean* (Balendres et al. 2016). Tetrose and pentose sugar alcohols were found to accumulate in plants of the Rosacea family inoculated with *Gymnosporangium asiaticum*, a biotrophic pathogenic fungus which altered the host amino acid and sugar metabolism (Lee et al. 2016). Metabolomics can also be used to detect molecules determinants of host resistance or susceptibility (López-Gresa et al. 2011; Mills et al. 2015). For example, the detection of metabolites, like phytoalexins, produced in response to infecting agents (Finnegan et al. 2016). Ishiga et al. (2015) showed that medicarpin acted as a defense metabolite in non-host resistance of *Medicago truncatula* to *Phakopsora pachyrhizi*, which also inhibited uredinospore germination *in vitro*. Furthermore, pipecolic acid, isoflavones and isoflavonoids were produced in higher levels by soybean plants infected with the causal agent of sudden death syndrome, *Fusarium virguliforme* (Abeysekara et al. 2016). Since metabolites are the result of metabolic pathways coordinated by genes and their related products (Peyraud et al. 2017), detecting them and measuring their accumulation in a plant tissue can corroborate transcriptomic and proteomic data, providing thus data systems-level understanding of plant-pathogen interactions.

Changes in genes, DNA, messenger RNA (mRNA) and proteins are studied by genomic, transcriptomic and proteomic analysis, respectively. The products of these gene-centered mechanisms can be also regulated by metabolites (Gemperline et al. 2016). Thus, metabolomics influences and is influenced by the other omics (Peyraud et al. 2017); the interactions between them result in the final plant phenotype (Figure 2).

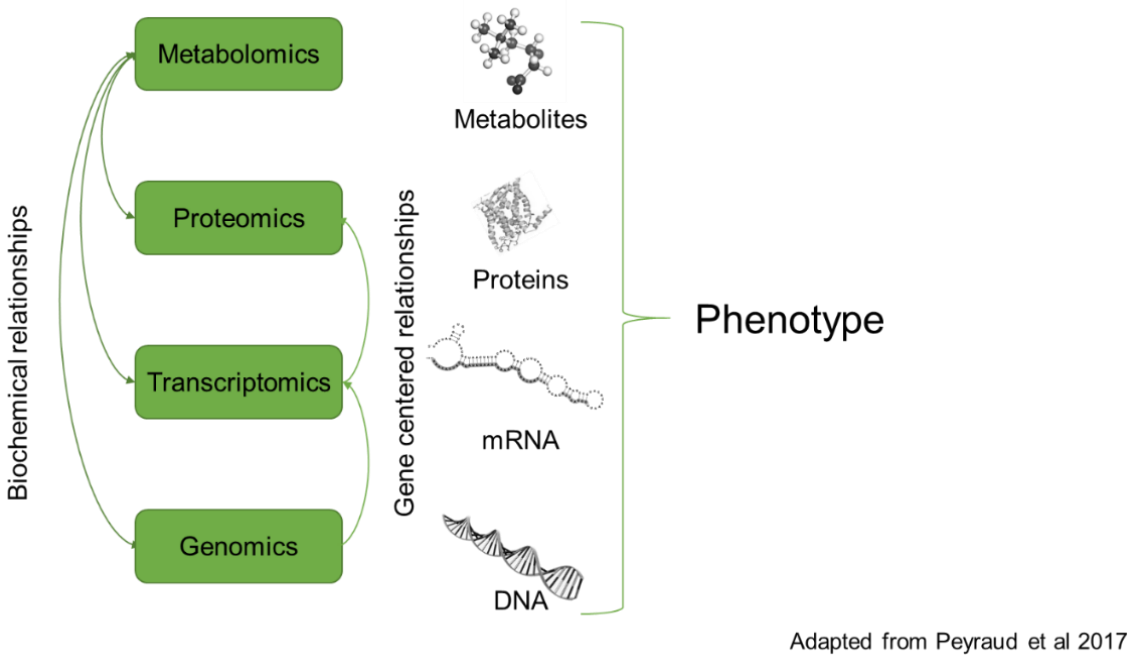


Figure 2. The products of genomics (DNA), transcriptomics (mRNA), proteomics (proteins) and metabolomics (metabolites) analysis and their relationships, resulting in the plant phenotype.

There are two approaches used in metabolomics: untargeted and targeted analysis. Untargeted metabolomics identifies all detectable metabolites, measuring as many molecules as possible among different samples (Vinayavekhin and Saghatelian 2010) whereas targeted metabolomics is used to detect and quantify a defined set of chemically annotated metabolites in a sample using known compounds as standards for quantification (Patti et al. 2012). Untargeted analysis can be performed by gas chromatography coupled with mass spectrometry (GC-MS), which is a robust and precise analytical method (Goodacre et al. 2004). Metabolites analyzed by gas chromatography are volatilized at high temperatures, separated and carried by an inert gas (usually helium) to the mass spectrometer, where they are ionized and then identified by their mass/charge ratios (m/z). The resulting data can be normalized using

an internal standard which allows metabolite comparison by their relative abundances. Differently, targeted metabolomics specifically quantifies of a part of the metabolome, giving more sensitive and robust data for biological studies (Boughton et al. 2011). This approach can be performed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Unlike GC, LC uses liquid solvents to carry the molecules to the MS, which are set in tandem (multiple steps of MS) to break down ionized molecules into smaller ions, generating a very specific m/z (Lee et al. 2010).

There are very few studies on sugarcane metabolomics. Some work has been done regarding sugar composition by Glassop et al. (2010) who identified 32 different types of sugars in the stem, diverging in abundance as well as in location. A metabolomics approach was used to identify phenolic compounds and flavonoids from different sugarcane genotypes, aiming to study the resistance towards pathogens (Coutinho et al. 2016). The varieties considered more resistant to orange rust and mosaic virus had higher amounts of luteolin and tricin derivatives. Additionally, a fingerprinting study on three sugarcane varieties suggested that flavonoids might be involved in plant defense, acting as phytoalexins (Leme et al. 2014). Further, plants subjected to environmental stress had higher activity of enzymes related to the isoprenoid and phenylpropanoid pathways (França et al. 2001). However, enzyme annotation based on gene sequence can be misleading because of the evolutionary flexibility of secondary metabolism in plants (Dixon 2001). There is not yet such study regarding Lxx and its only natural host, sugarcane. More studies need to explore sugarcane metabolic response to biotic and abiotic stresses to find alternative selection methods for more resistant and productive plants. Phenolics are secondary metabolites from the shikimate, phenylpropanoid and flavonoid pathways (Lattanzio et al. 2006). Phenolic compounds and flavonoids derive directly from the shikimate pathway, while terpenes derive from the mevalonate pathway. Products from those two pathways interact to synthesize stilbenes (Figure 3). Phenolic, polyphenols and flavonoids are known to have antimicrobial, more specifically, antibacterial activity (Su et al. 2014; Lou et al. 2011; Lee et al. 2006; Cushnie and Lamb 2005; Nascimento et al. 2000; Basile et al. 1999).

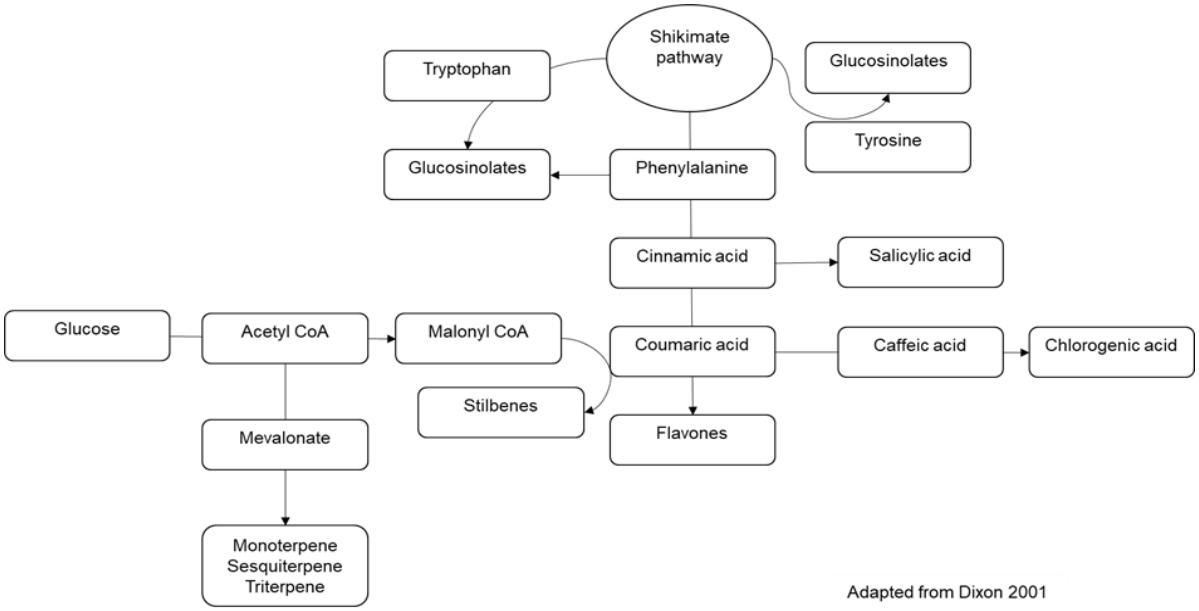


Figure 3. Secondary metabolism pathways – the shikimate pathway leads to the production of glucosinolates, phenolic compounds (cinnamic acid, coumaric acid, caffeic and chlorogenic acid), flavones and terpenes (mono, sesqui and triterpenes).

Even though susceptible varieties of sugarcane sustain higher levels of Lxx than resistant ones, there is not yet a comprehensive explanation of the mechanisms that hinders Lxx growth. Metabolomic profiling carried out in this study intended to elucidate part of this enigma, revealing metabolites differentially accumulated in a susceptible and a resistant variety due to Lxx inoculation. To confirm the activity of potentially bacterial inhibitors, selected compounds were tested for their capacity to inhibit Lxx growth. Therefore, it would be interesting to determine potential bacterial inhibitors or growth promoters on sugarcane and check their effect on Lxx activity in vitro to start unveiling this complex pathosystem and provide better tools for sugarcane breeding against RSD.

1.5. Research Goal

The main objective of this study was to define metabolomic targets that will be compared with existing proteomic and transcriptomic data to define a core target (proteins, genes, and metabolites) that can be further tested as markers of resistance in a collection of sugarcane varieties. To pursue that goal, a comparative metabolomics analysis of a resistant and a susceptible variety of sugarcane inoculated or not with Lxx was undertaken. Chapter 2 describes the metabolite profiling in sugarcane leaves

and xylem sap of a susceptible (CB49-260) and a resistant (SP80-3280) variety. Leaves and xylem sap samples were collected 30 and 120 days after inoculation with Lxx; untargeted metabolomics was performed in the samples collected at 120 DAI to obtain the global metabolic profile from the samples and determine a set of differentially represented metabolites among treatments. Then, targeted metabolomics was applied in the samples collected at both times to quantify the set of metabolites defined by the untargeted analysis. In chapter 3, a set of metabolites was chosen and tested for they antimicrobial action against Lxx *in vitro* to validate those molecules as potential inhibitors of bacterial growth in planta.

References

Abeysekara, N. S., Swaminathan, S., Desai, N., Guo, L., and Bhattacharyya, M. K. 2016. The plant immunity inducer pipecolic acid accumulates in the xylem sap and leaves of soybean seedlings following Fusarium virguliforme infection. Plant Sci. 243:105–114

Balendres, M. A., Nichols, D. S., Tegg, R. S., and Wilson, C. R. 2016. Metabolomes of Potato Root Exudates: Compounds That Stimulate Resting Spore Germination of the Soil-Borne Pathogen *Spongospora subterranea*. J. Agric. Food Chem. 64:7466–7474

Basile, A., Giordano, S., López-Sáez, J. A., and Cobianchi, R. C. 1999. Antibacterial activity of pure flavonoids isolated from mosses. Phytochemistry. 52:1479–1482

Bennett, R. N., and Wallsgrove, R. M. 1994. Secondary metabolites in plant defence mechanisms. New Phytol. 127:617–633

Boughton, B. A., Callahan, D. L., Silva, C., Bowne, J., Nahid, A., Rupasinghe, T., Tull, D. L., Mcconville, M. J., Bacic, A., and Roessner, U. 2011. Comprehensive Profiling and Quantitation of Amine Group Containing Metabolites. :7523–7530

Brumbley, S. M., Petrasovits, L. A., Hermann, S. R., Young, A. J., and Croft, B. J. 2006. Recent advances in the molecular biology of *Leifsonia xyli* subsp. *xyli* , causal organism of ratoon stunting disease. Australas. Plant Pathol. 35:681

Brumbley, S. M., Petrasovits, L. A., Murphy, R. M., Nagel, R. J., Candy, J. M., and Hermann, S. R. 2004. Establishment of a functional genomics platform for Leifsonia xyli subsp. xyli. Mol. Plant-Microbe Interact. 17:175–183

Buckeridge, M. S., de Souza, A. P., Arundale, R. A., Anderson-Teixeira, K. J., and Delucia, E. 2012. Ethanol from sugarcane in Brazil: A “midway” strategy for increasing ethanol production while maximizing environmental benefits. GCB Bioenergy. 4:119–126

Carvalho, G. 2012. Proteome and antioxidant system analysis of sugarcane in response to Leifsonia xyli subsp. xyli, causal agent of ratoon stunting disease.

Cia, M. C. 2014. Análise transcritômica de plantas de cana de açúcar inoculadas com Leifsonia xyli subsp. xyli, agente causal do raquitismo das soqueiras.

Comstock, J. C. 2002. *Ratoon stunting disease*.

Comstock, J. C. C., Shine Jr, J. M. M., Davis, M. J. J., and Dean, J. L. L. 1996. Relationship between resistance to Clavibacter xyli subsp. xyli colonization in sugarcane and spread of ratoon stunting disease in the field. Plant Dis. 80:704–708

CONAB. 2017. *Companhia Nacional de Abastecimento*.

Coutinho, I. D., Baker, J. M., Ward, J. L., Beale, M. H., Creste, S., and Cavalheiro, A. J. 2016. Metabolite Profiling of Sugarcane Genotypes and Identification of Flavonoid Glycosides and Phenolic Acids. J. Agric. Food Chem. 64:4198–4206

Croft, B. J. 2002. A method for rating sugarcane cultivars for resistance to ratoon stunting disease based on an enzyme-linked immunoassay. Australas. Plant Pathol. 31:63

Cushnie, T. P. P. T., and Lamb, A. J. 2005. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents. 26:343–356

Dal-Bianco, M., Carneiro, M. S., Hotta, C. T., Chapola, R. G., Hoffmann, H. P., Garcia, A. A. F., and Souza, G. M. 2012. Sugarcane improvement: How far can we go? Curr. Opin. Biotechnol. 23:265–270

Damann, Jr., K. E. 1983. Evaluation of Commercial Heat-Treatment Methods for Control of Ratoon Stunting Disease of Sugarcane. Plant Dis. 67:966

Davis, M. J., Dean, J. L., and Harrison, N. A. 1988a. Distribution of Clavibacter xyli subsp. xyli in stalks of sugarcane cultivars differing in resistance to ratoon stunting disease. Plant Dis. 72:443–448

Davis, M. J., Dean, J. L., and Harrison, N. A. 1988b. Quantitative variability of Clavibacter xyli subsp. xyli populations in sugarcane cultivars differing in resistance to ratoon stunting disease. Phytopathology. 78:462–468

Davis, M. J., Gillaspie, A. G., Harris, R. W., and Lawson, R. H. 1980. Ratoon stunting disease of sugarcane: isolation of the causal bacterium. Science. 210:1365–1367

Davis, M. J., Gillaspie JR, A. G., Vidaver, A. K., and Harris, R. W. 1984. Clavibacter : a New Genus Containing Some Phytopathogenic Coryneform Bacteria , Including Clavibacter xyli subsp . xyli sp . nov ., subsp . nov . and Clavibacter xyli subsp . cynodontis subsp . nov . Pathogens That Cause Ratoon Stunting Disease of Sugarca. Int. J. Syst. Bacteriol. 34:107–117

Debibakas, S., Rocher, S., Garsmeur, O., Toubi, L., Roques, D., D'Hont, A., Hoarau, J. Y., and Daugrois, J. H. 2014. Prospecting sugarcane resistance to Sugarcane yellow leaf virus by genome-wide association. Theor. Appl. Genet. 127:1719–1732

Dixon, R. A. 2001. Natural products and plant disease resistance. Nature. 411:843–847

Evtushenko, L. I., Dorofeeva, L. V, Subbotin, S. A., Cole, J. R., and Tiedje, J. M. 2000. Leifsonia poae gen. nov., sp. nov., isolated from nematode galls on Poa annua, and reclassification of "Corynebacterium aquaticum" Leifson 1962 as Leifsonia aquatica (ex Leifson 1962) gen. nov., nom. rev., comb. nov. and Clavibacter xyli Davis et al. 1984. Int. J. Syst. Evol. Microbiol. :371–380

Fegan, M., Croft, B. J., Teakle, D. S., Hayward, A. C., and Smith, G. R. 1998. Sensitive and specific detection of Clavibacter xyli subsp. xyli, causal agent of ratoon stunting disease of sugarcane, with a polymerase chain reaction-based assay. Plant Pathol. 47:495–504

Ferro, M. I. T., Barros, N. M., Dabbas, K. M., Laia, M. L., Kupper, K. C., Moraes, V. A., Oliveira, J. C. F. de, Ferro, J. A., and Zingaretti, S. M. 2007. Análise do perfil de expressão dos genes da cana-de-açúcar envolvidos na interação com Leifsonia xyli subsp. xyli. Summa Phytopathol. 33:157–166

Finnegan, T., Steenkamp, P. A., Piater, L. A., Dubery, I. A., Flors, V., Glawischnig, E., and Meijden, E. van der. 2016. The lipopolysaccharide-induced metabolome signature in Arabidopsis thaliana reveals dynamic reprogramming of phytoalexin and phytoanticipin pathways S. Osorio-Algar, ed. PLoS One. 11:e0163572

França, S. C., Roberto, P. G., Marins, M. A., Puga, R. D., Rodrigues, A., and Pereira, J. O. 2001. Biosynthesis of secondary metabolites in sugarcane. Genet. Mol. Biol. 24:243–250

Fu, H. Y., Sun, S. R., Wang, J. Da, Ahmad, K., Wang, H. B., Chen, R. K., and Gao, S. J. 2016. Rapid and Quantitative Detection of Leifsonia xyli subsp. Xyli in Sugarcane Stalk Juice Using a Real-Time Fluorescent (TaqMan) PCR Assay. Biomed Res. Int. 2016

Gazaffi, R., Oliveira, K. M., Souza, A. P. De, and Garcia, A. A. F. 2014. Sugarcane: breeding and genetic mapping. Pages 203–216in:Sugarcane Bioethanol,

Gemperline, E., Keller, C., and Li, L. 2016. Mass spectrometry in plant-omics. Anal. Chem. 88:3422–3434

Glassop, D., Ryan, L. P., Bonnett, G. D., and Rae, A. L. 2010. The complement of soluble sugars in the Saccharum complex. Trop. Plant Biol. 3:110–122

Goodacre, R., Vaidyanathan, S., Dunn, W. B., Harrigan, G. G., and Kell, D. B. 2004. Metabolomics by numbers: Acquiring and understanding global metabolite data. Trends Biotechnol. 22:245–252

Grisham, M. 1991. Effect of ratoon stunting disease on yield of sugarcane grown in multiple three-year plantings. Phytopathology. 91

Haapalainen, M., Mattinen, J., and Metzler, M. 2000. The growth of a plant-parasitic bacterium, Clavibacter xyli subsp. cynodontis, is enhanced by xylem fluid components. Physiol. Mol. Plant Pathol. 56:147–155

Hoy, J. W., Grisham, M. P., and Damann, K. E. 1999. Spread and Increase of Ratoon Stunting Disease of Sugarcane and Comparison of Disease Detection Methods. Plant Dis. 83:1170–1175

INT. 2017. Instituto Nacional de Tecnologia (National Institute of Technology). Available at: http://www.int.gov.br/noticias/8067-int-uma-historia-de-inovacao-desde-1921 [Accessed March 6, 2017].

Ishiga, Y., Uppalapati, S. R., Gill, U. S., Huhman, D., Tang, Y., and Mysore, K. S. 2015. Transcriptomic and metabolomic analyses identify a role for chlorophyll catabolism and phytoalexin during Medicago nonhost resistance against Asian soybean rust. Sci. Rep. 5:13061

Lattanzio, V., Lattanzio, V. M. T., Cardinali, A., and Amendola, V. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects.

Lee, D.-K., Ahn, S., Cho, H. Y., Yun, H. Y., Park, J. H., Lim, J., Lee, J., and Kwon, S. W. 2016. Metabolic response induced by parasitic plant-fungus interactions hinder amino sugar and nucleotide sugar metabolism in the host. Sci. Rep. 6:37434

Lee, D. Y., Bowen, B. P., and Northen, T. R. 2010. Mass spectrometry-based metabolomics, analysis of metabolite-protein interactions, and imaging. Biotechniques. 49:557–65

Lee, H. C., Jenner, A. M., Low, C. S., and Lee, Y. K. 2006. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. Res. Microbiol. 157:876–884

Leme, G. M., Coutinho, I. D., Creste, S., Hojo, O., Carneiro, R. L., Bolzani, V. da S.,

and Cavalheiro, A. J. 2014. HPLC-DAD method for metabolic fingerprinting of the phenotyping of sugarcane genotypes. Anal. Methods. 6:7781–7788

Li, W.-F. F., Shen, K., Huang, Y.-K. K., Wang, X.-Y. Y., Yin, J., Luo, Z.-M. M., Zhang, R.-Y. Y., and Shan, H.-L. L. 2014. Incidence of sugarcane ratoon stunting disease in the major cane-growing regions of China. Crop Prot. 60:44–47

López-Gresa, M. P., Torres, C., Campos, L., Lisón, P., Rodrigo, I., Bellés, J. M., and Conejero, V. 2011. Identification of defence metabolites in tomato plants infected by the bacterial pathogen Pseudomonas syringae. Environ. Exp. Bot. 74:216–218

Lou, Z., Wang, H., Zhu, S., Ma, C., and Wang, Z. 2011. Antibacterial activity and mechanism of action of chlorogenic acid. J. Food Sci. 76

Malinowski, R., Novák, O., Borhan, M. H., Spíchal, L., Strnad, M., and Rolfe, S. A. 2016. The role of cytokinins in clubroot disease. Eur. J. Plant Pathol. 145:543–557

MAPA. 2017. Ministério da Agricultura, Pecuária e Abastecimento. Available at: http://www.agricultura.gov.br/ [Accessed April 17, 2017].

Marques, J. P. R., Appezzato-da-Glória, B., Piepenbring, M., Massola, N. S., Monteiro-Vitorello, C. B., Vieira, M. L. C., Massola Jr, N. S., Monteiro-Vitorello, C. B., and Vieira, M. L. C. 2016. Sugarcane smut: shedding light on the development of the whip-shaped sorus. Ann. Bot. 119:mcw169

McFarlane, S. 2002. The relationship between extent of colonisation by Leifsonia xyli subsp. xyli and yield loss in different sugarcane varieties. Proc S Afr Sug Technol Ass. 76:281–284

Mills, C. E., Tzounis, X., Oruna-Concha, M.-J., Mottram, D. S., Gibson, G. R., and Spencer, J. P. E. 2015. In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth. Br. J. Nutr. 113:1220–7

Monteiro-Vitorello, C. B., Camargo, L. E. A., Van Sluys, M. A., Kitajima, J. P., Truffi, D., Do Amaral, A. M., Harakava, R., De Oliveira, J. C. F., Wood, D., De Oliveira, M. C., Miyaki, C., Takita, M. A., Da Silva, A. C. R., Furlan, L. R., Carraro, D. M.,

Camarotte, G., Almeida, N. F., Carrer, H., Coutinho, L. L., El-Dorry, H. A., Ferro, M. I. T., Gagliardi, P. R., Giglioti, E., Goldman, M. H. S., Goldman, G. H., Kimura, E. T., Ferro, E. S., Kuramae, E. E., Lemos, E. G. M., Lemos, M. V. F., Mauro, S. M. Z., Machado, M. A., Marino, C. L., Menck, C. F., Nunes, L. R., Oliveira, R. C., Pereira, G. G., Siqueira, W., De Souza, A. A., Tsai, S. M., Zanca, A. S., Simpson, A. J. G., Brumbley, S. M., and Setúbal, J. C. 2004. The genome sequence of the gram-positive sugarcane pathogen Leifsonia xyli subsp. xyli. Mol. Plant-Microbe Interact. 17:827–836

Nascimento, G. G. F., Locatelli, J., Freitas, P. C., Silva, G. L., and Piracicaba, U. M. De. 2000. Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic-. Brazilian J. Microbiol. 31:247–256

Núñez, O., Gallart-Ayala, H., Martins, C. P. B., Lucci, P., and Busquets, R. 2013. State-of-the-art in fast liquid chromatography–mass spectrometry for bio-analytical applications. J. Chromatogr. B. 927:3–21

Ogden-Brown, J. 2015. A healthy crop starts with clean seed. Sugar Res. Aust. :1

Osakabe, Y., Arinaga, N., Umezawa, T., Katsura, S., Nagamachi, K., Tanaka, H., Ohiraki, H., Yamada, K., Seo, S.-U., Abo, M., Yoshimura, E., Shinozaki, K., and Yamaguchi-Shinozaki, K. 2013. Osmotic stress responses and plant growth controlled by potassium transporters in Arabidopsis. Plant Cell. 25:609–624

Patti, G. J., Yanes, O., Siuzadak, G., and Siuzdak, G. 2012. Metabolomics: the apogee of the omics triology. Nat. Rev. Mol. Cell Biol. 13:263–269

Peyraud, R., Dubiella, U., Barbacci, A., Genin, S., Raffaele, S., and Roby, D. 2017. Advances on plant-pathogen interactions from molecular toward systems biology perspectives. Plant J. 90:720–737

Pieretti, I., Pesic, A., Petras, D., Royer, M., Süssmuth, R. D., and Cociancich, S. 2015. What makes Xanthomonas albilineans unique amongst xanthomonads? Front. Plant Sci. 6:1–7

Powell, A. E., and Lenhard, M. 2012. Control of Organ Size in Plants. Curr. Biol. 22:R360–R367

Quecine, M. C., Silva, T. M., Carvalho, G., Saito, S., Mondin, M., Teixeira-Silva, N. S., Camargo, L. E. A., and Monteiro-Vitorello, C. B. 2016. A stable Leifsonia xyli subsp. xyli GFP-tagged strain reveals a new colonization niche in sugarcane tissues. Plant Pathol. 65:154–162

Root, P., Sood, S., Comstock, J. C., Gilbert, R. A., Sandhu, H. S., Rott, P., Sood, S., Comstock, J. C., Gilbert, R. A., and Sandhu, H. S. 2002. Sugarcane ratoon stunting disease. Florida Sugarcane Handb. :1–3

Su, Y., Ma, L., Wen, Y., Wang, H., and Zhang, S. 2014. Studies of the in vitro antibacterial activities of several polyphenols against clinical isolates of methicillin-resistant Staphylococcus aureus. Molecules. 19:12630–12639

Teakle, D. S. S., Smith, P. M., and Steindl, D. R. L. R. L. 1975. *Ratton stunting disease of sugarcane: possible correlation of resistance with vascular anatomy*.

Teakle, D., Smith, P., and Steindl, D. 1973. Association of a small coryneform bacterium with the ratoon stunting disease of sugar-cane. Aust. J. Agric. Res. 24:869

Tsuge, T., Harimoto, Y., Akimitsu, K., Ohtani, K., Kodama, M., Akagi, Y., Egusa, M., Yamamoto, M., and Otani, H. 2013. Host-selective toxins produced by the plant pathogenic fungus Alternaria alternata. FEMS Microbiol. Rev. 37:44–66

UNICA. 2017. UNICA - União da Industria de Cana-de-açúcar. Available at: http://www.unica.com.br/ [Accessed April 17, 2017].

Urashima, A. S., and Marchetti, L. B. L. 2013. Incidence and Severity of Leifsonia xyli subsp. xyli Infection of Sugarcane in Sao Paulo State, Brazil. J. Phytopathol. 161:478–484

Urashima, A. S., Silva, M. F., Correa, J. J. J. J., Moraes, M. C., Singh, A. V. A. V., Smith, E. C. E., and Sainz, M. B. M. 2017. Prevalence and Severity of Ratoon Stunt in Commercial Brazilian Sugarcane Fields. Plant Dis. :PDIS-07-16-1030

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., and van Sinderen, D. 2007. Genomics of Actinobacteria: tracing the evolutionary

history of an ancient phylum. Microbiol. Mol. Biol. Rev. 71:495–548

Vinayavekhin, N., and Saghatelian, A. 2010. Untargeted Metabolomics. Pages Unit 30.1.1-24 in: Current Protocols in Molecular Biology, John Wiley & Sons, Inc., Hoboken, NJ, USA.

Young, A. J. 2016a. Possible origin of ratoon stunting disease following interspecific hybridization of *Saccharum* species. Plant Pathol. 65:1403–1410

Young, A. J. 2016b. Seedbed Inspections Underestimate the Overall Incidence of Ratoon Stunting Disease. Pages 112–119in:Proc Aust Soc Sugar Cane Technol,

Young, A., Lokes, S., Davis, W., and Aitken, B. O. B. 2012. Reassessing Rsd: Insights From Harwood. Proc Aust Soc Sugar Cane Technol. 34:1–7

Zhang, X.-Q., Liang, Y.-J., Zhu, K., Wu, C.-X., Yang, L.-T., and Li, Y.-R. 2017. Influence of inoculation of Leifsonia xyli subsp. xyli on photosynthetic parameters and activities of defense enzymes in sugarcane. Sugar Tech. 19:394–401

Zhang, X., Chen, M., Liang, Y., Xing, Y., Yang, L., Chen, M., Comstock, J. C., Li, Y., and Yang, L. 2016. Morphological and physiological responses of sugarcane to Leifsonia xyli subsp. xyli infection. Plant Dis. 100:1–8

Zhao, D., Glynn, N. C., Glaz, B., Comstock, J. C., and Sood, S. 2011. Orange Rust Effects on Leaf Photosynthesis and Related Characters of Sugarcane. Plant Dis. 95:640–647

2. METABOLOMIC ANALYSIS OF SUGARCANE LEAVES AND XYLEM SAP REVEALS METABOLITES INVOLVED IN THE RESPONSES TO RATOON STUNTING DISEASE

ABSTRACT

The gram-positive and xylem-limited bacterium Leifsonia xyli subsp. xyli (Lxx) is the causal agent of ratoon stunting disease (RSD), a major infection of sugarcane. The RSD is worldwide distributed, and reported losses in biomass production may reach 80% due to the generalized poor growth of diseased plants characterized by shorter internodes and thinner stalks. Breeding for resistance to RSD, although not in practice, is a viable control measure. Since sugarcane varieties differ in relation to their degree of colonization by Lxx and losses are directly related to population densities of the pathogen in the plant, a promising breeding strategy would be to select for genotypes that are resistant to bacterial multiplication. Thus, knowledge on the responses of sugarcane to RSD at the "omics" level is an essential starting step to identify key metabolic targets for breeding resistant varieties. A metabolomic study was performed to compare the metabolic profiling of two sugarcane varieties, one susceptible (CB49-260) and the other resistant (SP-80-3280) to RSD mock or Lxx-inoculated leaves and xylem sap obtained through guttation drops, collected at 30 and 120 days after inoculation (DAI). Ultimately, these results will be compared to previous proteomics and transcriptomics studies to establish a relationship between metabolites, proteins and transcripts that differentially responded to Lxx-infection. This pool of knowledge will establish a set of disease-related molecules that can be used as selection biomarkers for resistance against RSD. Lxx titer was detected with Real-Time Polymerase Chain Reaction (qPCR) and the results confirmed CB49-260 as the more susceptible genotype, because it had the highest bacterial titers. Contrarily, SP80-3280 was able to control endophytic levels of the bacterium even with artificial inoculation. Untargeted profiling was performed by Gas Chromatography-Mass Spectrometry (GC-MS) and highlighted the main chemical groups in sugarcane leaves and sap. It revealed that the two varieties have different metabolic profiles, but those distinctions are more quantitative than qualitative. Targeted metabolomics was performed using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) to quantify amino acids, sugars, sugar alcohols, organic acids, phosphorylated compounds, phenolic acids, flavonoids and hormones. Susceptible non-inoculated plants were found to accumulate more amino acids, sugars and organic acids, while the resistant ones had more phenolics. Inoculation with Lxx resulted in higher accumulation of amino and organic acids, phosphorylated compounds and some phenolics. This study also highlighted a key amino acid for Lxx survival in the leaves and a phenolic compound with known plant defense activity in the sap.

Keywords: Plant pathogenic bacterium; Metabolite; Untargeted and targeted metabolomics; Host response; Biomarker

2.1. Introduction

Ratoon stunting disease (RSD) occurs worldwide and is a serious disease affecting all sugarcane producing countries (Li et al. 2014). The state of São Paulo is the major sugar and ethanol producer in Brazil where incidences of RSD in commercial fields have been reported to be as high as 80% (Urashima and Marchetti 2013). The major symptoms of RSD are shorter internodes and thinner stalks that develop only in ratoon plants leading to a marked reduction in biomass production over the years. Diagnosis of RSD based on symptoms is difficult as they can be easily mistaken for symptoms caused by many environmental factors that affect plant growth. The disease is caused by *Leifsonia xyli* subsp. *xyli*, a gram-positive and nutritionally fastidious bacterium that so far has been found to colonize the xylem vessels and bundle sheath cells surrounding the vascular system of sugarcane (Quecine et al. 2016; Young 2016b; Monteiro-Vitorello et al. 2004b). Lxx is regarded as an obligatory endophytic organism that grows to parasitic levels depending on yet poorly understood biotic and abiotic factors (Zavaglia et al. 2016).

The only method for controlling RSD is to subject propagative material to heat treatment to eliminate the bacterium, but the efficacy of this method is variable and it also may affect the germinability of the buds (Urashima and Grachet 2012). Genetic control of RSD using resistant varieties is a promising strategy, since sugarcane genotypes vary in relation to the levels of Lxx colonization of their tissues (Davis et al. 1988b; Li et al. 2013). Thus, since losses due to RSD are related to bacterial densities in the plant (Davis et al. 1988b; McFarlane 2002), a breeding strategy would be to select genotypes that restrict the multiplication of the bacterium. However, phenotypic selection of resistant genotypes based on inoculation trials is not feasible because quantification of Lxx *in planta* requires serological or molecular methods which would add significant costs to the breeding programs. For instance, it is required to screen approximately 250 thousand seedlings in order to develop one commercial variety just by selecting for higher sugar content alone and not considering other traits (Dal-Bianco et al. 2012).

Studies developed by Cia (2014) and Carvalho (2012) revealed that plants inoculated with Lxx presented altered expression of proteins and genes involved in the cell-cycle, in responses to stress (e.g. trehalose synthase and phosphatase), and in the metabolism of hormones (ABA, ethylene, gibberellic acid, and jasmonic acid).

Noteworthy are the genes of the cell cycle that were all down regulated, which is consistent with the major symptom of RSD. These results were corroborated by Zhang et al. (2016) who demonstrated that Lxx infection increases abscisic acid and decreases auxin and gibberellin levels. Interestingly, a methionine synthase encoding gene was up-regulated in Lxx inoculated plants. This finding suggests an increase of the synthesis of this amino acid, providing a nice example of host manipulation by a pathogen since Lxx is a probable auxotroph for methionine due to mutations in two genes essential for its biosynthesis (metE and metF). Also, the increased production of this amino acid could be linked to the enhanced expression of genes involved in the synthesis of ethylene (1aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase). These studies highlighted metabolic pathways that should be further investigated to assess their potential role in controlling the multiplication of the pathogen in the host. Nevertheless, research on sugarcane response to Lxx is scarce and therefore we lack information on the effects of RSD at the metabolic level.

The main objective of this study was to identify potential mechanisms of resistance to Lxx. For this, metabolomic data will be integrated with existing proteomic and transcriptomic data to identify potential mechanisms of resistance to Lxx. A comparative metabolomic study was conducted on leaves and xylem sap samples from a susceptible and a resistant variety of sugarcane to RSD, which were subjected to untargeted and targeted metabolic profiling. The plants were inoculated with Lxx and mock-inoculated as controls. Samples were collected at 30 and 120 days after inoculation and were evaluated using untargeted and targeted metabolomics to compare their metabolic profiles. Distinct profiles were detected between varieties and pathogen inoculated treatments.

2.2. Material and methods

2.2.1. Bacterial strains and growth conditions

The CTCB07 strain of Lxx was used. A 1 mL volume of a bacterial suspension obtained from a 25% glycerol inoculum was cultivated in 250 mL flasks containing 50 mL of modified sugarcane New (MSC-New) medium (Davis et al. 1984; Monteiro-Vitorello et al. 2004b). The culture was incubated at 28 ºC and 400 rpm agitation until it reached the optical density (O.D) of 0.7 (approximately 7 days; λ=600). To prepare

cell suspensions for inoculations, the cultured medium was centrifugated at 3,000 rpm for 20 min at room temperature to obtain a bacterial pellet, which was resuspended in 0.85% saline solution to O.D = 0.7.

2.2.2. Plant material and inoculation

One-eyed stem cuttings (setts) of the susceptible CB49-260 and of the moderately resistant SP80-3280 varieties were obtained from greenhouse-grown ratoons generated from *in vitro* cultured plants. The setts were germinated in commercial substrate (Basaplant, BASE) in a germination chamber at 25ºC and photoperiod of 12 h until the germinated buds reached 1.5 cm in height (approximately 6 days) and then were transplanted to 22L plastic pots, each pot containing 20 L of substrate (Basaplant, BASE) supplemented with 25 g of NPK (8-28-16). Plants were inoculated by placing 50 µL of the cell suspension (0.85% NaCl solution) of Lxx ($O.D._{600}$= 0.7) on top of the surface of the germinated buds that were cut just above the meristem with a sterile blade. Control plants were mock-inoculated with the same volume of 0.85% NaCl. The inoculum was serially diluted and plated on solid MSC-New medium for determination of its concentration.

The experiment was conducted in a greenhouse and consisted of 4 treatments: two varieties inoculated or not with Lxx. Each treatment had four biological replicates consisting of one pot containing a single plant; samples from leaves and from the xylem were collected at two time points (30 and 120 days after inoculation), comprising 64 samples.

2.2.3. Lxx quantification

Bacterial titers were quantified in the stem fluid 180 days after inoculation (DAI) by qPCR as in Carvalho et al., (2016) with. The fluid was collected by pressing pieces of the stem with a Lineman’s plier; 500 µL aliquots were used for DNA extraction.

2.2.4. Sample collection

The first leaves with a visible dewlap (+1 leaves) were collected at 30 and 120 DAI and flash-frozen. The samples were kept at -80ºC until they were ground in liquid

nitrogen using a pestle and a mortar, and lyophilized for 48 hours in Eppendorf tubes. The samples were kept at room temperature in the tubes inside sealed bags with silica gel until use. Xylem sap samples were pipetted from the hydathodes in the form of guttation droplets every 30 min for 3 hours starting at 6 AM, and stored in sterile tubes kept on ice. The samples were flash-frozen and stored at -80ºC until they were aliquoted in 200 µL subsamples into eppendorf tubes, lyophilized for 48 hours, and preserved under the same conditions as the leaf samples.

2.2.5. Metabolomic analyses

2.2.5.2. Untargeted metabolomics

Metabolomic analyses were performed at the Center for Applied Plant Sciences Targeted Metabolomics Laboratory at The Ohio State University. Untargeted metabolic profiling was performed only in leaf and xylem sap samples collected at 120 DAI to identify the main classes of metabolites present in these tissues. Four biological replicates from four treatments (2 varieties x 2 inoculation procedures) and two tissue types were used in this analysis.

Metabolites were extracted from the leaves with -20 ºC water/methanol/chloroform solvent for at least 60 min. Approximately 5 mg of lyophilized tissue were weighted and the exact weight was recorded for data normalization. One mL of cold extraction solvent (chloroform/methanol/water at the ratio 1:2.5:1; v/v/v) was added to each sample as well as 10 µL of 20 mM [U-$^{13}C_2$]-glycine as an internal standard. The samples were vortexed at 4ºC for 5 min and centrifuged at 17,000 RCF (g) for 2 min. The upper colorless (polar) phase of the supernatant was collected and transferred to a new tube and then lyophilized. Dry samples were resuspended in 250 µL methanol/water (50:50; v/v) and cleaned through a 3 kDa Amicon filtering device at 14,000 x g for 45 min.

Lyophilized sap samples were resuspended in 250 µL of -20 ºC extraction solvent (methanol/water at the ratio 2:8; v/v) for at least 60 min. Then, 3 µL of 2 mM [U-$^{13}C_2$]--glycine standard solution was added as an internal standard. The samples were vortexed at 4ºC for 5 min and centrifuged at 17,000 x g for 2 min. The supernatant was collected and cleaned through a 0.2 µm filtering device at 17,000 x g for 10 min.

For both leaf and sap samples, the filtered extracts were lyophilized and resuspended in methylene chloride. The extracts were dried under a flow of nitrogen gas and then derivatized with 20mg/mL methoxyamine hydrochloride in pyridine and N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1 % trimethylchlorosilane (MSTFA + 1% TMCS). The derivatized samples were subjected to gas chromatography-mass spectrometry (GC-MS) analyses as previously described by Tsogtbaatar et al. (2015), using a Thermo Trace 1310 gas chromatograph coupled to an ISQ single quadrupole mass spectrometer. The derivatized samples were separated using a TG-5MS capillary (30 m x 0.25 mm x 0.50 μm) column from Thermo Scientific. To avoid carry-overs in the xylem sap sample runs, a blank (100% acetonitrile) was injected between each sample. The Xcalibur software v. 4.0 (Thermo Scientific) was used for data acquisition and processing of the GC-MS data; XCMS Online (Tautenhahn et al. 2012) was used for chromatogram alignment and peak integration. The main classes of compounds were identified using the National Institute of Standards and Technology (NIST 14) mass spectral library. Relative abundances were calculated by normalizing the area of each individual detected metabolite by the one of the ^{13}C-internal standard and the weight the leaf sample or the volume of sap extract.

2.2.5.3. Targeted metabolomics

For the targeted analysis of leaf extracts, amino acids, sugars, sugar alcohols, organic acids, phosphorylated metabolites, phenolic compounds, flavonoids and hormones were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) for all treatments, biological replicates, evaluation timepoints and tissue types (2 varieties x 2 inoculation conditions x 2 evaluation times x 4 replicates x 2 sample types) using a UHPLC 1290 (Agilent) for LC separation and QTRAP 5500 linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer (AB Sciex Instruments) for tandem MS.

Amino acids, sugars and sugar alcohols, organic acids and phosphorylated compounds were extracted with boiling water as in Cocuron et al. (2014). 10 µL of a mix containing 10 mM [U-$^{13}C_6$]-glucose, 1 mM [U-$^{13}C_4$]-fumarate, and 5 mM [U-$^{13}C_2$]-glycine were added to each sample as internal standards during the extraction. After lyophilization, extracts were resuspended in 350 µL of water, vortexed, and filtered

through a 0.2 µm nanosep MF centrifugal device. For amino acid analyses, 8 µL of extract was added in a vial containing 192 µL of water with 1mM HCl and 10 µL of the sample was injected into a Hypercarb column (100 x 2.1 mm, 5µL pore; Thermo Fisher Scientific). For sugars and sugar alcohols, 10 µL of extracts were added in a vial containing 190 µL of acetonitrile/water (60:40 v/v), and 5 µL was injected into a Shodex Asahipak NH2P-50 2D column (2.0 x 150 mm) with a Shodex Asahipak NH2P-50G 2A guard column (Showa Denko America). For organic acids and phosphorylated compounds, 10 µL of the extracts were added to a vial containing 90 µL of water and 5 µL of the sample was injected into a IonPac AS11 column (250 x 2 mm) with a Guard column AG11 (50 x 2 mm; Dionex).

Phenolics were extracted using 1 mL of methanol:water (40:60 v/v) per sample of 10 mg of dried leaf. 10 µL of 10 mM ^{13}C-benzoic acid was added as an internal standard to each sample during the extraction. The samples were shaken in a bead beater for 5 minutes then sonicated at 30 Hz for 10 minutes. After centrifuging the samples for 15 minutes at 17,000 x *g* at room temperature, 0.5 mL of the supernatant was cleaned up using 3kDa Amicon filtering device for 45 min at 14,000 x g at room temperature. 50 µL of the samples was added to a vial containing 50 µL of water:methanol (60:40 v/v) and 10 µL was injected into the LC-MS/MS. Phenolic compounds, flavonoids and hormones were separated using a reverse phase C18 Symmetry column (4.6 x 75 mm; 3.5 mm) associated with a Symmetry C18 pre-column (3.9 x 20 mm; 5 mm; Waters).

No extraction was performed for the analysis of xylem sap metabolites: sap samples were lyophilized, resuspended in 250 µL water:methanol (1:1 v/v), and then the different classes of compounds were analyzed by LC-MS/MS and processed as above.

Data acquisition and processing were performed with Analyst v. 1.1.6 software. Metabolite quantification was performed by correlating the resulting peak area of each metabolite with its corresponding standard as previously described by Cocuron et al. (2014). First, the total amount of a sample in each run was estimated according to its dilution. Then, each metabolite peak area was corrected to the peak area of the ^{13}C-internal standard. Finally, each area was correlated to the concentration and estimated area of the corresponding external standard. The list of standards used and their respective concentrations can be found in the supplementary material section (Table S 1).

2.2.6. Statistical analysis

Comparisons of mean bacterial titers (cells/mL) among treatments were performed by one-way anova and Tukey's test ($p<0.05$) with MetaboAnalyst version 3.0 (Xia et al. 2009) available online (http://www.metaboanalyst.ca).

Comparisons of metabolite abundances were performed with MetaboAnalyst version 3.0 (Xia et al. 2009) as well. The data was normalized using Log transformation and then it was mean-centered. Mean metabolite concentrations were compared between treatments with t-Test and $p<0.05$. Principal component analysis (PCA) was used to visualize differences among treatments, using principal component 1 (PC1) and principal component 2 (PC2). Three treatment comparisons were defined *a priori* to address different questions. The first one compared the metabolite concentrations of the susceptible and the resistant mock-inoculated plants (S x R) at each time to identify constitutive metabolites that could inhibit or enhance Lxx growth. The second compared inoculated and mock-inoculated plants of the susceptible variety (S x SI) at each time to identify compounds induced by Lxx that could affect the plant metabolism and explain the genesis of the symptoms of RSD before they appear. The third compared inoculated and mock-inoculated plants of the resistant variety (R x RI) at each time to identify induced metabolites possibly related to resistance to LxxResults.

2.2.7. Lxx quantification revealed higher bacterial titers in the susceptible variety CB49-260

The susceptible (CB49-260) and resistant (SP80-3280) varieties were inoculated with Lxx or mock-inoculated with a NaCl solution (0.85%) for metabolomic profiling at 30 and 120 days after inoculation (DAI). The concentration of the inoculum was 6.5×10^{10} CFU mL^{-1} as estimated by plate counting of the serial dilution (data not shown). The plants regenerated well after inoculation (Figure 4).

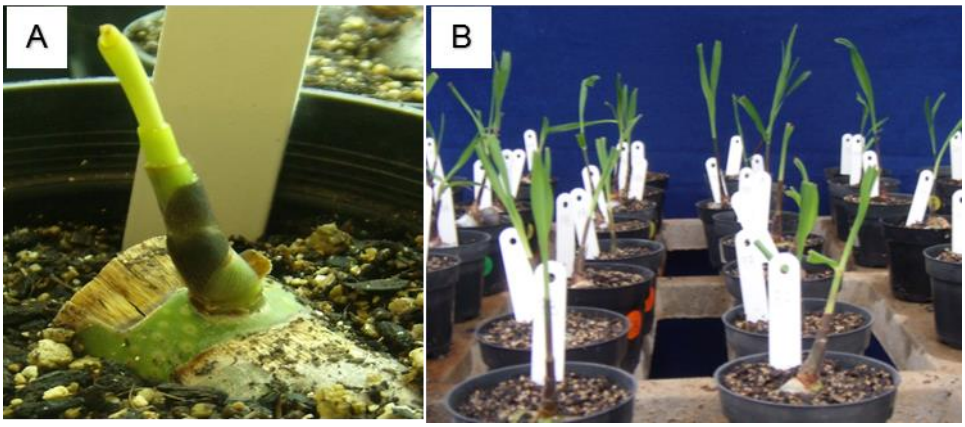


Figure 4. Regenerated Plants at 3 (A) and 10 (B) DAI

Quantitative PCR of the vascular fluid collected from the 180 DAI stems using primers specific for Lxx showed 8-fold higher bacterial titers ($p<0.05$) in the inoculated plants of the susceptible variety compared to the mock-inoculated control ones, whereas for the resistant one there was no significant increase in titers in the Lxx-inoculated plants compared to the mock-inoculated (Figure 5).

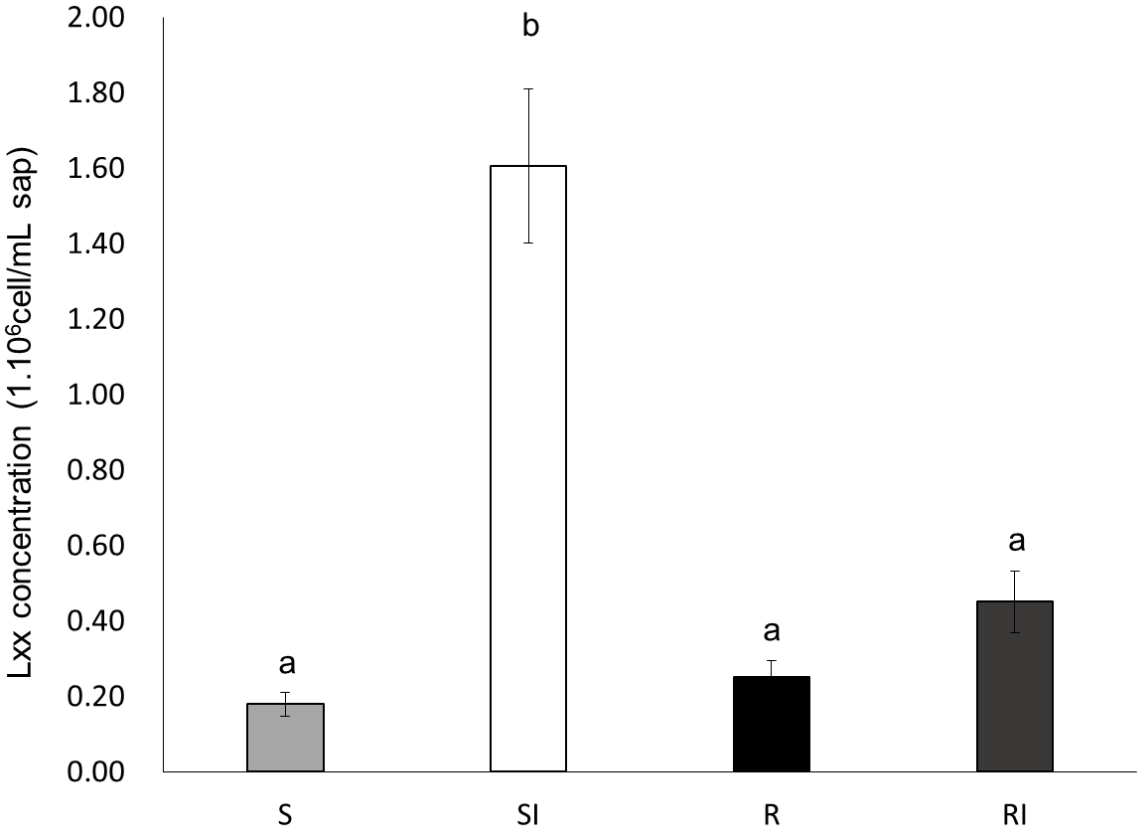


Figure 5. Lxx concentration (x10^6 of bacterial cells/mL of sugarcane juice) at 180 DAI in mock inoculated or inoculated plants of the susceptible (S and SI, respectively) and resistant (R and RI, respectively) varieties. Different letters above bars indicate significant differences between concentration means according to the Tukey test ($p< 0.05$). Bars represent average Lxx concentration ± standard error (n=4).

2.2.8. Untargeted metabolomics detected amino acids, sugars, organic acids and phenolic compounds in sugarcane tissues

Untargeted metabolomics was performed in leaves and xylem sap samples collected at 120 DAI from the CB49-260 (S) and SP80-3280 (R) varieties inoculated or not with Lxx to compare their global metabolic profiles. A total of 114 and 63 metabolites were detected in the leaves and in the sap, respectively (Supplementary material, Table S 2 and S 3).

Principal component analysis (PCA) showed that the global metabolic profiles of the leaves of mock-inoculated plants of the S and R varieties were similar (Figure 6A). However, Lxx-inoculated leaves from the susceptible and resistant varieties were in separate clusters, suggesting distinct metabolic features between CB40-260 and SP80-3280 (Figure 6A). Differences between S and R varieties can be seen even

clearer in the sap, as the distance between clusters from those samples is greater than in leaves (Figure 6B). On the other hand, mock-inoculated and Lxx-inoculated samples were grouped in the same cluster, for both leaf and sap samples, indicating that differences in the global metabolic profiles were larger between varieties than between inoculated and non-inoculated samples (Figure 6A and B).

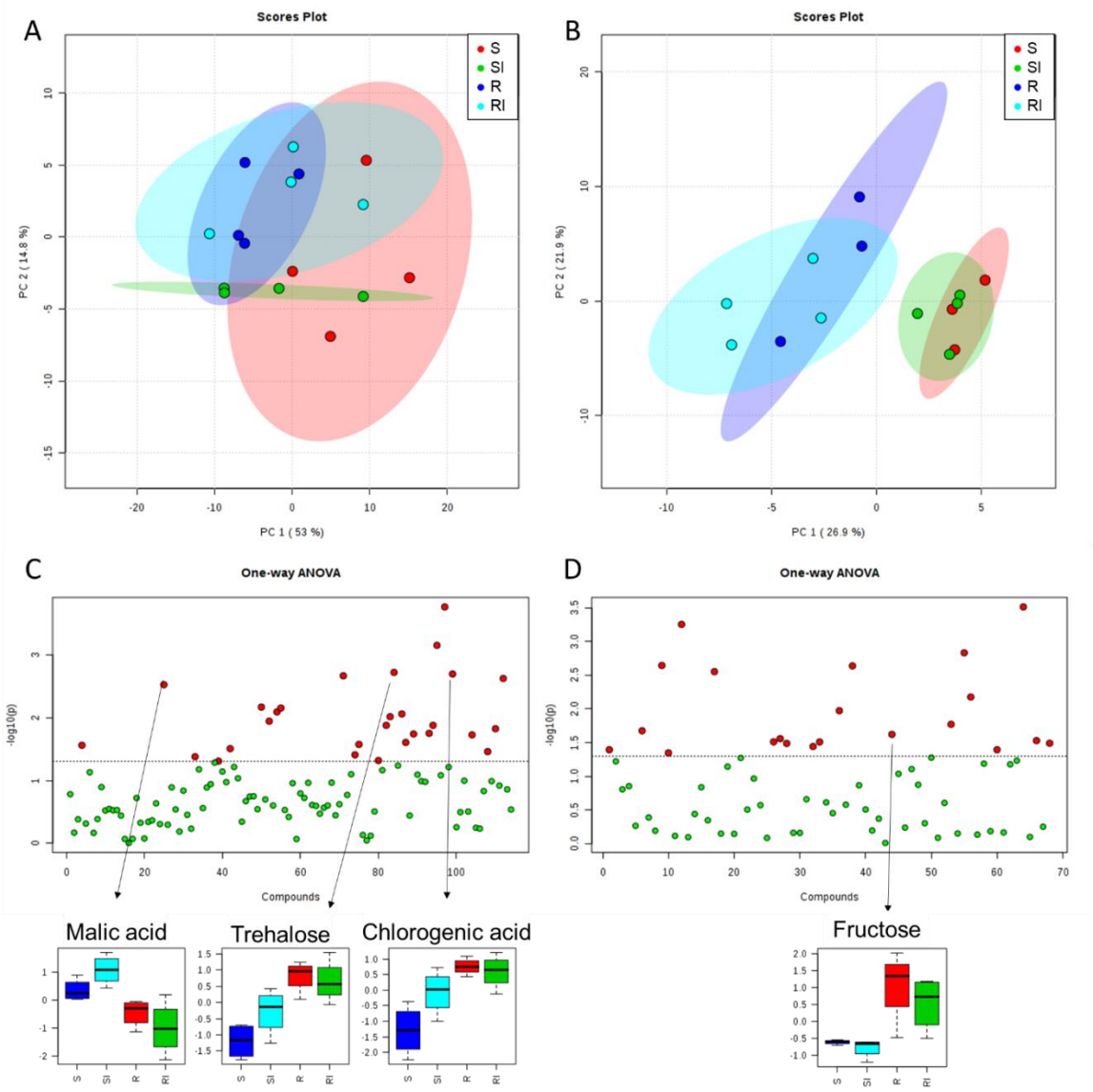


Figure 6. PCA clustering of leaf (A) and xylem sap (B) metabolites from two sugarcane varieties inoculated with Lxx or mock-inoculated analyzed at 120 DAI. Differentially accumulated metabolites identified by the ANOVA (n = 4, $p<0.05$) in the leaves (C) and in the sap (D) are represented by red dots. Normalized abundances of malic acid, trehalose, chlorogenic acid and fructose are depicted as examples. S and SI are susceptible mock-inoculated and Lxx-inoculated,respectively. R and RI are resistant mock-inoculated and Lxx-inoculated, respectively.

One-way ANOVA showed that from all the metabolites, 28 and 22 had differential relative abundances in at least one comparison ($p<0.05$) in the leaves and in the sap, respectively (Figure 6C and 6D). Some of these metabolites were identified using the NIST mass spectral library, and classified into distinct chemical groups, such as sugars, organic acids and phenolics (Table 1). In the leaves, organic acids were detected at higher abundances in the susceptible genotype whereas sugars were more abundant in the resistant (Table 1; Figure 6 and 7). The most striking difference was the higher level of phenolics in the resistant variety when compared to the susceptible. In the sap, even though only a few metabolites were detected and identified, a higher abundance of fructose was observed in the resistant variety, especially in mock-inoculated plants (Table 1; Figure 6 and 7).

Untargeted analysis revealed the main family of compounds found in leaves and xylem of sugarcane plants. However, as the differences among treatments were quantitative rather than qualitative (Figure 7, Table S 2 and S 3), a targeted approach was used to determine the absolute levels of amino acids, sugars, sugar alcohols, organic acids, phosphorylated compounds, phenolics, flavonoids and hormones in the leaves. It is important to note that only sugars and phenolics were targeted in the sap because the detection levels of other metabolites were low.

Table 1. Chemical classes of metabolites differentially represented in sugarcane leaves and xylem sap of mock-inoculated and Lxx-inoculated plants of the susceptible (S and SI, respectively) and resistant (R and RI, respectively) varieties at 30 and 120 DAI detected by untargeted metabolomics. The m/z, retention time (RT), and average relative abundance ± standard errors of the metabolites were determined by the NIST library engine with at least 20% probability. Treatment means were compared by Test-t ($p<0.05$).

Leaves														
class	m/z	RT (min)	Metabolite	S			SI			R			RI		
sugars	361.2	76.95	sucrose	8.13E-03	±	1.88E-03	1.55E-02	±	3.46E-03	3.62E-02	±	4.32E-03	4.07E-02	±	1.38E-02
	361.2	68.83	threalose	2.05E-02	±	2.90E-03	3.21E-02	±	8.06E-03	9.68E-03	±	1.49E-03	1.12E-02	±	2.59E-03
organic acids	147.1	32.68	malic acid	2.67E-02	±	3.16E-03	4.10E-02	±	6.10E-03	1.71E-02	±	2.15E-03	1.37E-02	±	3.73E-03
	317.2	50.68	sebacic acid	8.63E-04	±	2.39E-04	1.11E-03	±	2.44E-04	3.49E-03	±	1.09E-03	3.49E-03	±	7.24E-04
	273.1	45.35	pentaric acid	3.42E-02	±	1.91E-02	7.97E-02	±	2.59E-02	6.85E-02	±	7.51E-03	4.64E-03	±	6.71E-04
	147.1	42.48	aconitic acid	1.79E-02	±	4.81E-03	4.70E-02	±	9.85E-03	1.87E-02	±	1.67E-03	1.45E-02	±	4.28E-03
	292.2	43.68	gluconic acid	4.43E-04	±	1.08E-04	9.21E-04	±	1.51E-04	1.00E-03	±	1.61E-04	8.96E-04	±	2.06E-04
phenolics	371.1	71.40	d-glucopyranosiduronic acid	3.38E-04	±	6.04E-05	6.97E-04	±	1.33E-04	1.59E-03	±	1.41E-04	1.56E-03	±	3.56E-04
	293.2	48.58	coumaric acid	4.09E-04	±	9.80E-05	8.43E-04	±	2.00E-04	1.26E-03	±	1.36E-04	1.02E-03	±	1.76E-04
	345.2	73.98	feruloylquinic acid	5.08E-03	±	1.40E-03	1.24E-02	±	3.20E-03	1.94E-02	±	1.76E-03	1.71E-02	±	4.60E-03
	345.2	73.20	chlorogenic acid	3.54E-02	±	8.13E-03	8.00E-02	±	2.23E-02	1.09E-01	±	9.89E-03	1.06E-01	±	2.14E-02
Xylem sap															
sugar	103.1	47.73	fructose	1.65E-02	±	1.37E-03	1.35E-02	±	2.49E-03	5.71E-01	±	3.70E-01	1.77E-01	±	7.58E-02
organic acid	159.2	13.32	methylmalonic monoamide	3.58E-01	±	8.16E-03	6.15E-01	±	9.53E-02	4.27E-01	±	8.73E-02	6.00E-01	±	6.05E-02

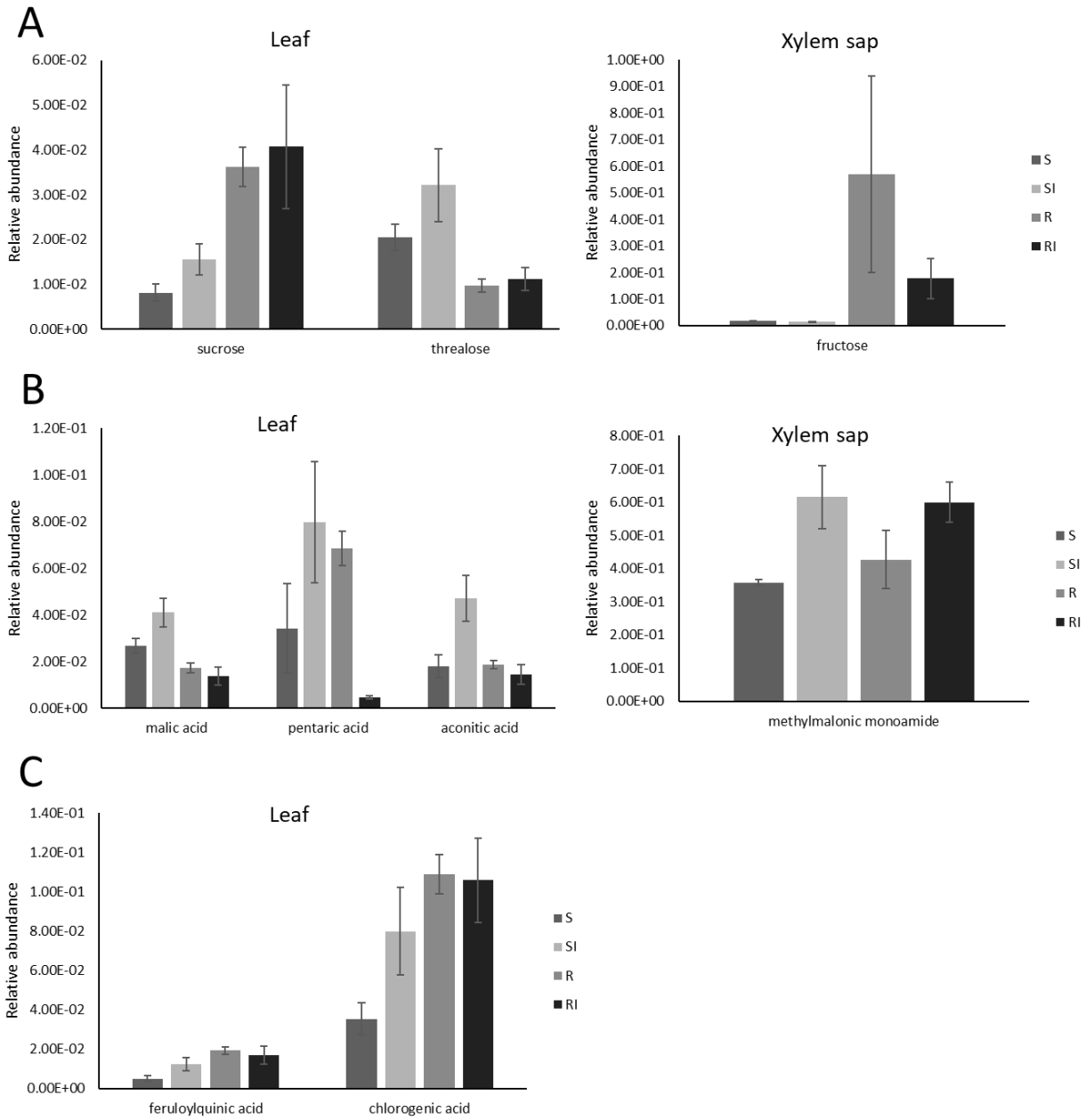


Figure 7. Differentially accumulated sugars (A), organic acids (B) and phenolics (C) detected by untargeted metabolomics of leaf and xylem sap from two sugarcane varieties inoculated with Lxx or mock-inoculated analyzed at 120 DAI. Bars represent metabolites identified by anova ± standard error (n = 4, $p<0.05$). S and SI are susceptible mock-inoculated and Lxx-inoculated,respectively. R and RI are resistant mock-inoculated and Lxx-inoculated, respectively.

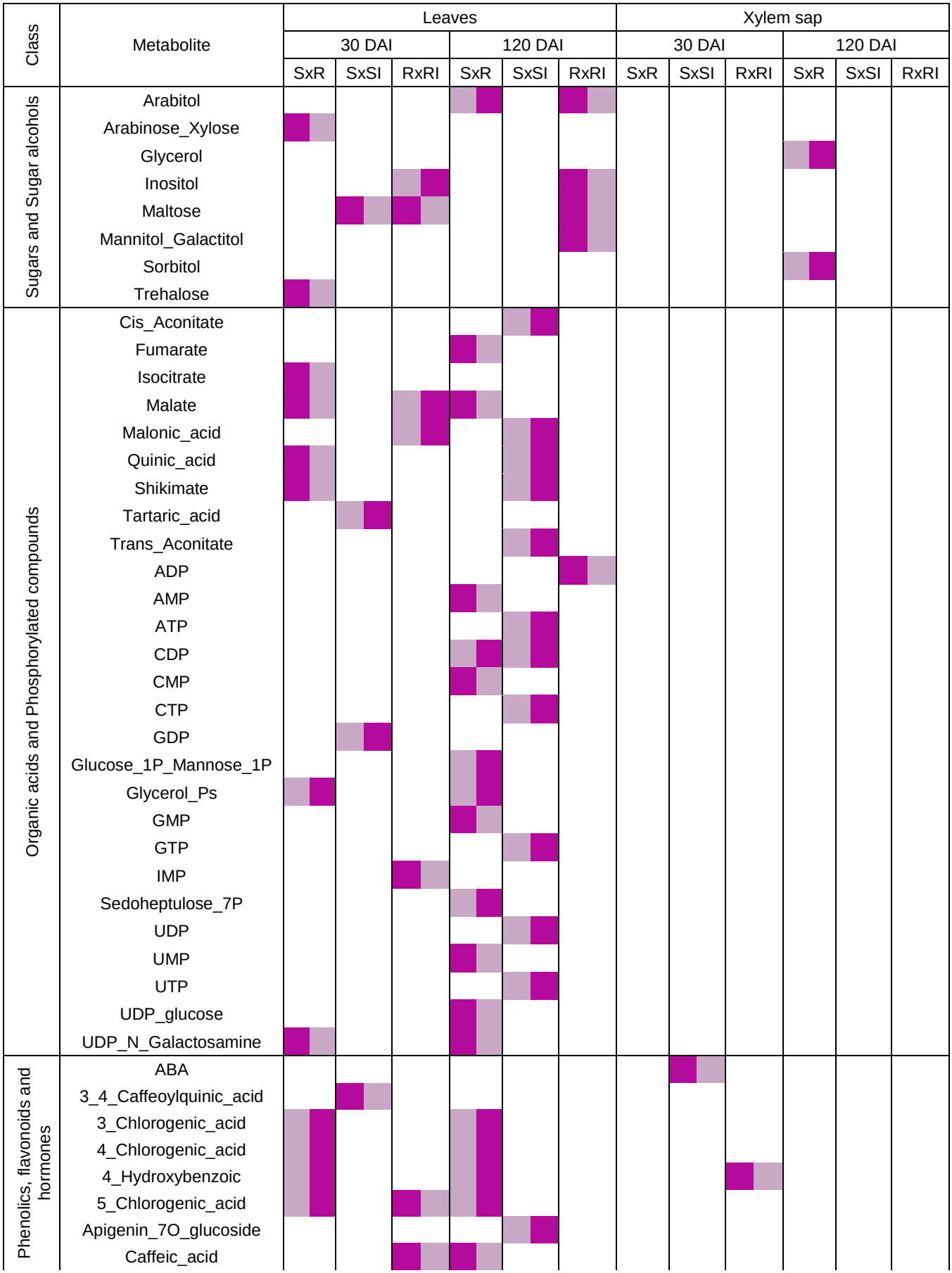
2.2.9. Targeted metabolomics revealed compounds potentially related to resistance and susceptibility

The targeted LC-MS/MS analyses were performed on samples from sugarcane plants mock-inoculated or inoculated with Lxx collected at 30 and 120 DAI

to detect and quantify amino acids, sugars, sugar alcohols, organic acids, phosphorylated compounds, phenolic compounds, flavonoids and hormones. In total, 112 metabolites were quantified in the leaves (supplementary material, Table S 4) and from those, 67 were differentially accumulated in at least one comparison, being 17 amino acids, 6 sugars and sugar alcohols, 27 organic acids and phosphorylated compounds and 17 phenolic compounds, flavonoids and hormones (Table 2). In the xylem sap, 29 metabolites were quantified of which 12 were differentially accumulated, including two sugar alcohols and 10 phenolic compounds, flavonoids and hormones (Table S 5, Table 2). In all cases, PCA indicated greater metabolic differences between DAI than between bacterial or mock-inoculated treatments, as indicated by the formation of distinct clusters for each timepoint of analysis. These results reflect metabolic changes related to the developmental stages of the plant, underlining the need to consider the responses to infection at each stage individually (Figure 8).

Table 2. Differentially accumulated metabolites in sugarcane leaves and xylem sap of the susceptible (S) and resistant (R) varieties mock-inoculated or inoculated (I) at 30 and 120 days after inoculation (DAI) quantified by targeted metabolomics using LC-MS/MS. Treatment means were compared by Test-t ($p<0.05$). Dark purple cells indicate significantly higher and light purple cells indicate significantly lower metabolite accumulation (n=4).

Class	Metabolite	Leaves						Xylem sap					
		30 DAI			120 DAI			30 DAI			120 DAI		
		SxR	SxSI	RxRI	SxR	SxSI	RxRI	SxR	SxSI	RxRI	SxR	SxSI	RxRI
Amino acids	Alanine												
	Citrulline												
	Cysteine												
	GABA												
	Glutamate												
	Glycine												
	Histidine												
	Homoserine												
	Isoleucine												
	Leucine												
	Lysine												
	OHProline												
	Proline												
	Serine												
	Threonine												
	Tyrosine												
	Valine												


Class
Metabolite
Leaves
Xylem sap
30 DAI
120 DAI
30 DAI
120 DAI
SxR
SxSI
RxRI
SxR
SxSI
RxRI
SxR
SxSI
RxRI
SxR
SxSI
RxRI
Sugars and Sugar alcohols
Arabitol
Arabinose_Xylose
Glycerol
Inositol
Maltose
Mannitol_Galactitol
Sorbitol
Trehalose
Organic acids and Phosphorylated compounds
Cis_Aconitate
Fumarate
Isocitrate
Malate
Malonic_acid
Quinic_acid
Shikimate
Tartaric_acid
Trans_Aconitate
ADP
AMP
ATP
CDP
CMP
CTP
GDP
Glucose_1P_Mannose_1P
Glycerol_Ps
GMP
GTP
IMP
Sedoheptulose_7P
UDP
UMP
UTP
UDP_glucose
UDP_N_Galactosamine
Phenolics, flavonoids and hormones
ABA
3_4_Caffeoylquinic_acid
3_Chlorogenic_acid
4_Chlorogenic_acid
4_Hydroxybenzoic
5_Chlorogenic_acid
Apigenin_7O_glucoside
Caffeic_acid

Class	Metabolite	Leaves						Xylem sap					
		30 DAI			120 DAI			30 DAI			120 DAI		
		SxR	SxSI	RxRI	SxR	SxSI	RxRI	SxR	SxSI	RxRI	SxR	SxSI	RxRI
Phenolics, flavonoids and hormones	ConiferylOH_H2O												
	Coumaric_acid												
	Eriodictyol												
	Ferulic_acid												
	FQA_2												
	FQA_3												
	Isoorientin												
	Isovitexin_Vitexin												
	Luteolin_7_O_glc												
	Maysin												
	Orientin												
	pCoQA_1												
	pCoQA_2												
	Rhamisoorientin												
	Salicylic_acid												
	Sinapaldehyde												
	Syringic_acid												

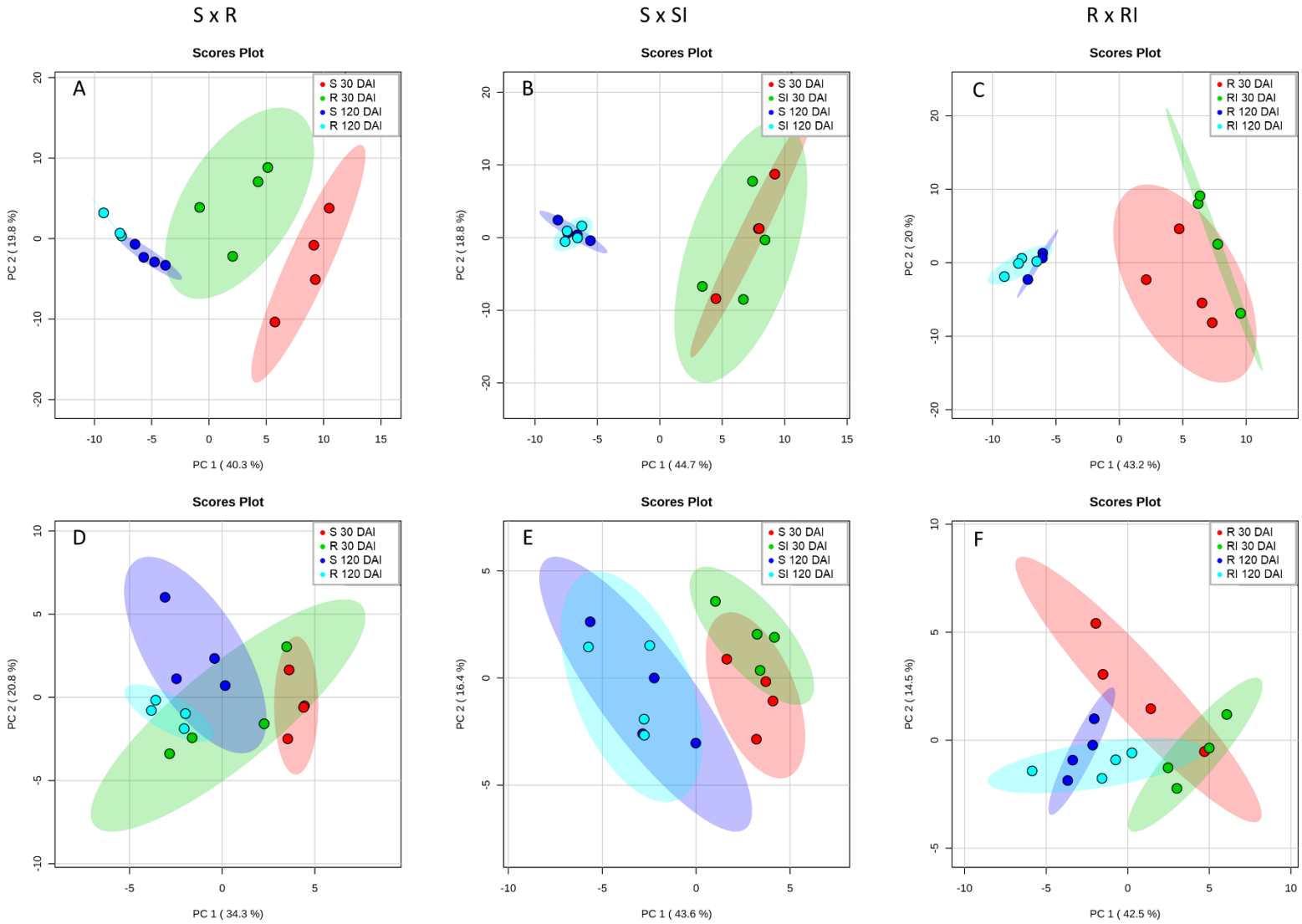


Figure 8. PCA clusters of leaf (A, B, C) and xylem sap (D, E, F) metabolites from sugarcane susceptible mock-inoculated and Lxx-inoculated (S and SI respectively) and resistant mock-inoculated and Lxx-inoculated (R and RI respectively) varieties at 30 and 120 DAI.

In a first comparison, non-inoculated plants of both varieties were contrasted to identify compounds that either stimulate or restrict colonization by the pathogen. PCA analysis of leaf samples showed that the two varieties are metabolically different at both times (Figure 8A) in contrast to the composition of their sap, which did not vary among varieties and DAI as much as in the leaves (Figure 8D). Nonetheless, a change in the composition of the sap was noticed between T30 and T120 in the S variety as the clusters of these treatments did not overlap (Figure 8D). The susceptible variety presented higher abundances of amino acids, sugars and most organic acids and phosphorylated compounds and lower abundances of phenolics in the leaves compared to the resistant one at both DAI (Table 2). All differentially represented amino acids were more abundant in S samples at both timepoints. At 30 DAI, the levels of arabinose, xylose and trehalose were higher in S samples whereas glycerol phosphate was the only type of phosphorylated compound less accumulated in this genotype. At 120 DAI, arabitol was the only sugar differentially accumulated in the leaves, and it was less abundant in the susceptible variety. Additionally, at this time a more diverse set of phosphorylated compounds was detected, including adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), uridine monophosphate (UMP) and uridine diphosphate glucose (UDP-glucose) which were more abundant in the susceptible variety, and glucose-1-phosphate, mannose-1-phosphate and sedoheptulose-7-phosphate which were more abundant in the resistant. Of the phenolics, caffeic acid was the only compound detected at higher levels in the leaves of the susceptible genotype and it was only differentially accumulated at 120 DAI. All other 10 phenolics were present at higher levels in the resistant genotype. As in the leaves, phenolic compounds and flavonoids were less abundant in the sap of the S variety at both timepoints with the exception of luteolin-7-O-glucoside which was the only phenolic found in higher levels in this variety at 30 DAI. There were only two sugars (glycerol and sorbitol) and two phenolics (isovitexin and vitexin) differentially accumulated in the sap at 120 DAI in this comparison, all less abundant in the S genotype (Table 2).

A second comparison was made between Lxx inoculated and mock-inoculated plants of the susceptible variety (SI x S, respectively) at each DAI to find changes in the abundances of metabolites that could enhance the growth of Lxx. The profiles differed between DAI but there was no clear separation between mock and Lxx-inoculated plants within DAI in both the leaves and sap (Figure 8B and 8E). At 30 DAI,

there were no differences between S and SI plants regarding their amino acid composition in the leaves (Table 2). However, all organic acids and phosphorylated compounds that were differentially accumulated were detected at higher levels in SI plants, as did most phenolics, indicating an alteration in the metabolism of these compounds in response to higher bacterial titers as a consequence of inoculation. Maltose and 3,4-caffeoylquinic acid were respectively the only sugar and phenolic less abundant in SI plants. In this comparison, most differences were detected at 120 DAI (Table 2), when cysteine, lysine, apigenin-7-O-glucoside and 2-ferulyquinic acid (FQA-2) accumulated to higher levels and proline to lower levels in the leaves of SI plants. With respect to phenolics, bacterial inoculation resulted in higher levels of FQA-3 at both timepoints. In the 30 DAI sap, no significant differences were found in the amount of sugars or sugar alcohols. On the other hand, abscisic acid (ABA) and luteolin-7-O-glucoside were lower in SI samples, while coumaric acid was higher. At 120 DAI, the only difference related to S x SI was ferulic acid, which was more abundant in SI (Table 2).

Finally, to identify metabolites of the resistant variety that could account for the restricted growth of Lxx and therefore the greater resistance level of this variety to the bacterium, the mock and Lxx-inoculated treatments of this variety (R x RI, respectively) were compared at both 30 and 120 DAI. Similarly to the previous comparisons, leaf profiles differed between the two timepoints but there was no distinct separation between inoculated and non-inoculated treatments (Figure 8C). Contrarily, the xylem sap profiles did not contrast, as their clusters overlapped (Figure 8F). In the leaves, amino acids exhibited opposite patterns at 30 and 120 DAI (Table 2) since most of the compounds found more abundant in RI samples at 30 DAI were detected at lower levels at 120 DAI compared to R plants. As for sugars, organic acids and phenolics, only a few compounds were differentially accumulated in this comparison. At 30 DAI, alanine, glycine, homoserine, isoleucine, leucine, proline and valine were more accumulated in RI leaves (Table 2). Additionally, inositol, malate and malonic acid were also higher in RI samples while cysteine, maltose, inosine monophosphate (IMP), 5-chlorogenic acid, caffeic acid and salicylic acid were lower. At 120 DAI, in sharp contrast to 30 DAI, cysteine was detected at higher levels in RI leaves (Table 2). Also, the levels of citrulline, glutamate, homoserine, threonine, inositol, arabitol and mannitol/galactitol were lower. Adenosine diphosphate (ADP) was the only phosphorylated compound differentially accumulated in this comparison and it was

less abundant in RI leaf samples. In the sap of R and RI plants, there were no differences in the levels of sugars at both timepoints. At 30 DAI, 4-hydrobenzoic, syringic acid and sinapaldehyde were detected at lower levels in RI plants, whereas at 120 DAI ferulic acid was the only phenolic significantly higher in RI plants (Table 2).

Targeted analysis revealed that leaves and xylem sap have distinct metabolic profiles. Additionally, it was shown that amino acids, sugars and organic acids where more abundant in the susceptible genotype compared to the R one while phenolics presented the opposite trend. Higher bacterial titers resulting from inoculation had different effects on the varieties. Organic acid levels were higher in Lxx-inoculated susceptible plants. Amino acids were higher in RI plants at earlier times, notably inverting this pattern later on. Phenolic compounds, like ferulic acid and FQA-3, were more abundant when the plants were inoculated.

2.3. Discussion

Although RSD has been recognized for a long time as an important disease with significant impact on the sugarcane industry, little is known about the mechanisms that control its interaction with the host (Young et al. 2012). Because sugarcane breeding is extremely challenging due to the complex genetic nature of its polyploid genome (Raboin et al. 2006), breeding efforts have traditionally focused only on increasing sugar yields rather than resistance to pathogens. Therefore, metabolomic profiling is a suitable tool to identify plant metabolites that can inhibit bacterial growth, which makes it a suitable tool to find biomarkers to be used in the early selection of resistant genotypes to reduce losses in biomass due to RSD.

In this study, metabolic profiling of leaves and xylem sap of a susceptible and a resistant variety was undertaken to detect changes in metabolism due to Lxx inoculation. The resistance level of the varieties was confirmed by quantifying bacterial titers 180 DAI by real-time qPCR. Because xylem vessels are the principal niche of Lxx, quantification was done in vascular extracts only at the end of the end of the experiment, since it is destructive.

The endophytic behavior of Leifsonia xyli subsp. xyli challenges the search for constitutive response metabolites

Lxx was detected in all treatments, including the mock-inoculated plants Despite the fact that the originating setts came from *in vitro* cultured plants. This can be explained by the fact that that Lxx is not restricted to the xylem vessels as previously assumed (Quecine et al. 2016), so it can still be present in meristem cultured cells. Even though Lxx can colonize sugarcane asymptomatically as an endophyte, it can cause serious yield losses. High bacterial titers (Davis et al. 1988b) and the number of consecutive crops (Grisham 1991) are directly dependent on disease severity, which can be aggravated by environmental conditions, such as prolongated droughts (Ngaruiya et al. 2005; Rott et al. 2002). Other vascular bacteria closely related to Lxx are also characterized as endophytes that can potentially cause disease. For instance, *Clavibacter michiganensis* subsp. *michiganensis*, which causes bacterial canker in tomato, has an endophytic behavior at low titers, but becomes virulent when the population increases (Sen et al. 2015). *Leifsonia xyli* subsp. *cynodontis* is pathogenic to Bermuda grass, causing stunting just like Lxx (Monteiro-Vitorello et al. 2013). However, in other crops like rice, maize, oats and sugarcane itself, it lives as an endophyte (Li et al. 2007). Notwithstanding, bacterial densities in the susceptible genotype (CB49-260) were 8 times greater than in the resistant one and in the mock-inoculated plants, confirming its susceptibility to RSD and also the greater level of resistance of SP80-3280. Furthermore, plants of the resistant genotype were able to maintain low endophytic levels of Lxx as there was no significant differences between the mock and Lxx-inoculated treatments. Thus, differently than CB49-260, SP80-3280 has the capacity to control Lxx growth. However, having the bacterium in the plants previously to inoculation makes it impossible to define if the metabolites detected in the mock-inoculated treatments are preformed or induced by the Lxx. Therefore, this study alone could not determine if the mechanisms used to reduce Lxx growth were already present in the resistant plants or were induced by inoculation. On the other hand, previous transcriptomics results from the resistant variety inoculated with Lxx predicted a low number of genes related to defense at early stage after inoculation when compared to the mock (Cia 2014). These results combined with the present study suggest that the resistant plants have a naturally enhanced defense to Lxx

colonization; a large pool of defense metabolites could explain why the bacterial titer was maintained at an endophytic level even with inoculation.

Untargeted metabolomics of sugarcane leaves disclosed different metabolic profiles between varieties

Global profiling of sugarcane was performed at 120 DAI and identified the presence of major classes of compounds (amino acids, sugars, organic acids and phenolics) in the leaves. The susceptible variety had higher abundances of organic acids , whereas the resistant plants were found to have higher levels of sugars. The number of metabolic studies in sugarcane metabolome is still very limited, especially when it comes to plant-pathogen interaction. Nonetheless, the compound classes detected in the untargeted analysis of leaves agrees with previous studies on sugarcane and other grasses. For instance, increased levels of amino acids, sugars and organic acids were also found in sugarcane plants inoculated with *Sporisorium scitamineum*, the causal agent of smut disease (Schaker et al. 2017). The differences between a susceptible and a resistant rice lineages to *Rhizoctonia solani* also involved alterations in the accumulation of organic acids and phenolics (Suharti et al. 2016). A non-targeted approach on sorghum leaves from different lineages identified similar classes of compounds found in this study (Turner et al. 2016), as distinct lineages grouped according to amino acid and phenolic compositions in the leaves.

As for the analysis of the xylem fluid, fewer metabolites were found when compared to the leaves. Still, it was possible to determine higher fructose levels in the susceptible genotype opposed to what was found in the leaves. To this date, no studies on sugarcane guttation fluid have been performed and this is the first report that compares the metabolites of the xylem sap obtained from guttation drops with those from leaves of sugarcane plants inoculated with Lxx. Since the xylem vessels are the main niche of Lxx (Monteiro-Vitorello et al. 2004a; Li et al. 2014), we expected to find differences in metabolite composition, especially those related to plant defense and bacterial growth, due to higher titers of the bacteria. However, detecting metabolites in the xylem sap from guttation drops is challenging due to their low concentrations (most molecules were found to be close to the limits of detection). Another difficulty found in analyzing sap samples was the great variability among replicates. Our methodology involved collecting drops from the hydathodes, which required repetitive sampling

because the volume obtained in each collection was very small. As the guttation rate varies among plants it is possible that the collected drops had different concentrations of metabolites, thus leading to variation between plants, as suggested by Schurr (1998). While this study relates xylem sap with pathogenesis, most research involving vascular extract composition deals with abiotic stress, such as drought (Alvarez et al. 2008); salt (Fernandez-Garcia et al. 2011) and mineral deficiency (Rellán-Álvarez 2011). Interestingly, similar phenolic compounds were detected in maize plants under water stress, which exhibited higher abundances of most detected metabolites, especially ferulic and coumaric acids (Alvarez et al. 2008). However, increased levels of amino acids were found in maize compared to sugarcane. It is important to note that since guttation drops are exuded from the plant, the detected compounds might not represent the whole set of metabolites inside sugarcane, as molecules that are not in exceeding concentrations can remain undetected inside the xylem. As a matter of fact, phenolic analysis of grapevines infected with Pierce's disease (caused by *Xylella fastidiosa*, which is a vascular bacterium) revealed diversified composition between xylem compartments (Wallis and Chen 2012). Stilbenoids and procyanidins were found in xylem tissues while astringin and digalloylquinic acid were found in the xylem sap. Furthermore, most metabolites found in the sap were unknown to the NIST library, meaning that there are still potential biochemical compounds that need to to be identified. Similarly, another study that compared the global profiles of xylem saps from different plant species subjected to iron deficiency related that more than 70% of the detected compounds were unknown (Rellán-Álvarez 2011).

Even though there were differences among varieties, there were no significant changes related to inoculation. Additionally, those differences among profiles were more quantitative than qualitative, reinforcing the need of a more precise, sensitive and quantitative analysis. As the untargeted metabolomic profiling of leaves and sap detected sugars, organic acids and phenolics, these classes of metabolites were targeted by LC MS/MS and quantified from samples collected at 30 and 120 DAI.

Targeted metabolomics revealed that the susceptible CB49-260 variety has higher accumulation of potential bacterial growth enhancers and lower of potential inhibitors

Compared to samples from mock-inoculated resistant plants, susceptible plants showed greater content in many amino acids: alanine, citrulline, GABA,

glutamate, glycine, homoserine, OH-proline, proline, serine, threonine, tyrosine, and valine in the leaves. Amino acids are key elements for protein production and essential for regulating many bacterial processes (Radkov and Moe 2014), like quorum sensing (Whitehead et al. 2001) and cell wall integrity (Vollmer et al. 2008). Therefore, greater abundances in the plant could enhance bacterial growth. The sugars arabitol, arabinose/xylose and trehalose were also more abundant in susceptible. Actually, Lxx is cultivated *in vitro* with glucose, but since the bacterium has many ABC transporter homologs, it could use diverse sugars as carbon sources, such as arabinose, maltose, trehalose, and xylose (Evtushenko et al. 2015). Also, citrus plants more susceptible to Huanglongbing (HLB) showed higher abundances of amino acids in comparison to more resistant plants (Cevallos-Cevallos et al. 2012). The plants had significant higher levels of proline, serine and aspartic acid when compared to two other varieties, one tolerant and one resistant to HLB.

Mock-inoculated resistant plants had a higher accumulation of phenolic acids and flavonoids, when compared to the susceptible variety. Phenolics are known for their antimicrobial activity and for having a role in plant defense (Lou et al. 2011; Nascimento et al. 2000). For instance, the virulence of the causal agent of soft rot, *Dickeya dadantii*, is impaired by phenolic derivatives produced by the host (Li et al. 2015). They act by inhibiting the transcription factors that regulate the expression of the type III secretion system (T3SS), which is essential for pathogenesis. Also, glucosinolates and phenolic compounds suppressed the development of *Xanthomonas campestris* pv. *campestris*, which causes clubroot disease in *Brassicaceae* (Velasco et al. 2013). Some phenolics like sinapic acid have a constitutive effect against the bacterium when inoculated in *Brassica rapa*, while others such as 3-caffeoyl-quinnic acid and kaempferol-3-O-(sinapoyl)-sophoroside-7-O-glucoside are induced after inoculation with the pathogen. Moreover, potato constituents like cinnamic, syringic and coumaric acids were found to inhibit the growth of pectolytic bacteria, causal of soft rots in many fruits (Joshi et al. 2015). Additionally, the present study revealed that salicylic acid, a hormone involved in plant defense (Grant and Jones 2009), was more abundant in resistant leaf samples. Finally, xylem sap from the non-inoculated resistant variety also exhibited more phenolic compounds and flavonoids, which could be good marker candidates for RSD resistance.

Lxx inoculated plants accumulate more amino acids, organic acids, phosphorylated compounds and phenolics

Lxx-inoculated plants of the susceptible variety exhibited higher concentrations of the amino acids cysteine and lysine. Cysteine is a key compound for Lxx since the bacterium presents truncated sulfite/sulfate pathways to synthesize it, as cysteine synthase is not activated in Lxx (Monteiro-Vitorello et al. 2004a). Therefore, it has to be added *in vitro* for the cultivation of Lxx. The fact that these compounds are present in higher concentration in the CB49-260 inoculated plants suggests that Lxx stimulates its host to produce essential compounds that it cannot synthesize alone. Actually, transcriptomics studies revealed that methionine synthase is more expressed in plants inoculated with Lxx (Cia 2014). Because methionine synthesis is directly linked with cysteine metabolism (Brosnan and Brosnan 2006), these results are consistent with metabolomics findings. Interestingly, Lxx-inoculated leaves of the resistant plants had temporarily more amino acids at an early stage, reversing that pattern later except for cysteine. A possible interpretation is that even at a basal level of endophytic colonization, Lxx stimulates the plant to produce cycteine, since this metabolite is crucial for its survival. Also,reduction of other amino acids in R imply that later on those compounds that could enhance the bacterial growth are not available in greater quantities. Besides, amino acid pathways have been linked with plant immunity (Zeier 2013). For instance, pipecolic acid, which derives from lysine catabolism, is a key regulator of systemic acquired resistance and priming defense in Arabidopsis (Bernsdorff et al. 2016; Ding et al. 2016; Návarová et al. 2012). Initial higher amounts of amino acids in R could indicate more resistance related activity.

Most changes in susceptible plants were observed at 120 DAI, suggesting that the mock and the inoculated plants had more contrasting metabolism at a later time. There was a higher relative abundance of organic acids and phosphorylated compounds in inoculated plants, which indicates increased synthesis rates or reduced transportation of these metabolites to sink organs as a function of higher bacterial titers. Even though all plants were under the same conditions, measurements of the photosynthetic rate would have helped determining if higher levels of phosphorylated compounds were due to a higher photosynthetic performance. In sugarcane, photosynthesis tends to decline as the plant matures because sink organs have reduced demands of sugar production and therefore sucrose is more accumulated

over time (McCormick A J et al. 2005). There was no significant differentiation in sucrose content among infected and non-inoculated plants for both varieties, indicating that there were no higher demands from the plants themselves. Actually in another study, Lxx-inoculated plants were found to have decreased photosynthetic, stomatal conductance, transpiration rates and reduced intercellular CO_2 (Zhang et al. 2017a). Although this study seems to contradict the present findings, it is important to note that the measurements were made on a later period after inoculation (starting from 150 until 240 DAI) and the authors did not relate their findings with bacterial titers (which was only measured in the seedlings, on an unreported period of time). Also, despite analyzing three varieties, their measurements were pooled, which hindered the relationship between a possible resistance or susceptibility to Lxx inoculation which could show up if the three varieties were analysed separately.

Susceptible plants had more abundance of the phenolics apigenin-7-O-glucoside and ferulyquinic acid at 120 DAI, when compared to mock-inoculated plants. Resistant plants inoculated with Lxx, on the other hand, did not show any differentially accumulation regarding phenolics compared to the mock at a later stage. This can represent a differential response to inoculation between the two varieties, with a late response from the susceptible plants. Because resistant plants have naturally higher amounts of phenolics, their production might not be longer necessary if the plants manage to maintain the bacterial titers on a basal limit. Susceptible plants, on the other hand, did not repress Lxx growth, possibly because they have lower phenolic contents at an earlier stage after inoculation, increasing the amount of phenolics only later on. Actually, two varieties of Eucalyptus responded in a similar way to *Phytophthora cinnamomic* infection (Cahill and McComb 1992). The more resistant variety had a greater amount of total phenolics when compared to the susceptible one, increasing its levels right after inoculation. The susceptible plants, on the other hand, delayed the increase the total phenolic content, which was only observed at a later time. Also, phenolic content was associated with leaf disease resistance in black currant (Vagiri et al. 2017). Higher accumulations of flavonols like quercetin-3-O-glucoside and catechin and phenolics, such as chlorogenic acid were directly correlated with more resistance to powdery mildew, rust, septoria and anthracnose.

Inoculated resistant plants had few metabolic changes on primary and secondary metabolism

Only a few sugars and organic acids were differentially represented in the resistant genotype when inoculated plants were compared to the mock-inoculated. At 30 DAI, the only three phenolic acids detected in this comparison were less accumulated in the inoculated leaves, and three other also were less abundant in the sap. Interestingly, these findings are consistent with previous transcriptomics and proteomics results (Cia 2014; Carvalho 2012). Genes related to plant defense mechanisms, like 4-coumarate CoA ligase, were down-regulated in SP80-3280 plants inoculated with Lxx. Additionally, a protein related to salicylic acid (SA) stimulation, calreticulin, was found to be less abundant in Lxx-inoculated plants, which is in agreement with the lower abundance of SA in resistant inoculated plants in the current study, since calreticulin accumulation is directly related to SA biosynthesis (Qiu et al. 2012). Transcriptomics results did not show any SA related gene differentially expressed (Cia 2014), which indicates that this metabolite is not induced by Lxx inoculation.

Higher bacterial titers is associated with higher ferulic acid and lower ABA content in the sap

Regarding inoculation, there were no significant differences between the metabolic profiles of the saps from the two varieties under investigation. Consistent with this study, xylem sap from two varieties of maize inoculated with growth-promoting bacteria exhibited similar metabolic profiles, showing more quantitative differences than qualitative (Rozier 2016).

At an earlier stage, sap samples from Lxx-inoculated susceptible plants revealed lower levels of ABA, an important hormone related to plant defense (Baccelli and Mauch-Mani 2016; Pandey et al. 2016; AbuQamar et al. 2017; Krishnan et al. 2016). This could indicate defense suppression by Lxx inside the tissue where this bacterium is preponderant. Intriguingly, previous transcriptomics results from the resistant variety reported the higher expression of genes related to ABA synthesis and defense at a later stage after inoculation (Cia 2014). Even though the tissues analyzed were leaves (instead of vascular fluids), it highlights a late response to inoculation.

Furthermore, ferulic acid was increased at 120 DAI in both inoculated varieties, suggesting its production as a response to inoculation. Nonetheless, the resistant genotype presented higher abundance of this compound. Ferulic acid is a highly oxidized phenolic: when applied to different strains of Gram-positive and negative human pathogenic bacteria, it caused damage to the bacterial membranes, resulting in ion leakage (Borges et al. 2013). Another study showed that ferulic and gallic acid also inhibited biofilm formation in the same bacterial group (Borges et al. 2012). However, this same metabolite was found to interact positively with *Ralstonia solanacearum*, as it enhanced its infection in tomato and tobacco by inducing the expression of the type III secretion system (Zhang et al. 2017b). Higher accumulation of ferulic acid was also observed in the sap of drought stressed maize plants (Alvarez et al. 2008). Our findings suggest that this metabolite is involved in biotic stress response as well, but additional experiments are needed to understand the mechanism involved in this interaction. Enzymatic studies on Lxx morphology and metabolism can define if ferulic acid addition is detrimental to sugarcane defense. Additionally, it is an important component of sugarcane cell wall (Xu et al. 2005) by improving its rigidity (Kumar and Pruthi 2014). Higher accumulations of ferulic acid due to inoculation suggest that the plant is reinforcing its cell walls to avoid deterioration, which could be possible as Lxx has 6 genes related to host cell degradation (Monteiro-Vitorello et al. 2004a). Interestinlgy, anatomical features in the xylem vessels were previously associated with Lxx titers (Davis et al. 1988a; Teakle et al. 1975). Increased branching in the nodal region of the xylem vessels in more resistant varieties resulted in a reduced Lxx concentration when compared to more susceptible plants that have less branching. These facts suggest that sugarcane plants composed of xylem vessels with more structural branching and rigid cell walls are more resistant to Lxx colonization.

2.4. Conclusion and perspectives

Lxx inoculation and detection confirmed that the variety CB49-260 had higher bacterial titers, which reflects its susceptibility to RSD. Accordingly, the variety SP80-3280 had low bacterial titers, that did not increase significantly above the endophyte basal level even with artificial inoculation. This fact confirms that this variety is the more resistant genotype. Untargeted and targeted metabolomic profiling of leaf and sap revealed that amino acids, sugars and sugar alcohols, organic acids, phosphorylated

compounds, phenolic acids, flavonoids and hormones accumulated to distinct levels according to each variety and inoculation with Lxx. While the susceptible plants had higher abundances of amino acids, sugars and organic acids, the resistant plants had more phenolics. When inoculated, plants from both genotypes accumulated more amino and organic acids, however, they had less sugars and sugar alcohols. The accumulation of phenolics varied among the genotypes and tissues.

Further experiments would be needed to check if a selection of metabolites can be used as markers of resistance to RSD. Since the shikimic acid phenylpropanoid and flavonoid synthetic pathways have been elucidated, gene expression and enzymatic studies can be performed to enlighten the direction and production of phenolics by Lxx-inoculated sugarcanes and the differences among distinct varieties. Additionally, *in vitro* experiments can further demonstrate the effect of the resistance related compounds on the bacterium, by adding them to its culture medium and measuring the effects on growth, viability, cell-membrane, and osmotic stability. The knowledge resulting from these studies will clarify much of the unveiled mechanisms between Lxx and its natural host, sugarcane. Determining resistance-related molecules and establishing biomarkers will facilitate the breeding selection for more resistant genotypes to RSD, a necessary tool to reduce the crop losses caused by this disease that is present in all sugarcane producing regions.

References

AbuQamar, S., Moustafa, K., and Tran, L. S. 2017. Mechanisms and strategies of plant defense against *Botrytis cinerea*. Crit. Rev. Biotechnol. 37:262–274

Alvarez, S., Marsh, E. L., Schroeder, S. G., and Schachtman, D. P. 2008. Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant. Cell Environ. 31:325–340

Baccelli, I., and Mauch-Mani, B. 2016. Beta-aminobutyric acid priming of plant defense: the role of ABA and other hormones. Plant Mol. Biol. 91:703–711

Bernsdorff, F., Döring, A.-C., Gruner, K., Schuck, S., Bräutigam, A., and Zeier, J. 2016. Pipecolic acid orchestrates plant systemic acquired resistance and defense priming via salicylic acid-dependent and -independent pathways. Plant Cell. 28:102–29

Borges, A., Ferreira, C., Saavedra, M. J., and Simões, M. 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb. Drug Resist. 19:256–265

Borges, A., Saavedra, M. J., and Simões, M. 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. Biofouling. 28:755–767

Brosnan, J. T., and Brosnan, M. E. 2006. The sulfur-containing amino acids: an overview. J. Nutr. 136:1636S–1640S

Cahill, D. M., and McComb, J. A. 1992. A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of Eucalyptus calophylla (field resistant) and E. marginata (susceptible) when infected with Phytophthora cinnamomi. Physiol. Mol. Plant Pathol. 40:315–332

Carvalho, G. 2012. Proteome and antioxidant system analysis of sugarcane in response to Leifsonia xyli subsp. xyli, causal agent of ratoon stunting disease.

Carvalho, G., da Silva, T. G. E. R., Munhoz, A. T., Monteiro-Vitorello, C. B., Azevedo, R. A., Melotto, M., and Camargo, L. E. A. 2016. Development of a qPCR for Leifsonia xyli subsp. xyli and quantification of the effects of heat treatment of sugarcane cuttings on Lxx. Crop Prot. 80:51–55

Cevallos-Cevallos, J. M., Futch, D. B., Shilts, T., Folimonova, S. Y., and Reyes-De-Corcuera, J. I. 2012. GC-MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing. Plant Physiol. Biochem. 53:69–76

Cia, M. C. 2014. Análise transcritômica de plantas de cana de açúcar inoculadas com Leifsonia xyli subsp. xyli, agente causal do raquitismo das soqueiras.

Cocuron, J. J.-C., Anderson, B., Boyd, A., and Alonso, A. P. A. 2014. Targeted metabolomics of Physaria fendleri, an industrial crop producing hydroxy fatty acids. Plant Cell Physiol. 55:620–33

Dal-Bianco, M., Carneiro, M. S., Hotta, C. T., Chapola, R. G., Hoffmann, H. P., Garcia, A. A. F., and Souza, G. M. 2012. Sugarcane improvement: How far can we go? Curr. Opin. Biotechnol. 23:265–270

Davis, M. J., Dean, J. L., and Harrison, N. A. 1988. Quantitative variability of Clavibacter xyli subsp. xyli populations in sugarcane cultivars differing in resistance to ratoon stunting disease. Phytopathology. 78:462–468

Davis, M. J., Gillaspie JR, A. G., Vidaver, A. K., and Harris, R. W. 1984. Clavibacter : a New Genus Containing Some Phytopathogenic Coryneform Bacteria , Including Clavibacter xyli subsp . xyli sp . nov ., subsp . nov . and Clavibacter xyli subsp . cynodontis subsp . nov . Pathogens That Cause Ratoon Stunting Disease of Sugarca. Int. J. Syst. Bacteriol. 34:107–117

Ding, P., Rekhter, D., Ding, Y., Feussner, K., Busta, L., Haroth, S., Xu, S., Li, X., Jetter, R., Feussner, I., and Zhang, Y. 2016. Characterization of a pipecolic acid biosynthesis pathway required for systemic acquired resistance. Plant Cell. 28:2603–2615

Evtushenko, L., Dorofeeva, L. V, Subbotin, S. A., Cole, J. R., and Tiedje, J. M. 2015. Leifsonia. Pages 1–32in:Bergey’s Manual of Systematics of Archaea and Bacteria,

Fernandez-Garcia, N., Hernandez, M., Casado-Vela, J., Bru, R., Elortza, F., Hedden, P., and Olmos, E. 2011. Changes to the proteome and targeted metabolites of xylem sap in Brassica oleracea in response to salt stress. Plant. Cell Environ. 34:821–836

Grant, M. R., and Jones, J. D. G. 2009. Hormone (Dis)harmony Moulds Plant Health and Disease. Science (80-.). 324:750–752

Grisham, M. 1991. Effect of ratoon stunting disease on yield of sugarcane grown in multiple three-year plantings. Phytopathology. 91

Joshi, J. R., Burdman, S., Lipsky, A., and Yedidia, I. 2015. Effects of plant antimicrobial phenolic compounds on virulence of the genus Pectobacterium. Res. Microbiol. 166:535–545

Krishnan, H. B., Alaswad, A. A., Oehrle, N. W., and Gillman, J. D. 2016. Deletion of the *sacpd-c* locus alters the symbiotic relationship between *Bradyrhizobium japonicum* usda110 and soybean, resulting in elicitation of plant defense response and nodulation defects. Mol. Plant-Microbe Interact. 29:862–877

Kumar, N., and Pruthi, V. 2014. Potential applications of ferulic acid from natural sources. Biotechnol. Reports. 4:86–93

Li, T. Y., Zeng, H. L., Ping, Y., Lin, H., Fan, X. L., Guo, Z. G., and Zhang, C. F. 2007. Construction of a stable expression vector for Leifsonia xyli subsp. cynodontis and its application in studying the effect of the bacterium as an endophytic bacterium in rice. FEMS Microbiol. Lett. 267:176–183

Li, W.-F. F., Shen, K., Huang, Y.-K. K., Wang, X.-Y. Y., Yin, J., Luo, Z.-M. M., Zhang, R.-Y. Y., and Shan, H.-L. L. 2014. Incidence of sugarcane ratoon stunting disease in the major cane-growing regions of China. Crop Prot. 60:44–47

Li, W.-F., Shen, K., Huang, Y.-K., Wang, X.-Y., Luo, Z.-M., Ying, X.-M., Yin, J., Ma, L., Shan, H.-L., and Zhang, R.-Y. 2013. PCR detection of ratoon stunting disease pathogen and natural resistance analysis in sugarcane core germplasms. Crop Prot. 53:46–51

Li, Y., Hutchins, W., Wu, X., Liang, C., Zhang, C., Yuan, X., Khokhani, D., Chen, X., Che, Y., Wang, Q., and Yang, C. H. 2015. Derivative of plant phenolic compound inhibits the type III secretion system of Dickeya dadantii via HrpX/HrpY two-component signal transduction and Rsm systems. Mol. Plant Pathol. 16:150–163

Lou, Z., Wang, H., Zhu, S., Ma, C., and Wang, Z. 2011. Antibacterial activity and mechanism of action of chlorogenic acid. J. Food Sci. 76

McCormick A J, Cramer M C, and Watt D A. 2005. Sink strength affects assimilate distribution and photosynthesis in sugarcane. Proc. South African Sugar Technol. 79

McFarlane, S. 2002. The relationship between extent of colonisation by Leifsonia xyli subsp. xyli and yield loss in different sugarcane varieties. Proc S Afr Sug Technol Ass. 76:281–284

Monteiro-Vitorello, C. B., Camargo, L. E. A., Van Sluys, M. A., Kitajima, J. P., Truffi, D., Do Amaral, A. M., Harakava, R., De Oliveira, J. C. F., Wood, D., De Oliveira, M. C., Miyaki, C., Takita, M. A., Da Silva, A. C. R., Furlan, L. R., Carraro, D. M., Camarotte, G., Almeida, N. F., Carrer, H., Coutinho, L. L., El-Dorry, H. A., Ferro, M. I. T., Gagliardi, P. R., Giglioti, E., Goldman, M. H. S., Goldman, G. H., Kimura, E. T., Ferro, E. S., Kuramae, E. E., Lemos, E. G. M., Lemos, M. V. F., Mauro, S. M. Z., Machado, M. A., Marino, C. L., Menck, C. F., Nunes, L. R., Oliveira, R. C., Pereira, G. G., Siqueira, W., De Souza, A. A., Tsai, S. M., Zanca, A. S., Simpson, A. J. G., Brumbley, S. M., and Setúbal, J. C. 2004a. The genome sequence of the gram-positive sugarcane pathogen Leifsonia xyli subsp. xyli. Mol. Plant-Microbe Interact. 17:827–836

Monteiro-Vitorello, C. B., Zerillo, M. M., Sluys, V., Camargo, L. E. A. 2004b. Genome sequence-based insights into the biology of the sugarcane pathogen *Leifsonia xyli* subsp. *xyli*. in: Plant Pathogenic Bacteria - Genomics and Molecular Biology.

Monteiro-Vitorello, C. B., Zerillo, M. M., Sluys, M.-A. Van, Eduardo, L., Camargo, A., Kitajima, J. P., Monteiro-Vitorello, C. B., Zerillo, M. M., Van Sluys, M.-A., Camargo, L. E. A., and Kitajima, J. P. 2013. Complete genome sequence of Leifsonia xyli subsp. cynodontis strain DMS46306, a gram positive bacterial a pathogen of grasses. Genome Announc. 1:2000–2001

Nascimento, G. G. F., Locatelli, J., Freitas, P. C., Silva, G. L., and Piracicaba, U. M. De. 2000. Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic-. Brazilian J. Microbiol. 31:247–256

Návarová, H., Bernsdorff, F., Döring, A.-C., and Zeier, J. 2012. Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. Plant Cell. 24:5123–41

Ngaruiya, P. N., Shipton, W. A., and Coventry, R. 2005. Ratoon stunting disease of sugarcane as influenced by environmental stressors. Proc Aust Soc Sugar Cane Technol. 27:324–333

Pandey, D., Rajendran, S. R. C. K., Gaur, M., Sajeesh, P. K., and Kumar, A. 2016. Plant Defense Signaling and Responses Against Necrotrophic Fungal Pathogens. J. Plant Growth Regul. 35:1159–1174

Qiu, Y., Xi, J., Du, L., Roje, S., and Poovaiah, B. W. 2012. A dual regulatory role of Arabidopsis calreticulin-2 in plant innate immunity. Plant J. 69:489–500

Quecine, M. C., Silva, T. M., Carvalho, G., Saito, S., Mondin, M., Teixeira-Silva, N. S., Camargo, L. E. A., and Monteiro-Vitorello, C. B. 2016. A stable Leifsonia xyli subsp. xyli GFP-tagged strain reveals a new colonization niche in sugarcane tissues. Plant Pathol. 65:154–162

Raboin, L.-M., Oliveira, K. M., Lecunff, L., Telismart, H., Roques, D., Butterfield, M., Hoarau, J.-Y., and D'Hont, A. 2006. Genetic mapping in sugarcane, a high polyploid, using bi-parental progeny: identification of a gene controlling stalk colour and a new rust resistance gene. Theor. Appl. Genet. 112:1382–1391

Radkov, A. D., and Moe, L. A. 2014. Bacterial synthesis of d-amino acids. Appl. Microbiol. Biotechnol. 98:5363–5374

Rellán-Álvarez, R. 2011. Metabolite profile changes in xylem sap and leaf extracts of strategy I plants in response to iron deficiency and resupply. Front. Plant Sci. 2:1–18

Rott, P., Sood, S., Comstock, J. C., Gilbert, R. A., and Sandhu, H. S. 2002. *Sugarcane Ratoon Stunting*.

Rozier, C. 2016. Xylem sap metabolite profile changes during phytostimulation of maize by the plant growth-promoting Rhizobacterium, *Azospirillum lipoferum* CRT1. J. Postgenomics Drug Biomark. Dev. 6

Schaker, P. D. C., Peters, L. P., Cataldi, T. R., Labate, C. A., Caldana, C., and Monteiro-Vitorello, C. B. 2017. Metabolome dynamics of smutted sugarcane reveals mechanisms involved in disease progression and whip emission. Front. Plant Sci. 8:882

Schurr, U. 1998. Xylem sap sampling—new approaches to an old topic. Trends Plant Sci. 3:293–298

Sen, Y., Van Der Wolf, J., Visser, R. G. F., and Van Heusden, S. 2015. Bacterial canker of tomato: current knowledge of detection, management, resistance and interactions. Plant Dis. 99

Suharti, W. S., Nose, A., and Zheng, S. H. 2016. Metabolomic study of two rice lines infected by Rhizoctonia solani in negative ion mode by CE/TOF-MS. J. Plant Physiol. 206:13–24

Tautenhahn, R., Patti, G. J., Rinehart, D., and Siuzdak, G. 2012. XCMS Online: A Web-Based Platform to Process Untargeted Metabolomic Data. Anal. Chem. 84:5035–5039

Tsogtbaatar, E., Cocuron, J.-C. C., Sonera, M. C., and Alonso, A. P. 2015. Metabolite fingerprinting of pennycress (Thlaspi arvense L.) embryos to assess active pathways during oil synthesis. J. Exp. Bot. 66:4267–4277

Turner, M. F., Heuberger, A. L., Kirkwood, J. S., Collins, C. C., Wolfrum, E. J., Broeckling, C. D., Prenni, J. E., and Jahn, C. E. 2016. Non-targeted metabolomics in diverse sorghum breeding lines indicates primary and secondary metabolite profiles are associated with plant biomass accumulation and photosynthesis. Front. Plant Sci. 7:953

Urashima, A. S., and Grachet, N. G. 2012. Métodos de detecção de Leifsonia xyli subsp. xyli e efeito da termoterapia na brotação das gemas de diferentes variedades de cana-de-açúcar. Trop. Plant Pathol. 37:57–64

Urashima, A. S., and Marchetti, L. B. L. 2013. Incidence and Severity of Leifsonia xyli subsp. xyli Infection of Sugarcane in Sao Paulo State, Brazil. J. Phytopathol. 161:478–484

Vagiri, M., Johansson, E., and Rumpunen, K. 2017. Phenolic compounds in black currant leaves – an interaction between the plant and foliar diseases? J. Plant Interact. 12:193–199

Velasco, P., Lema, M., Francisco, M., Soengas, P., Elena Cartea, M., and Cartea, M. 2013. In Vivo and in vitro effects of secondary metabolites against Xanthomonas campestris pv. campestris. Molecules. 18:11131–11143

Vollmer, W., Blanot, D., and De Pedro, M. A. 2008. Peptidoglycan structure and architecture. FEMS Microbiol. Rev. 32:149–167

Wallis, C. M., and Chen, J. 2012. Grapevine phenolic compounds in xylem sap and tissues are significantly altered during infection by Xylella fastidiosa. Phytopathology. 102:816–826

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L., and Salmond, G. P. C. 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. 25:365–404

Xia, J., Psychogios, N., Young, N., and Wishart, D. S. 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 37:W652-60

Xu, F., Sun, R.-C., Sun, J.-X., Liu, C.-F., He, B.-H., and Fan, J.-S. 2005. Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse. Anal. Chim. Acta. 552:207–217

Young, A. J. 2016. Seedbed Inspections Underestimate the Overall Incidence of Ratoon Stunting Disease. Pages 112–119in:Proc Aust Soc Sugar Cane Technol,

Young, A., Lokes, S., Davis, W., and Aitken, B. O. B. 2012. Reassessing Rsd: Insights From Harwood. Proc Aust Soc Sugar Cane Technol. 34:1–7

Zavaglia, A. C., Cia, M. C., Popin, R. V., Eduardo, L., Camargo, A., and Camargo, L. E. A. 2016. No alternative hosts of the sugarcane pathogen Leifsonia xyli subsp. xyli were identified among grass and non-grass species using novel PCR primers. Trop. Plant Pathol. 41:336–339

Zeier, J. 2013. New insights into the regulation of plant immunity by amino acid metabolic pathways. Plant. Cell Environ. 36:2085–2103

Zhang, X.-Q., Liang, Y.-J., Zhu, K., Wu, C.-X., Yang, L.-T., and Li, Y.-R. 2017a. Influence of inoculation of Leifsonia xyli subsp. xyli on photosynthetic parameters and activities of defense enzymes in sugarcane. Sugar Tech. 19:394–401

Zhang, X., Chen, M., Liang, Y., Xing, Y., Yang, L., Chen, M., Comstock, J. C., Li, Y., and Yang, L. 2016. Morphological and physiological responses of sugarcane to Leifsonia xyli subsp. xyli infection. Plant Dis. 100:1–8

Zhang, Y., Li, J., Zhang, W., Wang, R., Qiu, Q., Luo, F., Hikichi, Y., Ohnishi, K., and Ding, W. 2017b. Ferulic acid, but not all hydroxycinnamic acids, is a novel T3SS inducer of Ralstonia solanacearum and promotes its infection process in host plants under hydroponic condition. Front. Plant Sci. 8:1595

3. THE ROLE OF DIFFERENTIALLY REPRESENTED PHENOLICS ON THE *IN VITRO* GROWTH OF *LEIFSONIA XYLI* SUBSP. *XYLI*

ABSTRACT

Phenolic acids, flavonoids and terpenes are secondary metabolites produced by plants during defense against biotic and abiotic factors. The present study aimed to identify the effects of phenolics on the Gram-positive, fastidious bacterium *Leifsonia xyli* subsp. *xyli* (Lxx), the causal agent of ratoon stunting disease (RSD) in sugarcane. RSD is a severe disease that affects all sugarcane growing regions. Even though it has been described for more than 70 years, the mechanisms involved in the bacterial-host interaction have not been completely elucidated. Vascular extracts from one susceptible and one resistant sugarcane plant were added to Lxx culture medium to check the effect of plant components on bacterial growth. Additionally, a set of phenolic compounds was selected from a previous metabolomic profiling study, which compared a susceptible and a resistant variety of sugarcane to RSD. Phenolics were added to the bacterial culture medium and after incubation, Lxx growth was evaluated by spectrophotometry. The compounds were added individually and in combination, to assess a possible additive effect. The results from this report indicated that *in vitro* conditions to test for the phenolic antimicrobial activity on Lxx must be improved. Extracts from the susceptible plants enhanced the bacterial growth in comparison to both the control and the resistant extract. From all the phenolics tested, only one had an inhibitory effect on bacterial growth. Also, there was no detrimental decrease in growth with combinatory additions.

Keywords: Bacterial plant pathogen; Ratoon stunting; Secondary metabolism; Sugarcane

3.1. Introduction

Phenolics are products of the plant secondary metabolism. They are not considered essential for growth but play a fundamental role in protection against biotic and abiotic stresses (Luckner 1984; Verpoorte and Alfermann 2000). Phenolics comprise a chemically diverse group of compounds, including terpenoids, flavonoids and modified aromatic amino acids whose synthesis occur through the shikimate biosynthetic pathway (Bennett and Wallsgrove 1994). Plant defense performed by these compounds can be constitutive or induced (Lattanzio et al. 2006). Stored preformed phenols can reduce pathogen attack by inhibiting their penetration or growth

(Wittstock and Gershenzon 2002). In grapevine, for instance, hydroxycinnamic acids and flavonols are constitutive barriers against *Plasmopara viticola*, slowing down its infection (Latouche et al. 2013). Interestingly, potato varieties resistant to *Phytophthora infestans* present higher hydroxycinnamic acid content and higher expression of genes related to the phenolic pathway (Pushpa et al. 2014). Other defense metabolites, like phytoalexins, are either synthesized *de novo* or are present in small amounts but have their production increased upon pathogen attack (Shalaby and Horwitz 2015; Sharma et al. 2015; Bengtsson et al. 2014). An example of how defense compounds can be constitutive and also induced was shown by Salla et al. (2016), who discovered that production of gallic and chlorogenic acids are constitutive in Eucalyptus against *Botrytis cinerea*. However, other phenolics like caffeic and benzoic acids were induced after inoculation. *Phakopsora pachyrhizi* was found to induce genes related to the phenolic and flavonoid pathways, and medicarpin, an isoflavonoid derivative, inhibited the germination of its spores (Ishiga et al. 2015). Plant defense can also be organ specific such as in maize against Colletotrichum graminicola, where flavonoids (e.g. eriodictyol, naringenin and genkwanin) accumulate in the roots, and chlorogenic acid and 5-feruloyl-quinic acid in the leaves (Balmer et al. 2013).

Ratoon stunting disease (RSD) of sugarcane is caused by the bacterium Leifsonia xyli subsp. xyli (Lxx), which colonizes the xylem vessels, the meristem (Brumbley et al. 2004), and the mesophyll and the bundle sheath cells of the host (Quecine et al. 2016). This disease has been described in all sugarcane growing regions (Young and Nock 2017) and it can result in yield losses up to 80%, depending on environmental conditions (Li et al. 2014). Although there are varieties with different degrees of resistance to bacterial growth (Croft et al. 2012), the mechanisms involved are not yet fully understood. Branched vascular bundles found in the nodes of resistant varieties are hypothesized to restrict the bacterial colonization (Teakle et al. 1978), because the smaller pores of the xylem vessels could be easily blocked by a gum supposedly produced by sugarcane. To this date, no biochemical defense mechanisms against Lxx, either constitutive or induced, have been described in sugarcane, but this can be expected given that Lxx induces physiological alterations well before the blocking of the xylem vessels takes place (Zhang et al. 2016).

Metabolomics is the identification of compounds produced in a biological system (Heuberger et al. 2014) and as such it can be used to characterize the responses of plants to pathogens. In sugarcane, metabolic fingerprinting from different

genotypes identified a set of secondary metabolites from leaf extracts, including phenolics (Coutinho et al. 2016). C-glycoside flavonoids were related to plant defenses, as they were found in higher amount in genotypes resistant to orange rust and to *sugarcane mosaic virus*. Rutin and luteolin-7-glucoside were effective on growth inhibition of *Verticillium dahlia*, a fungal pathogen that infects the vascular system of olive trees (Báidez et al. 2007). The vascular pathogen *Fusarium virguliforme* induced the accumulation of isoflavones and isoflavonoids phytoalexins in the xylem in soybean plants (Abeysekara et al. 2016). In our previous study, the comparative metabolomic profiling of a resistant and a susceptible variety to Lxx identified some phenolics that were more abundant in the resistant variety, suggesting a possible role in restricting the growth of the pathogen. This chapter reports on the inhibitory effects of these phenolics on the *in vitro* growth of Lxx in an attempt to identify potential metabolic markers of resistance. Additionally, sugarcane vascular extracts of a resistant and susceptible variety were also tested.

3.2. Material and methods

3.2.1. Bacterial strain and growth conditions

The CTCB07 strain of Lxx was used. A 1 mL volume of a bacterial suspension obtained from a 25% glycerol inoculum were cultivated in 250 mL flasks containing 50 mL of modified sugarcane New (MSC-New) medium (Davis et al. 1984; Monteiro-Vitorello et al. 2004b). The culture was incubated at 28 ºC and 400 rpm agitation until it reached the optical density (O.D) of 0.7 (approximately 7 days; λ=600).

3.2.2. Effect of vascular extracts on Lxx growth

Vascular extracts were obtained from the susceptible CB49-260 and the resistant SP80-3280 varieties from plants of first year ratoons grown in the greenhouse originated from *in vitro* culture plants. The extracts were obtained by cutting off and pressing a piece of stem from the top portion of the plant using a Lineman's plier. The extracts were filtered through 0.22 µm syringe filters with the medium supplement components and then added on a 10% proportion (v/v) to Lxx-inoculated MSC-New culture medium (1 mL of Lxx inoculum at O.D. of 0.7, λ=600 was added to 44 mL of

MSC-New); 1.5 mL of each treatment was added to the wells of MasterBlock 96-well sterile plates (Greiner). Lxx-inoculated medium with no sugarcane extract additions was used as a control and non-inoculated medium was used as a blank. The plates were sealed with Breathseal (Greiner) permeable sterile seals and incubated at 28 °C with orbital agitation (570 rpm) for approximately 6 days, until the control reached an O.D. of 0.7 (λ=600), which was checked by daily measurements. Each treatment had 8 biological replicates arranged in columns in the plates.

3.2.3. Effect of phenolics and flavonoids on Lxx growth

All the phenolic standards used in this study were purchased from Merck-Sigma. The following phenolic compounds and concentrations were tested individually on the growth of Lxx: caffeic, chlorogenic, 4-hydrobenzoic, 4-hydroxy-2-methoxycinnamic and ferulic acids were added to a final concentration of 4 mM and eriodictyol, luteolin-7-glucoside and orientin were added to a final concentration of 0.4 mM. Also, the inhibitory effect of 4-hydrobenzoic acid (4mM) was tested in combination with caffeic acid (4 mM) and eriodictyol and orientin (0.4mM). The metabolites were added to the medium supplement which was filtered through 0.22 µm syringe filters and added to Lxx-inoculated MSC-New culture medium (1 mL of Lxx inoculum at O.D. of 0.7, λ=600 was added to 44 mL of MSC-New); 1.5 mL of each treatment was placed in the wells of MasterBlock 96-well sterile plates (Greiner). Lxx-inoculated medium without phenolic compounds was used as the control and non-inoculated medium was used as a blank. The plates were sealed with Breathseal (Greiner) permeable sterile seals and incubated at 28 °C and 570 rpm agitation for approximately 6 days, until the control reached an O.D. of 0.7 (λ=600), which was checked by daily measurements. Each treatment had 8 biological replicates arranged in columns in the plates.

3.2.4. Lxx growth evaluation and statistics

Bacterial growth from the control was assessed daily; other treatments were measured at approximately 6 days after inoculation by measuring the culture O.D. in a spectrophotometer (λ=600). The mean optical densities were compared among treatments using Assistat (http://www.assistat.com) and Tukey's test ($p<0.05$).

3.3. Results

3.3.1. Vascular extracts alter Lxx growth

Extracts of the sugarcane varieties SP80-3280 (R) and CB49-260 (S) were added to the bacterial culture medium to investigate their effect on Lxx growth. Both extracts significantly enhanced bacterial growth compared to the control, however, S extracts induced higher Lxx growth when compared to R (Figure 9).

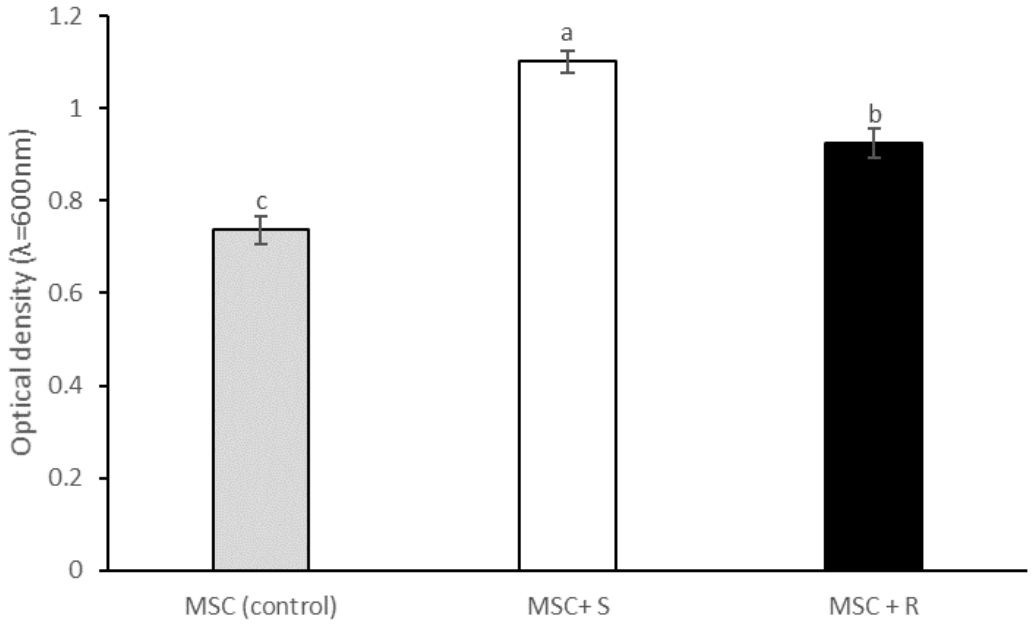


Figure 9. Average Lxx growth in MSC-New with no additions (control) and with the addition of susceptible and resistant sugarcane extracts (S and R respectively). Different letters above bars indicate significant differences between optical density means according to the Tukey test ($p < 0.05$). Bars represent average optical density +/- standard error, n=8.

3.3.2. Hydrobenzoic acid inhibits Lxx growth

A group of phenolic compounds and flavonoids differentially accumulated in the resistant variety SP80-3280 were selected and added to MSC-New culture media inoculated with Lxx to study their effect on bacterial growth. Out of 8 compounds tested in this study, 2 had a significant impact on Lxx growth. Chlorogenic acid significantly increased the bacterial growth when compared to the control, whereas hydrobenzoic acid significantly decreased Lxx growth (Figure 10). Caffeic acid, orientin, eriodictyol and luteolin-7-glucoside somewhat inhibited Lxx growth, while 4-hydroxy-2-methoxycinnamic acid and ferulic acid did not alter bacterial growth when compared to the control (Figure 10).

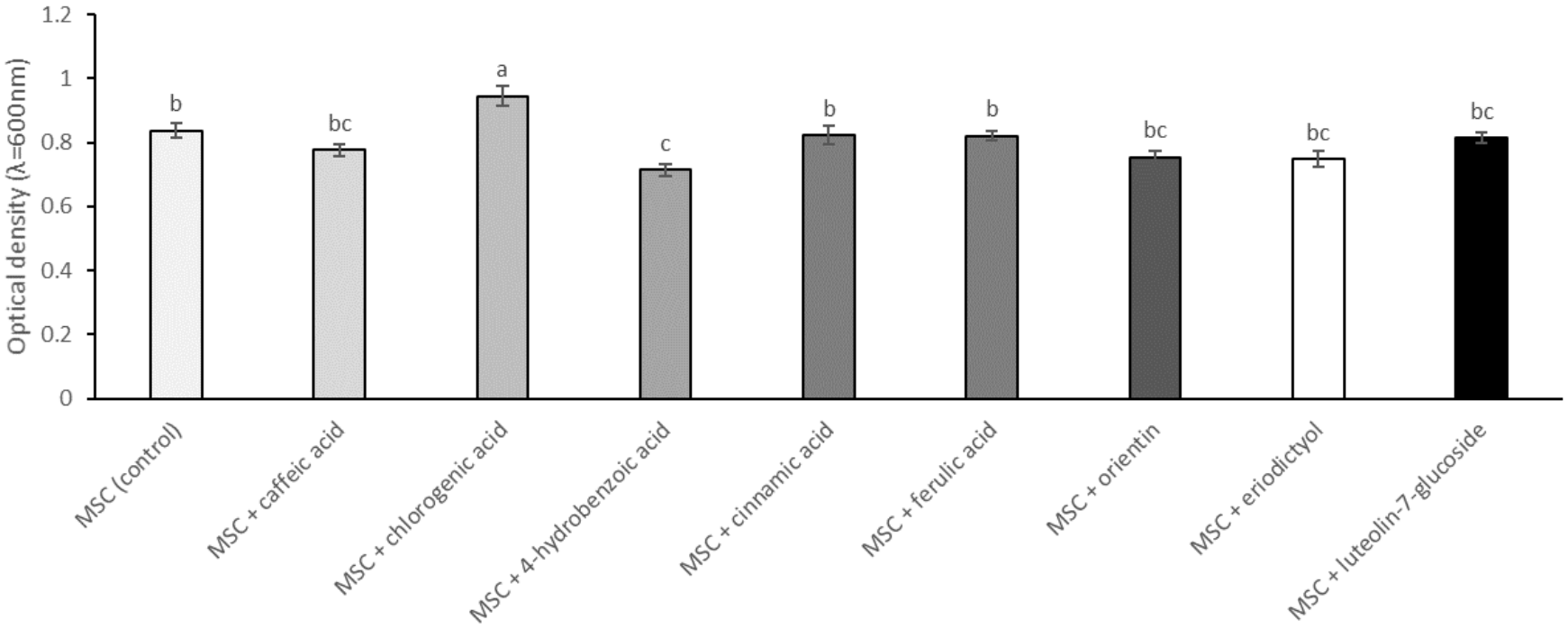


Figure 10. Average Lxx growth in MSC-New with no additions (control) and with the addition of caffeic acid, chlorogenic acid, 4-hydrobenzoic acid, 4-hydroxy-2-methoxycinnamic, ferulic acid (4mM) and orientin, eriodictyol and luteolin-7-glucoside (0.4 mM). Different letters above bars indicate significant differences between optical density means according to the Tukey test ($p < 0.05$). Bars represent average optical density +/- standard error, n=8.

3.3.3. The combination of phenolics does not have an additive effect on Lxx growth

Because hydrobenzoic acid caused a significant inhibition on Lxx growth, it was tested in combination with orientin, eriodictyol and caffeic acid, which caused moderate inhibition, to verify any additive effect. When combined with caffeic acid, hydrobenzoic acid significantly decreased the bacterial growth, compared to the control (Figure 11). However, it is at lower extend than the addition of hydrobenzoic acid alone (Figure 10). On the other hand, when orientin and eriodictyol were combined, there was a significant increase on Lxx growth compared to the control (Figure 11), which contrasts with the addition of hydrobenzoic acid alone (Figure 10).

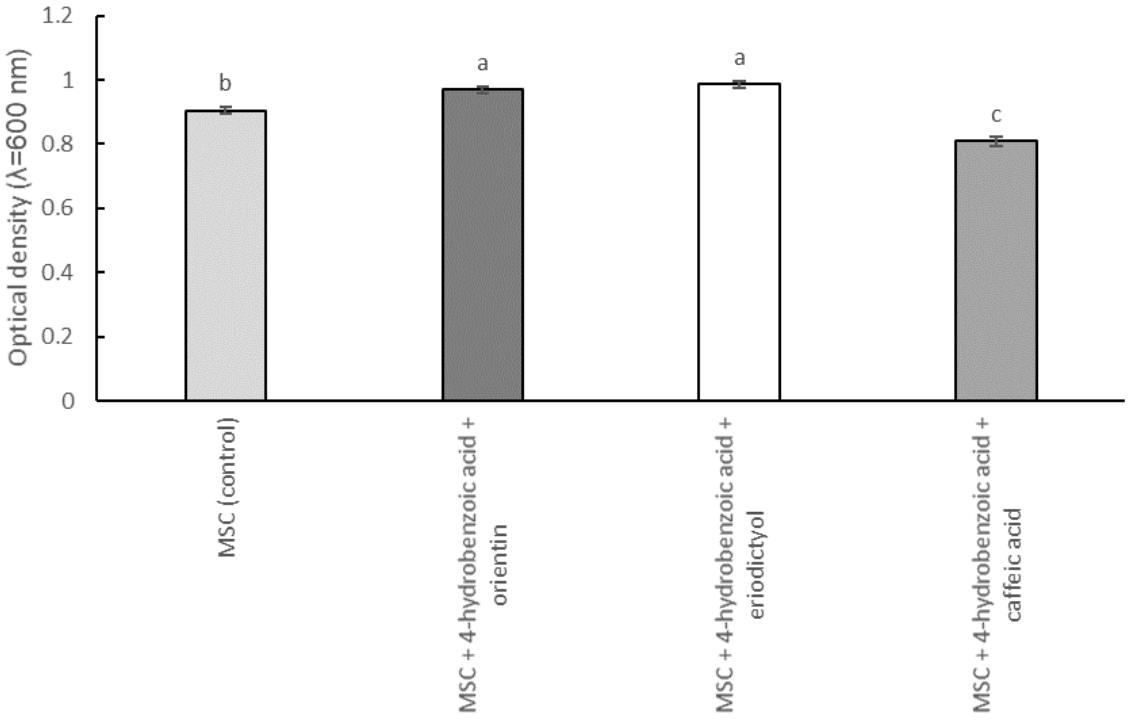


Figure 11. Average Lxx growth in MSC-New with no additions (control) and with the addition of 4-hydrobenzoic acid (4 mM) combined with orientin, eriodictyol (0.4 mM) and caffeic acid (4 mM). Different letters above bars indicate significant differences between optical density means according to the Tukey test ($p < 0.05$). Bars represent average optical density +/- standard error, n=8.

3.4. Discussion

Vascular extracts from two varieties, one susceptible (S; CB49-260) and one resistant (R; SP80-3280) to Lxx, and phenolics were added to Lxx culture medium to evaluate their effect on the bacterial growth. Even though there was a significant

difference between S and R extract addition, both treatments had a positive effect on bacterial growth when compared to the control. An explanation for this finding is that some components present in both vascular extracts provided additional nutrients to the bacterium. However, the extract from susceptible plants caused Lxx to grow significantly more than the extracts from the resistant genotype. This result could be explained by the higher levels of phenolics in the resistant variety SP80 3280(Chapter 2). Phenolic compounds and flavonoids are secondary metabolites produced by plants that have some antimicrobial activity, and play roles in defense (Maddox et al. 2010). Even though there was no growth inhibition with the addition of chlorogenic, 4-hydroxy-2-methoxycinnamic and ferulic acids, a moderate growth reduction was observed when caffeic acid, orientin, eriodictyol, luteolin glucoside and hydrobenzoic acid, were added, the latest causing significant inhibition when compared to the control. Those compounds were reported to inhibit other plant pathogens, such as the rice pathogens *Xanthomonas oryzae* pv. *oryzae*, *Pyricularia oryzae* and *Rhizoctonia solani* (Padmavati et al. 1997); the opportunistic pathogen *Pseudomonas aeruginosa* (Vandeputte et al. 2011) and the human pathogens *Staphylococcus aureus*, *Bacillus subtilis* and *E. coli* (Lou et al. 2011). Some phenolics also reduce plant pathogenic bacteria virulence instead of inhibiting their growth. For instance, *Pectobacterium aroidearum* and *P. carotovorum* ssp. *Brasiliense*, which cause blackleg of potato, had lower expression of genes related to quorum sensing when exposed to cinnamic and salicylic acids, resulting in lower production of signaling molecules key to successful infection in plants (Joshi et al. 2016). Additionally, coumaric and cinnamic acids can reduce the expression of genes linked to the production of type III secretion system in Dickeya dadantii, impairing virulence (Charkowski et al. 2012). Also, tomato plants, transformed to produce higher levels of chlorogenic acid, had lower incidence of *Pseudomonas syringae* infection (Niggeweg et al. 2004), confirming the negative effect of phenolic compounds on plant pathogenic microorganisms. These results also indicate that growth inhibition should not be the only measured characteristic when testing the activity of phenolics on plant pathogens. Nonetheless, the addition of phenolic acids and flavonoids to Lxx liquid culture medium did not inhibit bacterial growth completely. One explanation would be that the culture medium provides cysteine and methionine, both sulphur-containing molecules that are highly oxidizing molecules and that could react with the phenolic acids and flavonoids, thus masking their antibacterial activity (Atmaca 2004). Additionally, soy peptone, which has a high

protein content, is also a componente of the medium and previous studies demonstrated that proteins can react with flavonoids, reducing their antimicrobial capacities (Arts et al. 2002). Unfortunately, there is no minimal culture medium for Lxx because it is a fastidious bacterium with high nutritional requirements (Brumbley et al. 2006b; Monteiro-Vitorello et al. 2004a). Therefore, another method for testing the effects of phenolics against Lxx in vitri medium must be developed, like disc, agar diffusion and agar dilution tests (Baydar et al. 2006; Boyanova et al. 2005). Interestingly, a study on phenolics inhibition of the pathogenic bacterium *Xylella fastidiosa* used a plate dilution method (Maddox et al. 2010). The authors argued that an agar disc diffusion would not be suitable because the bacterium grows slowly on culture medium.

Rather than measuring growth, other physiological effects could be monitored. For instance, viability and the malolactic enzyme activity were measured in *Leuconostoc oenos*, a lactic bacterium used in wine industry, after the addition of phenolic acids and anthocyanins (Vivas et al. 1997). Moreover, the action of ferulic acid against human pathogenic bacteria was evaluated by measuring the integrity of their membrane and ion leakage, apart from determining the minimum inhibitory and lethal concentrations (Borges et al. 2013).

3.5. Conclusion and future perspectives

Metabolomic profiling of the susceptible (CB49-260) and resistant (SP80-3280) varieties to RSD reported higher phenolic content in the latter, suggesting that the phenolic compounds and flavonoids differentially accumulated in the resistant genotype may play a role in sugarcane defense. To validate those results *in vitro*, an improved methodology must be developed to test the inhibitory effects of phenolics on Lxx growth, as their addition to the culture medium did not have the critical negative effect that was expected. Since Lxx is fastidious and nutrient-demanding, there is no minimal culture medium established for this pathogen. Further efforts are needed to design a better methodology to study the effects of phenolics on the bacterial growth, viability, cell wall and membrane integrity and possibly, enzymatic activity. Accomplishing these goals will permit establishing specific phenolics as detrimental to Lxx, which may be used as biomarkers to select more resistant varieties to RSD.

References

Abeysekara, N. S., Swaminathan, S., Desai, N., Guo, L., and Bhattacharyya, M. K. 2016. The plant immunity inducer pipecolic acid accumulates in the xylem sap and leaves of soybean seedlings following Fusarium virguliforme infection. Plant Sci. 243:105–114

Arts, M. J. T. J., Haenen, G. R. M. M., Wilms, L. C., Beetstra, S. A. J. N., Heijnen, C. G. M., Voss, H.-P., and Bast, A. 2002. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. J. Agric. Food Chem. 50:1184–7

Atmaca, G. 2004. Antioxidant Effects of Sulfur-Containing Amino Acids. Yonsei Med. J. 45:776–788

Báidez, A. G., Gómez, P., Del Río, J. A., and Ortuño, A. 2007. Dysfunctionality of the xylem in Olea europaea L. plants associated with the infection process by Verticillium dahliae Kleb. role of phenolic compounds in plant defense mechanism. J. Agric. Food Chem. 55:3373–3377

Baydar, N. G., Sagdic, O., Ozkan, G., and Cetin, S. 2006. Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. Int. J. Food Sci. Technol. 41:799–804

Bennett, R. N., and Wallsgrove, R. M. 1994. Secondary metabolites in plant defence mechanisms. New Phytol. 127:617–633

Borges, A., Ferreira, C., Saavedra, M. J., and Simões, M. 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb. Drug Resist. 19:256–265

Boyanova, L., Gergova, G., Nikolov, R., Derejian, S., Lazarova, E., Katsarov, N., Mitov, I., and Krastev, Z. 2005. Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. J. Med. Microbiol. 54:481–483

Brumbley, S. M., Petrasovits, L. A., Hermann, S. R., Young, A. J., and Croft, B. J. 2006. Recent advances in the molecular biology of Leifsonia xyli subsp. xyli , causal organism of ratoon stunting disease. Australas. Plant Pathol. 35:681

Brumbley, S. M., Petrasovits, L. A., Murphy, R. M., Nagel, R. J., Candy, J. M., and Hermann, S. R. 2004. Establishment of a functional genomics platform for Leifsonia xyli subsp. xyli. Mol. Plant-Microbe Interact. 17:175–183

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., Hugouvieux-Cotte-Pattat, N., Solanilla, E. L., Low, D., Moleleki, L., Pirhonen, M., Pitman, A., Perna, N., Reverchon, S., Rodríguez Palenzuela, P., San Francisco, M., Toth, I., Tsuyumu, S., van der Waals, J., van der Wolf, J., Van Gijsegem, F., Yang, C.-H., and Yedidia, I. 2012. The Role of Secretion Systems and Small Molecules in Soft-Rot *Enterobacteriaceae* Pathogenicity. Annu. Rev. Phytopathol. 50:425–449

Coutinho, I. D., Baker, J. M., Ward, J. L., Beale, M. H., Creste, S., and Cavalheiro, A. J. 2016. Metabolite Profiling of Sugarcane Genotypes and Identification of Flavonoid Glycosides and Phenolic Acids. J. Agric. Food Chem. 64:4198–4206

Croft, B. J., Green, J., and Braithwaite, K. S. 2012. Comparison of RSD assays for diagnosis and screening varieties for resistance. Int. Sugar J. 114:879–885

Davis, M. J., Gillaspie JR, A. G., Vidaver, A. K., and Harris, R. W. 1984. Clavibacter : a New Genus Containing Some Phytopathogenic Coryneform Bacteria , Including Clavibacter xyli subsp . xyli sp . nov ., subsp . nov . and Clavibacter xyli subsp . cynodontis subsp . nov . Pathogens That Cause Ratoon Stunting Disease of Sugarca. Int. J. Syst. Bacteriol. 34:107–117

Heuberger, A. L., Robison, F. M., Lyons, S. M. a, Broeckling, C. D., and Prenni, J. E. 2014. Evaluating plant immunity using mass spectrometry-based metabolomics workflows. Front. Plant Sci. 5:291

Ishiga, Y., Uppalapati, S. R., Gill, U. S., Huhman, D., Tang, Y., and Mysore, K. S. 2015. Transcriptomic and metabolomic analyses identify a role for chlorophyll catabolism and phytoalexin during Medicago nonhost resistance against Asian soybean rust. Sci. Rep. 5:13061

Joshi, J. R., Burdman, S., Lipsky, A., Yariv, S., and Yedidia, I. 2016. Plant phenolic acids affect the virulence of *P ectobacterium aroidearum* and *P . carotovorum* ssp. *brasiliense* via quorum sensing regulation. Mol. Plant Pathol. 17:487–500

Lattanzio, V., Lattanzio, V. M. T., Cardinali, A., and Amendola, V. 2006. *Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects*.

Li, W.-F. F., Shen, K., Huang, Y.-K. K., Wang, X.-Y. Y., Yin, J., Luo, Z.-M. M., Zhang, R.-Y. Y., and Shan, H.-L. L. 2014. Incidence of sugarcane ratoon stunting disease in the major cane-growing regions of China. Crop Prot. 60:44–47

Lou, Z., Wang, H., Zhu, S., Ma, C., and Wang, Z. 2011. Antibacterial activity and mechanism of action of chlorogenic acid. J. Food Sci. 76

Luckner, M. 1984. *Secondary metabolism in microorganisms, plants, and animals*. Springer-Verlag.

Maddox, C. E., Laur, L. M., and Tian, L. 2010. Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa. Curr. Microbiol. 60:53–58

Monteiro-Vitorello, C. B., Camargo, L. E. A., Van Sluys, M. A., Kitajima, J. P., Truffi, D., Do Amaral, A. M., Harakava, R., De Oliveira, J. C. F., Wood, D., De Oliveira, M. C., Miyaki, C., Takita, M. A., Da Silva, A. C. R., Furlan, L. R., Carraro, D. M., Camarotte, G., Almeida, N. F., Carrer, H., Coutinho, L. L., El-Dorry, H. A., Ferro, M. I. T., Gagliardi, P. R., Giglioti, E., Goldman, M. H. S., Goldman, G. H., Kimura, E. T., Ferro, E. S., Kuramae, E. E., Lemos, E. G. M., Lemos, M. V. F., Mauro, S. M. Z., Machado, M. A., Marino, C. L., Menck, C. F., Nunes, L. R., Oliveira, R. C., Pereira, G. G., Siqueira, W., De Souza, A. A., Tsai, S. M., Zanca, A. S., Simpson, A. J. G., Brumbley, S. M., and Setúbal, J. C. 2004a. The genome sequence of the gram-positive sugarcane pathogen *Leifsonia xyli* subsp. *xyli*. Mol. Plant-Microbe Interact. 17:827–836

Monteiro-Vitorello, C. B., Zerillo, M. M., Sluys, V., Camargo, L. E. A. 2004b. Genome sequence-based insights into the biology of the sugarcane pathogen *Leifsonia xyli* subsp. *xyli*. in: Plant Pathogenic Bacteria - Genomics and Molecular Biology.

Niggeweg, R., Michael, A. J., and Martin, C. 2004. Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat. Biotechnol. 22:746–54

Padmavati, M., Sakthivel, N., Thara, K. V. V., and Reddy, A. R. 1997. Differential sensitivity of rice pathogens to growth inhibition by flavonoids. Phytochemistry. 46:499–502

Pushpa, D., Yogendra, K. N., Gunnaiah, R., Kushalappa, A. C., and Murphy, A. 2014. Identification of late blight resistance-related metabolites and genes in potato through nontargeted metabolomics. Plant Mol. Biol. Report. 32:584–595

Quecine, M. C., Silva, T. M., Carvalho, G., Saito, S., Mondin, M., Teixeira-Silva, N. S., Camargo, L. E. A., and Monteiro-Vitorello, C. B. 2016. A stable Leifsonia xyli subsp. xyli GFP-tagged strain reveals a new colonization niche in sugarcane tissues. Plant Pathol. 65:154–162

Salla, T. D., Astarita, L. V., and Santarém, E. R. 2016. Defense responses in plants of Eucalyptus elicited by Streptomyces and challenged with Botrytis cinerea. Planta. 243:1055–1070

Teakle, D. S., Appleton, J. M., and Steindl, D. R. L. 1978. An anatomical basis for resistance of sugar cane to ratoon stunting disease. Physiol. Plant Pathol. 12:83–91

Vandeputte, O. M., Kiendrebeogo, M., Rasamiravaka, T., Stevigny, C., Duez, P., Rajaonson, S., Diallo, B., Mol, A., Baucher, M., El Jaziri, M., Stévigny, C., Duez, P., Rajaonson, S., Diallo, B., Mol, A., Baucher, M., and El Jaziri, M. 2011. The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in pseudomonas aeruginosa PAO1. Microbiology. 157:2120–2132

Verpoorte, R., and Alfermann, A. W. 2000. *Metabolic Engineering of Plant Secondary Metabolism*. Springer Netherlands.

Vivas, N., Lonvaud-Funel, A., and Glories, Y. 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. Food Microbiol. 14:291–299

Young, A. J., and Nock, C. J. 2017. Molecular Detection of Diverse *Leifsonia* Strains Associated With Sugarcane. Plant Dis. :PDIS-01-17-0016

Zhang, X., Chen, M., Liang, Y., Xing, Y., Yang, L., Chen, M., Comstock, J. C., Li, Y., and Yang, L. 2016. Morphological and physiological responses of sugarcane to Leifsonia xyli subsp. xyli infection. Plant Dis. 100:1–8

4. FINAL DISCUSSION AND PERSPECTIVES

4.1. RSD and its impact of sugarcane production

The gram-positive and xylem-limited bacterium *Leifsonia xyli* subsp. *xyli* (Lxx) is the causal agent of ratoon stunting disease (RSD), a serious disease of sugarcane. RSD is distributed in all sugarcane growing countries (Li et al. 2014) and losses in biomass production may reach 80% due to the generalized poor growth of diseased plants (Fu et al. 2016). However, diagnosing RSD based on symptoms is difficult, as these can be easily mistaken by symptoms of many environmental stresses that affect plant growth. To date, the only control method of RSD is to subject propagative material to heat treatment in an attempt to eliminate the bacterium. Unfortunately the efficacy of this method, which affects the germinability of buds, is limited (Young et al. 2012).

4.2. Contribution to the field

Our group previously performed proteomics and transcriptomics studies in sugarcane plants; SP80-3280 leaf samples from Lxx and mock-inoculated plants at 30 and 60 DAI were collected and analyzed (Cia 2014; Carvalho 2012). Inoculation resulted in altered expression of proteins and genes involved in the control of the cell-cycle (e.g.: cyclin dependent kinase and cyclins), which is consistent with RSD main symptom, plant growth reduction. Interestingly, a methionine synthase encoding gene was up-regulated in inoculated plants (Cia 2014). The upregulation of the synthesis of methionine in plants infected with Lxx provides a nice example of host manipulation by a pathogen, as the synthesis of this amino acid is possibly impaired in Lxx due to mutations in two essential genes, metE and metF (Monteiro-Vitorello et al. 2004a). Even though this metabolite was not conspicuous in the metabolomic profiling, methionine is directly related to cysteine biosynthesis (Brosnan and Brosnan 2006), which was more accumulated on Lxx-inoculated plants. Moreover, defense signaling was also altered by inoculation, as in ABA perception (e.g.: calreticulin, protein G, and alcohol dehydrogenase), and genes related to the synthesis of ethylene were upregulated (Cia 2014; Carvalho 2012). Additionally, there was a higer accumulation of the proteins phenylalanine ammonia lyase (PAL) and cytochrome P450 proteins at a later stage, both directly related to phenolics pathways (Carvalho 2012). These

results are consistent with the metabolomic findings described here, as inoculated plants with Lxx had higher accumulation of the phenolic compound ferulic acid in the sap at 120 DAI.

The main objective of this study was to define metabolomic targets that will be compared with existing proteomic and transcriptomic data to define a core target (proteins, genes, and metabolites) that can be further tested as markers of resistance in a collection of sugarcane varieties. To achieve this goal, a comparative metabolomics study was performed using two sugarcane varieties, one susceptible (CB49-260) and the other resistant (SP-80-3280) to RSD. The plants were mock or Lxx-inoculated, the leaves and xylem sap obtained through guttation drops were collected at 30 and 120 days after inoculation (DAI) to determine their metabolic profiles. Lxx titer was detected with Real-Time Polymerase Chain Reaction (qPCR) and the results confirmed that CB49-260 is the more susceptible genotype because it had the highest bacterial titers. Contrarily, SP80-3280 was able to control endophytic levels of the bacterium even with artificial inoculation. Untargeted profiling was performed by Gas Chromatography-Mass Spectrometry (GC-MS) and highlighted the main family of compounds present in sugarcane leaves and sap. It revealed that the two varieties have different metabolic profiles, but those distinctions are more quantitative than qualitative. Targeted metabolomics was performed using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) to quantify amino acids, sugars, sugar alcohols, organic acids, phosphorylated compounds, phenolic acids, flavonoids and hormones. Susceptible non-inoculated plants were found to accumulate more amino acids, sugars and organic acids, while the resistant ones had more phenolics. Inoculation with Lxx resulted in higher accumulation of amino and organic acids, phosphorylated compounds and some phenolics. Lxx-inoculated plants had higher abundances of cysteine in the leaves, a key amino acid for Lxx survival. Likewise, ferulic acid, a phenolic compound with known plant defense activity (YOU NEED A REF HERE), was more accumulated in the sap of inoculated plants. In an attempt to validate the metabolimics results, vascular extracts from one susceptible and one resistant sugarcane plant were added to Lxx culture medium to check the effect of plant components on bacterial growth. Additionally, a set of phenolic compounds was selected from the metabolomic profiling study and were added to the bacterial culture medium. After incubation, Lxx growth was evaluated by spectrophotometry. The compounds were added individually and in combination to assess a possible additive

effect. The results from this report indicated that although *in vitro* conditions to test for the phenolic antimicrobial activity on Lxx must be improved, extracts from the susceptible plants enhanced the bacterial growth in comparison to both the control and the resistant extract. From all the phenolics tested, only one had a significant inhibitory effect on bacterial growth. Also, there was no detrimental decrease in growth with combinatory additions.

4.3. Perspectives

Knowing that sugarcane genotypes vary in terms of levels of Lxx colonization of their tissues, genetic control of RSD using resistant varieties is a promising strategy. Since losses due to RSD are related to bacterial densities in the plant (McFarlane 2002; Davis et al. 1988b), a breeding strategy would be to select genotypes that allow low multiplication of the bacterium. However, phenotypic selection based on inoculation trials is not feasible because quantification of Lxx *in planta* requires serological or molecular methods. For instance, screening 250 thousand seedlings would be necessary to develop one commercial variety just by selecting for higher sugar content alone and not considering other traits (Dal-Bianco et al. 2012). Therefore, selecting sugarcane biochemical markers of resistance to Lxx would speed-up breeding programs. This metabolomic profiling study detected more amino acids in CB49-260 and higher levels of phenolics in SP80-3280, which might explain the different levels of resistance to RSD between those varieties. Also, ferulic acid was found to accumulate more in Lxx-inoculated plants. Determining how these metabolites act on Lxx development is necessary for validating the metabolomics results. Unlike previous studies on other pathogens where growth imparement and virulence reduction was observed (Abeysekara et al. 2016; Li et al. 2015; Velasco et al. 2013; Báidez et al. 2007), adding phenolics to Lxx culture medium resulted only in a moderate inhibition, indicating that an optimized methodology should be developed. Indeed, there is no minimal culture medium established for this fastidious, nutritionally-demanding bacterium, and the components from the complex MSC-New medium could react with the phenolic acids and flavonoids, masking their antimicrobial activity. Alternatively, cell viability, stress-related enzymes and genes, or ion leakage can also be measured in future assays to indicate how phenolics can affect Lxx. Moreover, the addition of the candidate bacterial growth enhancers found in the susceptible variety

should also be tested to determine biochemical markers that are related to RSD sensitivity.

Even though RSD has been present in sugarcane fields for more than 70 years, it is still unclear how Lxx colonizes its host and what mechanisms are involved in this interaction. Based on *in silico* analysis of the Lxx genome, it has been hypothesized that the pathogen produces a toxic compound, analogous to ABA, using a carotenoid as a precursor (Monteiro-Vitorello et al. 2004). Since reduced plant growth is a major characteristic of RSD, this compound would be a major contributor to the RSD symptom. The genome of Lxx contains a carotenoid operon (Monteiro-Vitorello et al. 2004a), similar to that present in other Actinobacteria (Tao et al. 2007) and it has been hypothesized that these genes could be involved in the synthesis of a precursor, analog of ABA. The secretion of this compound *in planta* would contribute to the stunting symptom of RSD. Previous studies determined that Lxx produces a toxic compound that inhibits germination of lettuce seeds, but the nature of this compound has not yet been established. There is some evidence suggesting that this inhibitor would result from the desaturation of a carotenoid pigment by the action of a delta-12 acyl-lipid desaturase encoded by the gene desA in Lxx (Xiong and Zhu 2003). This hypothesis was partially validated when fosmidomycin, an inhibitor of the DXP reductoisomerase of the non-mevalonate pathway of isoprenoid synthesis was added to a liquid culture of Lxx, resulting in a reduction of both the toxic effect of the supernatant extract and the content of isoprenoid pigments in Lxx cells (Castro 2012). Interestingly, stronger effects were observed when polyethylene glycol 6000 (PEG) was added to the culture medium to simulate osmotic stress (Figure 12): lower seed germination and reduced root length. RSD symptoms are more accentuated during drought season (Ngaruiya et al. 2005; Rott et al. 2002), which could indicate that the toxic compound is secreted in greater quantity during stress. Characterizing this compound would unveil how the bacterium exacerbates disease symptoms by producing a toxic compound that reduces plant growth by affecting negatively cell division and unbalancing the host hormone metabolism.

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Figure 12. Seedling germination after treatments. A (MSC) and B (MSC+PEG) refer to control treatments, without and with PEG, respectively; D (MSC+Lxx) and E (MSC+PEG+Lxx) refer to the extracts of Lxx grown without and with PEG, respectively. F (ABA 100 µM) refers to the ABA control and C refers to water control.

4.4. Applications

Biomarkers are chemical signals that can be measured and related to a biological response, such as environmental stress and pathogenesis (Strimbu and Tavel 2010). Most studies on biochemical markers for plants, including sugarcane are related to drought conditions (Kaur et al. 2014; Munawarti et al. 2014; Jangpromma et al. 2010; Zhao et al. 2010; Ireland et al. 2004). For instance, proline has been widely reported as a metabolic target to plant stress, especially drought (Hayat et al. 2012; Cevallos-Cevallos et al. 2011; Ashraf and Foolad 2007). On the other hand, this amino acid accumulates in plants under normal conditions as well, especially in reproductive organs (Mattioli et al. 2009). Moreover, glyoxalases, which is a set of enzymes involved in diverse pathways, were directly related to abiotic stress tolerance (Kaur et al. 2014). For sugarcane, proteomics have suggested that the proteins p18, an unknown 18-kDa protein and serine protease inhibitor, are possible candidates as drought-resistance marker because they were related to water stress tolerance (Barnabas et al. 2015; Jangpromma et al. 2010). Additionally, high levels of the protein SoDip22 have been linked to abiotic stress based on the transcript expression and protein level (Ferreira et al. 2017), which would make it a possible target as well. In this study, cysteine, a

key amino acid for Lxx survival (Monteiro-Vitorello et al. 2004a), was less abundant in the resistant variety at the earlier time of 30 DAI (Figure 13). Interestingly, this amino acid was more accumulated later in both susceptible and resistant inoculated plants at 120 DAI (Figure 14). This finding suggests that cysteine accumulation can be related to Lxx inoculation and it is a possible candidate for biochemical marker of susceptibility to RSD. Nonetheless, only a few studies relate specific targets to pathogenesis. Thaumatin like proteins, which are related to biotic stress, were found to be more abundant in the xylem sap of vines infected with *Xylella fastidiosa*, the causal agent of Pierce's disease (Katam et al. 2015). Also, a set of flavonoids were related to Huanglongbing resistance in some citrus varieties (Cevallos-Cevallos et al. 2012). In sugarcane, phenolic levels were also linked to disease resistance (Coutinho et al. 2016; Leme et al. 2014). Leaf metabolic profiling in one study identified phenolic acids and glycosylated flavones in 13 sugarcane genotypes. Among them, C-glycosylated apigenin and luteolin were more abundant in varieties resistant to mosaic virus and to *Puccinia kuehnii* (Coutinho et al. 2016). In a comparative study, the flavonoid luteolin-8-C-glucoside was also higher in a resistant sugarcane to *Puccinia kuehnii*, the causal agent of orange rust (Leme et al. 2014). The previous findings in sugarcane were consistent with this study because when mock-inoculated plants were compared, resistant leaf samples also had higher abundances of phenolic compounds and flavonoids. Chlorogenic, hydrobenzoic, ferulyquinic and ferulic acids, eriodictyol, isoorientin, luteolin-7-O-glucoside and others were present at higher level in the resistant genotype at both timepoints (Figure 13 and 14). Moreover, inoculation with Lxx was related to more accumulation of apigenin-7-O-glucoside and 2 and 3-ferulyquinic acids in susceptible plants (Figure 13 and 14). In order to validate those results, more sugarcane varieties should be tested to establish a set of markers for RSD resistance.

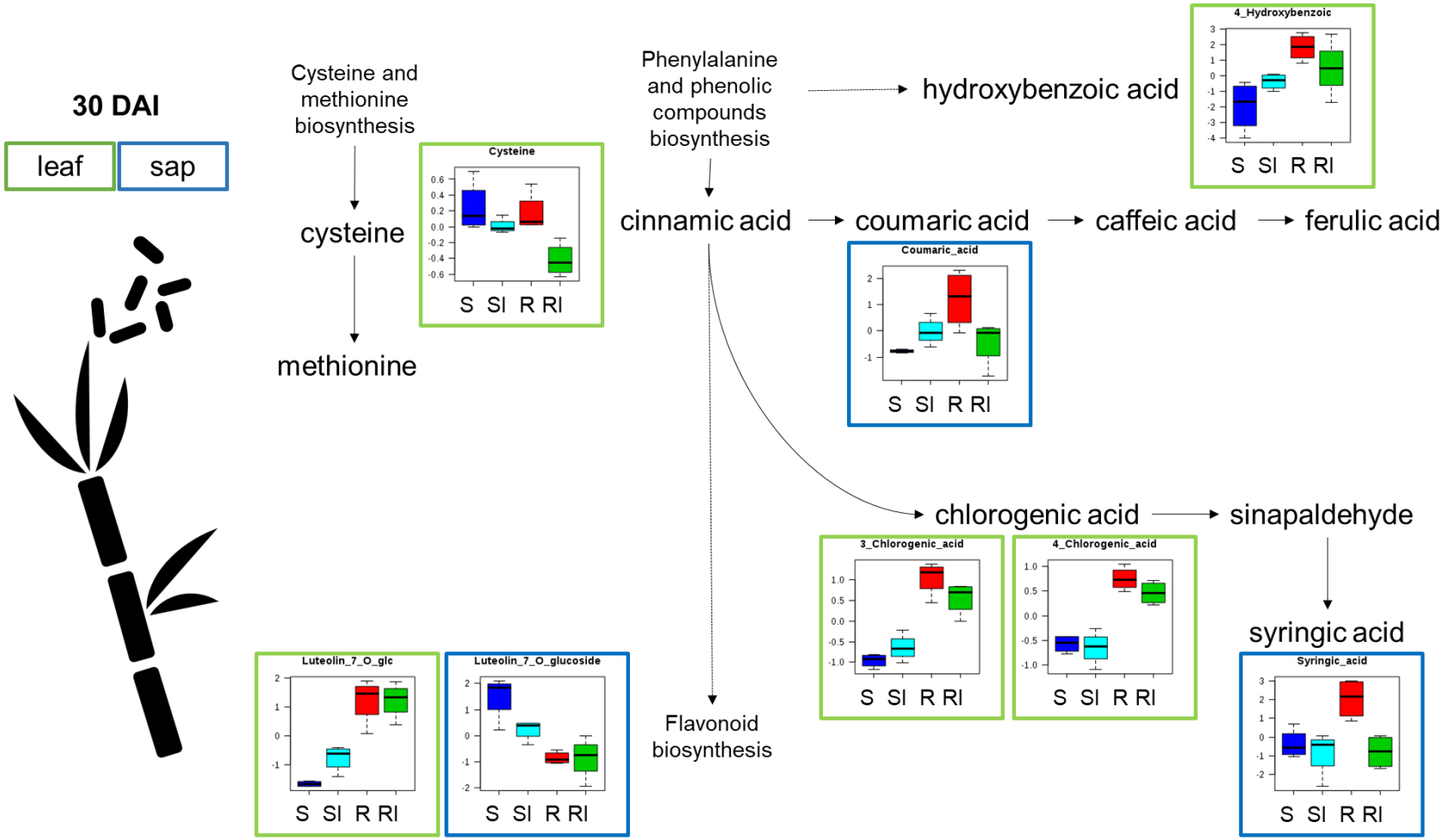

Figure 13. Most significant changes in sugarcane metabolome due to Lxx inoculation at 30 DAI. Box-plots represent normalized metabolite abundance in folds, from susceptible mock (S) and Lxx-inoculated (SI) and resistant mock (R) and Lxx-inoculatad (RI) leaf (green margin) and sap (blue margin). Pathways adapted from KEGG (Kanehisa et al. 2017).

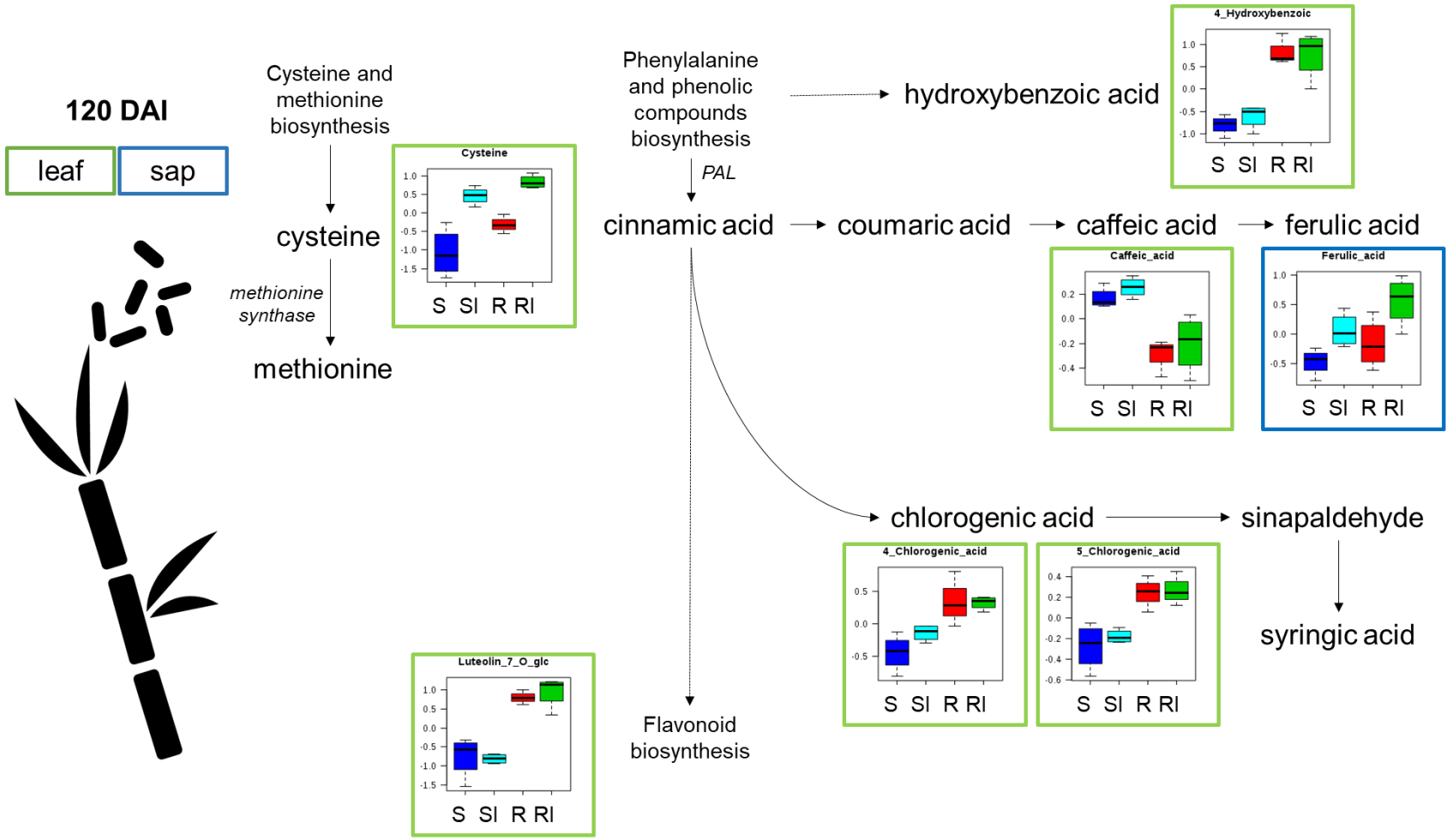

Figure 14. Most significant changes in sugarcane metabolome due to Lxx inoculation at 120 DAI. Box-plots represent normalized metabolite abundance in folds, from susceptible mock (S) and Lxx-inoculated (SI) and resistant mock (R) and Lxx-inoculatad (RI) leaf (green margin) and sap (blue margin). Methionine synthase and phenylalanine ammonia lyase (PAL) were more abundant in RI from previous proteomics studies. Pathways adapted from KEGG (Kanehisa et al. 2017).

References

Abeysekara, N. S., Swaminathan, S., Desai, N., Guo, L., and Bhattacharyya, M. K. 2016. The plant immunity inducer pipecolic acid accumulates in the xylem sap and leaves of soybean seedlings following Fusarium virguliforme infection. Plant Sci. 243:105–114

Ashraf, M., and Foolad, M. R. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59:206–216

Báidez, A. G., Gómez, P., Del Río, J. A., and Ortuño, A. 2007. Dysfunctionality of the xylem in Olea europaea L. plants associated with the infection process by Verticillium dahliae Kleb. role of phenolic compounds in plant defense mechanism. J. Agric. Food Chem. 55:3373–3377

Barnabas, L., Ramadass, A., Amalraj, R. S., Palaniyandi, M., and Rasappa, V. 2015. Sugarcane proteomics: An update on current status, challenges, and future prospects. Proteomics. 15:1658–1670

Brosnan, J. T., and Brosnan, M. E. 2006. The sulfur-containing amino acids: an overview. J. Nutr. 136:1636S–1640S

Carvalho, G. 2012. Proteome and antioxidant system analysis of sugarcane in response to Leifsonia xyli subsp. xyli, causal agent of ratoon stunting disease.

Castro, F. R. 2012. Biological characterization of the toxic effects of extracts of liquid cultures of Leifsonia xyli subsp. xyli on the germination of lettuce seeds .

Cevallos-Cevallos, J. M., Futch, D. B., Shilts, T., Folimonova, S. Y., and Reyes-De-Corcuera, J. I. 2012. GC-MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing. Plant Physiol. Biochem. 53:69–76

Cevallos-Cevallos, J. M., García-Torres, R., Etxeberria, E., and Reyes-De-Corcuera, J. I. 2011. GC-MS analysis of headspace and liquid extracts for metabolomic differentiation of citrus huanglongbing and zinc deficiency in leaves of “Valencia” sweet orange from commercial groves. Phytochem. Anal. 22:236–246

Cia, M. C. 2014. Análise transcritômica de plantas de cana de açúcar inoculadas com Leifsonia xyli subsp. xyli, agente causal do raquitismo das soqueiras.

Coutinho, I. D., Baker, J. M., Ward, J. L., Beale, M. H., Creste, S., and Cavalheiro, A. J. 2016. Metabolite Profiling of Sugarcane Genotypes and Identification of Flavonoid Glycosides and Phenolic Acids. J. Agric. Food Chem. 64:4198–4206

Dal-Bianco, M., Carneiro, M. S., Hotta, C. T., Chapola, R. G., Hoffmann, H. P., Garcia, A. A. F., and Souza, G. M. 2012. Sugarcane improvement: How far can we go? Curr. Opin. Biotechnol. 23:265–270

Davis, M. J., Dean, J. L., and Harrison, N. A. 1988. Quantitative variability of Clavibacter xyli subsp. xyli populations in sugarcane cultivars differing in resistance to ratoon stunting disease. Phytopathology. 78:462–468

Ferreira, T. H. S., Tsunada, M. S., Bassi, D., Araújo, P., Mattiello, L., Guidelli, G. V, Righetto, G. L., Gonçalves, V. R., Lakshmanan, P., and Menossi, M. 2017. Sugarcane water stress tolerance mechanisms and its implications on developing biotechnology solutions. Front. Plant Sci. 8:1077

Fu, H. Y., Sun, S. R., Wang, J. Da, Ahmad, K., Wang, H. B., Chen, R. K., and Gao, S. J. 2016. Rapid and Quantitative Detection of Leifsonia xyli subsp. Xyli in Sugarcane Stalk Juice Using a Real-Time Fluorescent (TaqMan) PCR Assay. Biomed Res. Int. 2016

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. 2012. Role of proline under changing environments: a review. Plant Signal. Behav. 7:1456–66

Ireland, H. E., Harding, S. J., Bonwick, G. A., Jones, M., Smith, C. J., and Williams, J. H. H. 2004. Evaluation of heat shock protein 70 as a biomarker of environmental stress in *Fucus serratus* and *Lemna minor*. Biomarkers. 9:139–155

Jangpromma, N., Kitthaisong, S., Lomthaisong, K., Daduang, S., Jaisil, P., Thammasirirak, S., Jangpromma, N., Kitthaisong, S., Lomthaisong, K., Daduang, S., Jaisil, P., and Thammasirirak, S. 2010. A proteomics analysis of drought stress-responsive proteins as biomarker for drought-tolerant sugarcane cultivars. Am. J. Biochem. Biotechnol. 6:89–102

Katam, R., Chibanguza, K., Latinwo, L. M., and Smith, D. 2015. Proteome biomarkers in xylem reveal Pierce's disease tolerance in grape. J. Proteomics Bioinform. 8:217–224

Kaur, C., Singla-Pareek, S. L., and Sopory, S. K. 2014. Glyoxalase and methylglyoxal as biomarkers for plant stress tolerance. CRC. Crit. Rev. Plant Sci. 33:429–456

Kanehisa, Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353-D361

Leme, G. M., Coutinho, I. D., Creste, S., Hojo, O., Carneiro, R. L., Bolzani, V. da S., and Cavalheiro, A. J. 2014. HPLC-DAD method for metabolic fingerprinting of the phenotyping of sugarcane genotypes. Anal. Methods. 6:7781–7788

Li, W.-F. F., Shen, K., Huang, Y.-K. K., Wang, X.-Y. Y., Yin, J., Luo, Z.-M. M., Zhang, R.-Y. Y., and Shan, H.-L. L. 2014. Incidence of sugarcane ratoon stunting disease in the major cane-growing regions of China. Crop Prot. 60:44–47

Li, Y., Hutchins, W., Wu, X., Liang, C., Zhang, C., Yuan, X., Khokhani, D., Chen, X., Che, Y., Wang, Q., and Yang, C. H. 2015. Derivative of plant phenolic compound inhibits the type III secretion system of Dickeya dadantii via HrpX/HrpY two-component signal transduction and Rsm systems. Mol. Plant Pathol. 16:150–163

Mattioli, R., Costantino, P., and Trovato, M. 2009. Proline accumulation in plants: not only stress. Plant Signal. Behav. 4:1016–8

McFarlane, S. 2002. The relationship between extent of colonisation by Leifsonia xyli subsp. xyli and yield loss in different sugarcane varieties. Proc S Afr Sug Technol Ass. 76:281–284

Monteiro-Vitorello, C. B., Camargo, L. E. A., Van Sluys, M. A., Kitajima, J. P., Truffi, D., Do Amaral, A. M., Harakava, R., De Oliveira, J. C. F., Wood, D., De Oliveira, M. C., Miyaki, C., Takita, M. A., Da Silva, A. C. R., Furlan, L. R., Carraro, D. M., Camarotte, G., Almeida, N. F., Carrer, H., Coutinho, L. L., El-Dorry, H. A., Ferro, M. I. T., Gagliardi, P. R., Giglioti, E., Goldman, M. H. S., Goldman, G. H., Kimura, E. T., Ferro, E. S., Kuramae, E. E., Lemos, E. G. M., Lemos, M. V. F., Mauro, S. M. Z., Machado, M. A., Marino, C. L., Menck, C. F., Nunes, L. R., Oliveira, R. C., Pereira, G. G., Siqueira, W., De Souza, A. A., Tsai, S. M., Zanca, A. S., Simpson, A. J. G., Brumbley, S. M., and Setúbal, J. C. 2004. The genome sequence of the gram-positive sugarcane pathogen Leifsonia xyli subsp. xyli. Mol. Plant-Microbe Interact. 17:827–836

Munawarti, A., Semiarti, E., Taryono, T., Semiarti, E., and Sismindari, S. 2014. Morphological and Biochemical Responses of Glagah (Saccharum spontaneum L .) Accessions to Drought Stress. J. Trop. Life Sci. 4:61–66

Ngaruiya, P. N., Shipton, W. A., and Coventry, R. 2005. Ratoon stunting disease of sugarcane as influenced by environmental stressors. Proc Aust Soc Sugar Cane Technol. 27:324–333

Rott, P., Sood, S., Comstock, J. C., Gilbert, R. A., and Sandhu, H. S. 2002. *Sugarcane Ratoon Stunting*.

Strimbu, K., and Tavel, J. A. 2010. What are biomarkers? Curr. Opin. HIV AIDS. 5:463–6

Tao, L., Yao, H., and Cheng, Q. 2007. Genes from a Dietzia sp. for synthesis of C40 and C50 β-cyclic carotenoids. Gene. 386:90–97

Velasco, P., Lema, M., Francisco, M., Soengas, P., Elena Cartea, M., and Cartea, M. 2013. In Vivo and in vitro effects of secondary metabolites against Xanthomonas campestris pv. campestris. Molecules. 18:11131–11143

Xiong, L., and Zhu, J. 2003. Update on Abscisic Acid Biosynthesis Regulation Regulation of Abscisic Acid Biosynthesis 1. Plant Physiol. 133:29–36

Young, A., Lokes, S., Davis, W., and Aitken, B. O. B. 2012. Reassessing Rsd: Insights From Harwood. Proc Aust Soc Sugar Cane Technol. 34:1–7

Zhao, D., Glaz, B., and Comstock, J. C. 2010. Sugarcane response to water-deficit stress during early growth on organic and sand soils. Am. J. Agric. Biol. Sci. 5:403–414

SUPPLEMENTARY MATERIAL

Table S 1. Standards used for LC-MS/MS analysis and their concentrations

Class	Analyte Peak Name	Concentration (µM)
Amino acids	Arginine	5
	Citrulline	5
	GABA	5
	Histidine	5
	Isoleucine	5
	Leucine	5
	Methionine	5
	OHProline	5
	Proline	5
	Serine	5
	Threonine	5
	Tryptophan	5
	Tyrosine	5
	Phenylalanine	5
	Valine	5
	Asparagine	5
	Alanine	5
	Aspartate	5
	Cysteine	5
	Glutamate	5
	Glutamine	5
	Glycine	5
	Ornithine	5
	Lysine	5
	Homoserine	5

(cont.) Table S 1. Standards used for LC-MS/MS analysis and their concentrations

Class	Analyte Peak Name	Concentration (µM)
Sugars and sugar alcohols	Arabinose	10
	Galactose	10
	Maltose	10
	Mannitol	0.5
	Arabitol	0.5
	Threitol	1
	Galactitol	0.5
	Ribitol	0.5
	Ribose	10
	Glucose	1
	Fructose	1
	Sucrose	1
	Erythritol	1
	Sorbitol	1
	Maltitol	1
	Inositol	1
	Xylitol	0.5
	Xylose	10
	Mannose	10
	Trehalose	1
	Glycerol	100

(cont.) Table S 1. Standards used for LC-MS/MS analysis and their concentrations

Class	Analyte Peak Name	Concentration (µM)
Organic acids	Alpha ketoglutarate	1
	Citrate	1
	Fumarate	1
	Isocitrate	1
	Malate	1
	Succinate	1
	Pyruvate	5
	cis-Aconitate	1
	trans-Aconitate	1
	Shikimate	1
	Ascorbic acid	10
	2-oxobutyric acid	10
	Mevalonic acid	0.5
	Quinic acid	0.5
	Maleic acid	0.5
	Glutaric acid	0.5
	Pimelic acid	0.5
	Suberic acid	0.5
	Ascorbic acid	10
	Sebacic acid	0.5
	Azelaic acid	0.5
	Adipic acid	0.5
	Propionic acid	0.5
	Malonic acid	0.5
	Gluconic acid	0.5
	Tartaric acid	0.5
	Chorismic acid	10

(cont.) Table S 1. Standards used for LC-MS/MS analysis and their concentrations

Class	Analyte Peak Name	Concentration (µM)
Phosphorylated compounds	Glycerol phosphate	0.1
	Sucrose 6-phosphate	0.1
	Trehalose 6-phosphate	0.1
	Phosphoenolpyruvate (PEP)	0.5
	Fructose 1,6 bis phosphate	1
	Ribulose 5-phosphate	5
	6-phosphogluconate	1
	CMP	1
	UMP	0.5
	AMP	0.5
	GMP	0.5
	UDP	0.5
	ADP	0.5
	GDP	0.5
	CTP	0.5
	UTP	1
	ATP	1
	GTP	1
	UDP-glucose	1
	ADP-glucose	1
	Deoxyxylulose 5-phosphate	1
	IMP	0.5
	IDP	0.5
	ITP	0.5
	S7P	0.5
	CDP	0.5
	2/3-PGA	0.1
	Ribulose 1,5-bis phosphate	5
	E4-phosphate	50
	Glucose-1-phosphate	0.1
	Mannose-1-phosphate	0.1
	Glucose-6-phosphate	0.1
	Fructose-6-phosphate	0.1
	Mannose-6-phosphate	0.1
	Ribose-1-phosphate	1
	Galactose-1-phosphate	0.1
	UDP-N-glucosamine	10
	UDP-N-galactosamine	10

(cont.) Table S 1. Standards used for LC-MS/MS analysis and their concentrations

Class	Analyte Peak Name	Concentration (µM)
Phenolic compounds	3-Chorogenic acid	1
	3,4-Dimethoxycinnamic acid	1
	4-Chlorogenic acid	1
	4-hydroxybenzoic	1
	5-Chlorogenic acid	1
	4,5-Di-O-Caffeoylquinic acid	10
	3,4-Di-O-Caffeoylquinic acid	10
	Benzoic acid	1
	Caffeic acid	1
	Cinnamic acid	1
	Coniferaldehyde	1
	Coniferyl-OH-H_2O	1
	Coumaric acid	1
	Ferulic acid	1
	Salicylic acid	1
	Sinapaldehyde	1
	Syringic acid	1
	Vanillic acid	1
	Vanillin	1
	Sinapic acid	1
	Sinapylalcohol	10
Flavonoids	Isoorientin	1
	isovitexin/Vitexin	1
	Kaempferol	1
	Luteolin	1
	Luteolin-7-O-glucoside	1
	Maysin	1
	Naringenin	1
	Orientin	0
	Quercetin	1
	Rhamisoorientin	1
	Apigenin	1
	Apigenin-7O-glucoside	1
	Dihydrokaempferol	1
	Dihydroquercetin	1
	Eriodictyol	1
Hormones	Caffeine	1
	Jasmonic acid	10
	Methyl-jasmonate	1
	GA3	1
	Salicylic acid	1
	Abscisic acid (ABA)	1

Table S 2. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in leaves of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI.

m/z	RT (min)	Leaves											
		S			SI			R			RI		
174	12.58	1.06E-03	±	4.30E-04	9.92E-04	±	1.76E-04	1.95E-03	±	9.20E-05	1.04E-03	±	3.75E-04
234.1	13.03	6.41E-04	±	2.37E-04	5.96E-04	±	1.35E-04	8.05E-04	±	6.46E-05	7.05E-04	±	1.40E-04
117	13.13	1.38E-03	±	3.16E-04	1.74E-03	±	4.36E-04	4.22E-03	±	2.16E-03	1.84E-03	±	4.04E-04
56	13.27	2.93E-04	±	3.76E-05	2.30E-04	±	2.89E-05	1.58E-04	±	1.91E-05	2.55E-04	±	3.45E-05
131.1	14.57	1.03E-03	±	3.72E-04	2.33E-03	±	1.10E-03	1.99E-03	±	5.12E-04	8.21E-04	±	1.17E-04
116.1	15.08	4.65E-04	±	9.00E-05	7.99E-04	±	2.23E-04	6.60E-04	±	1.44E-04	3.02E-04	±	1.68E-04
133	15.57	2.00E-03	±	6.76E-04	1.57E-03	±	3.36E-04	2.25E-03	±	2.31E-04	2.07E-03	±	3.52E-04
73	15.83	1.88E-03	±	6.15E-04	3.35E-03	±	1.04E-03	5.28E-03	±	1.42E-03	4.21E-03	±	2.22E-03
103.1	15.85	3.09E-03	±	8.33E-04	9.19E-03	±	2.82E-03	1.39E-02	±	3.96E-03	8.70E-03	±	5.61E-03
147.1	16.88	2.22E-03	±	5.12E-04	4.31E-03	±	9.91E-04	4.11E-03	±	7.48E-04	4.56E-03	±	1.64E-03
188.2	18.27	9.89E-05	±	5.00E-05	3.65E-04	±	1.19E-04	3.65E-04	±	1.46E-04	4.74E-04	±	1.93E-04
281.1	18.72	2.17E-03	±	3.26E-04	3.21E-03	±	5.65E-04	3.05E-03	±	2.67E-04	3.47E-03	±	7.38E-04
174.2	22.77	1.45E-03	±	3.37E-04	1.05E-03	±	7.58E-05	1.04E-03	±	2.62E-05	8.57E-04	±	9.51E-05
131.1	23.15	6.08E-05	±	2.23E-05	1.63E-04	±	7.69E-05	1.38E-04	±	4.96E-05	7.88E-05	±	9.35E-06
299.1	23.28	4.43E-01	±	2.44E-02	5.05E-01	±	6.31E-02	4.26E-01	±	9.11E-02	4.82E-01	±	1.24E-01
72.9	23.42	1.15E-01	±	1.95E-02	1.18E-01	±	1.65E-02	1.20E-01	±	1.93E-02	1.13E-01	±	1.47E-02
205.1	23.45	2.16E-01	±	3.81E-02	2.51E-01	±	4.04E-02	2.54E-01	±	3.71E-02	2.47E-01	±	3.88E-02
247.1	24.70	3.72E-03	±	1.33E-04	3.04E-03	±	6.46E-04	2.33E-03	±	9.68E-04	3.93E-03	±	1.36E-04
147.1	24.82	3.53E-03	±	6.81E-04	4.60E-03	±	8.36E-04	3.45E-03	±	2.40E-04	3.17E-03	±	3.38E-04
292.2	25.93	5.14E-03	±	9.18E-04	5.48E-03	±	9.58E-04	5.62E-03	±	6.54E-04	4.65E-03	±	8.23E-04
57	26.60	3.90E-04	±	2.26E-04	5.44E-04	±	1.97E-04	9.52E-04	±	1.20E-04	7.50E-04	±	3.04E-04
188.2	26.92	8.77E-05	±	3.22E-05	4.82E-04	±	1.58E-04	2.56E-04	±	7.96E-05	3.09E-04	±	1.70E-04
247.1	27.58	2.68E-04	±	3.60E-05	4.51E-04	±	1.01E-04	4.03E-04	±	4.68E-05	4.02E-04	±	9.09E-05
239.2	29.28	2.87E-04	±	7.52E-05	1.93E-04	±	8.13E-05	1.36E-04	±	6.28E-06	2.08E-04	±	1.08E-04
147.1	32.68	2.67E-02	±	3.16E-03	4.10E-02	±	6.10E-03	1.71E-02	±	2.15E-03	1.37E-02	±	3.73E-03

(cont.) Table S 2. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in leaves of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI.

m/z	RT (min)	Leaves											
		S			SI			R			RI		
159.1	33.15	1.39E-04	±	8.10E-05	2.51E-04	±	9.01E-05	3.01E-04	±	2.50E-05	2.74E-04	±	1.01E-04
156.1	33.68	5.41E-04	±	6.24E-05	1.22E-03	±	2.68E-04	9.92E-04	±	2.42E-04	8.20E-04	±	1.71E-04
292.2	35.90	2.75E-02	±	4.82E-03	4.06E-02	±	4.96E-03	3.94E-02	±	2.60E-03	4.04E-02	±	9.96E-03
186.1	36.07	2.08E-04	±	1.39E-04	1.02E-04	±	3.14E-05	8.97E-05	±	1.26E-05	6.58E-05	±	1.93E-05
213.1	36.43	9.70E-05	±	3.69E-05	1.94E-04	±	4.64E-05	2.38E-04	±	5.34E-05	1.82E-04	±	4.77E-05
246.2	37.75	8.67E-04	±	3.80E-04	4.47E-04	±	3.88E-04	1.05E-04	±	4.04E-05	2.29E-04	±	1.89E-04
157.1	40.68	2.61E-04	±	7.18E-05	2.97E-04	±	5.51E-05	1.71E-04	±	3.07E-05	1.91E-04	±	3.84E-05
147.1	42.48	1.79E-02	±	4.81E-03	4.70E-02	±	9.85E-03	1.87E-02	±	1.67E-03	1.45E-02	±	4.28E-03
297.2	42.77	1.40E-04	±	3.23E-05	2.77E-04	±	5.90E-05	2.58E-04	±	2.38E-05	2.33E-04	±	3.63E-05
355.2	43.00	4.74E-04	±	9.06E-05	8.32E-04	±	1.46E-04	7.46E-04	±	1.32E-04	7.87E-04	±	2.02E-04
357.2	43.37	8.32E-05	±	1.81E-05	1.34E-04	±	1.93E-05	1.94E-04	±	4.37E-05	1.68E-04	±	5.09E-05
217.1	43.53	4.06E-03	±	4.75E-04	7.64E-03	±	1.40E-03	7.16E-03	±	1.03E-03	7.54E-03	±	1.86E-03
293.2	43.62	1.69E-04	±	4.59E-05	3.09E-04	±	6.10E-05	3.99E-04	±	4.13E-05	3.37E-04	±	7.49E-05
292.2	43.68	4.43E-04	±	1.08E-04	9.21E-04	±	1.51E-04	1.00E-03	±	1.61E-04	8.96E-04	±	2.06E-04
292.2	43.97	2.66E-04	±	7.78E-05	5.30E-04	±	7.67E-05	5.54E-04	±	9.31E-05	4.66E-04	±	9.78E-05
204.2	44.90	1.01E-03	±	1.94E-04	2.24E-03	±	3.54E-04	2.53E-03	±	6.24E-04	1.90E-03	±	2.79E-04
273.1	45.35	3.42E-02	±	1.91E-02	7.97E-02	±	2.59E-02	6.85E-02	±	7.51E-03	4.64E-03	±	6.71E-04
245.1	45.38	4.66E-02	±	6.64E-03	6.95E-02	±	8.66E-03	5.17E-02	±	5.20E-03	4.07E-02	±	5.68E-03
345.2	46.92	4.56E-03	±	1.01E-03	1.15E-02	±	2.56E-03	1.23E-02	±	2.60E-03	1.16E-02	±	2.77E-03
221.1	47.10	2.09E-04	±	1.41E-04	6.66E-04	±	2.21E-04	4.60E-04	±	4.94E-05	6.99E-04	±	2.50E-04
103.1	47.45	6.41E-03	±	1.77E-03	1.22E-02	±	2.21E-03	6.88E-03	±	1.44E-03	5.69E-03	±	2.25E-03
217.2	47.80	1.45E-02	±	3.68E-03	2.76E-02	±	5.26E-03	1.49E-02	±	2.71E-03	1.31E-02	±	5.23E-03
319.2	48.08	3.09E-03	±	7.72E-04	4.08E-03	±	6.33E-04	2.45E-03	±	5.79E-04	2.21E-03	±	7.45E-04
320.2	48.18	3.60E-03	±	1.80E-03	1.42E-02	±	4.74E-03	9.85E-03	±	1.99E-03	6.63E-04	±	2.18E-04
159.1	33.15	1.39E-04	±	8.10E-05	2.51E-04	±	9.01E-05	3.01E-04	±	2.50E-05	2.74E-04	±	1.01E-04

(cont.) Table S 2. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in leaves of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI.

m/z	RT (min)	Leaves											
		S			SI			R			RI		
319.2	48.27	2.74E-02	±	8.12E-03	5.95E-02	±	1.24E-02	3.58E-02	±	6.91E-03	3.10E-02	±	1.13E-02
293.2	48.58	4.09E-04	±	9.80E-05	8.43E-04	±	2.00E-04	1.26E-03	±	1.36E-04	1.02E-03	±	1.76E-04
205.2	48.85	5.46E-03	±	1.68E-03	7.54E-03	±	9.96E-04	4.34E-03	±	9.29E-04	4.60E-03	±	8.50E-04
227.1	50.63	7.29E-04	±	2.10E-04	9.20E-04	±	1.90E-04	2.65E-03	±	8.06E-04	4.72E-04	±	8.31E-05
317.2	50.68	8.63E-04	±	2.39E-04	1.11E-03	±	2.44E-04	3.49E-03	±	1.09E-03	3.49E-03	±	7.24E-04
313.3	51.82	2.13E-03	±	7.64E-04	3.40E-03	±	1.28E-03	3.90E-03	±	2.40E-04	1.79E-03	±	3.51E-04
449.3	52.02	7.68E-04	±	3.47E-04	2.35E-03	±	1.19E-03	6.05E-04	±	3.18E-04	5.97E-04	±	2.20E-04
300.2	53.02	2.90E-04	±	9.55E-05	6.90E-04	±	2.28E-04	1.25E-03	±	3.62E-04	1.06E-03	±	3.52E-04
333.2	53.10	6.09E-04	±	2.50E-04	5.49E-04	±	1.05E-04	5.40E-04	±	2.20E-04	6.82E-04	±	2.03E-04
305.2	54.25	2.47E-02	±	5.06E-03	3.41E-02	±	4.20E-03	2.29E-02	±	3.16E-03	1.87E-02	±	3.64E-03
219.1	54.92	1.11E-02	±	3.14E-03	2.25E-02	±	5.42E-03	2.24E-02	±	4.12E-03	1.72E-02	±	3.70E-03
299.2	55.05	1.02E-03	±	4.62E-04	2.50E-03	±	6.45E-04	1.03E-03	±	4.96E-04	5.97E-04	±	3.90E-04
327.4	55.17	5.19E-04	±	3.27E-04	1.33E-03	±	6.59E-04	1.24E-03	±	7.78E-05	2.32E-04	±	4.08E-05
204.2	57.30	7.24E-04	±	2.14E-04	1.33E-03	±	4.26E-04	1.69E-03	±	3.22E-04	1.36E-03	±	4.32E-04
357.2	57.47	3.20E-04	±	1.13E-04	4.99E-04	±	1.14E-04	3.29E-04	±	1.11E-04	2.27E-04	±	8.06E-05
341.4	57.68	2.27E-03	±	1.01E-03	3.63E-03	±	1.48E-03	4.31E-03	±	3.03E-04	1.51E-03	±	3.47E-04
338.2	57.93	1.27E-04	±	4.08E-05	2.21E-04	±	4.87E-05	2.57E-04	±	4.40E-05	1.95E-04	±	4.82E-05
204.1	60.95	3.01E-02	±	6.50E-03	4.32E-02	±	1.04E-02	2.69E-02	±	6.14E-03	1.74E-02	±	5.96E-03
253.2	61.25	1.73E-04	±	9.88E-05	2.53E-04	±	1.26E-04	3.34E-04	±	2.90E-05	5.59E-05	±	2.08E-05
239.2	61.73	6.76E-04	±	3.37E-04	9.44E-04	±	4.34E-04	1.33E-03	±	8.94E-05	3.23E-04	±	7.94E-05
204.2	62.33	7.71E-04	±	1.59E-04	1.66E-03	±	3.54E-04	4.35E-04	±	1.17E-04	2.78E-04	±	1.14E-04
318.2	63.23	1.59E-04	±	5.26E-05	2.58E-04	±	4.54E-05	2.91E-04	±	6.94E-05	1.56E-04	±	4.23E-05
204.2	65.00	3.53E-04	±	1.57E-04	8.65E-04	±	2.48E-04	9.86E-04	±	1.92E-04	7.23E-04	±	2.03E-04
387.2	65.90	4.02E-05	±	2.39E-05	6.22E-05	±	9.42E-06	2.23E-04	±	7.12E-05	1.28E-04	±	3.13E-05
267.1	66.08	4.82E-05	±	1.67E-05	1.61E-04	±	5.72E-05	1.94E-04	±	3.89E-05	1.35E-04	±	2.62E-05

(cont.) Table S 2. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in leaves of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI.

m/z	RT (min)	Leaves											
		S			SI			R			RI		
307.2	67.32	2.56E-03	±	2.24E-04	3.30E-03	±	5.23E-04	2.72E-03	±	4.74E-04	3.06E-03	±	6.29E-04
361.2	67.37	4.98E-01	±	1.58E-01	3.36E-01	±	8.34E-02	4.11E-01	±	1.57E-01	3.69E-01	±	8.30E-02
215.1	67.45	2.41E-04	±	2.96E-05	1.85E-04	±	3.94E-05	2.39E-04	±	7.00E-05	2.13E-04	±	3.78E-05
217.1	67.57	3.63E-03	±	4.78E-04	5.64E-03	±	9.73E-04	5.20E-03	±	5.75E-04	4.93E-03	±	9.88E-04
259.2	68.42	1.05E-04	±	2.27E-05	2.20E-04	±	4.13E-05	2.86E-04	±	5.60E-05	2.21E-04	±	6.15E-05
355.1	68.68	1.02E-03	±	2.10E-04	2.66E-03	±	6.42E-04	1.81E-03	±	2.18E-04	1.94E-03	±	4.20E-04
361.2	68.83	2.05E-02	±	2.90E-03	3.21E-02	±	8.06E-03	9.68E-03	±	1.49E-03	1.12E-02	±	2.59E-03
242.1	68.85	1.94E-04	±	3.30E-05	3.56E-04	±	8.52E-05	6.83E-04	±	9.37E-05	6.32E-04	±	1.63E-04
217.1	69.12	4.75E-04	±	8.36E-05	9.07E-04	±	1.90E-04	1.82E-03	±	2.66E-04	1.70E-03	±	4.05E-04
267.1	69.62	8.63E-04	±	1.45E-04	1.62E-03	±	3.21E-04	1.91E-03	±	2.20E-04	1.85E-03	±	4.82E-04
297.2	69.93	1.92E-03	±	3.74E-04	3.80E-03	±	9.78E-04	5.97E-03	±	6.44E-04	5.79E-03	±	1.28E-03
253.1	69.97	3.84E-04	±	4.77E-05	3.91E-04	±	1.55E-04	1.46E-03	±	2.62E-04	1.56E-03	±	4.06E-04
267.2	70.35	8.99E-04	±	1.41E-04	1.71E-03	±	3.63E-04	1.78E-03	±	1.55E-04	1.59E-03	±	5.06E-04
384.2	70.37	1.86E-04	±	4.08E-05	4.07E-04	±	9.04E-05	5.31E-04	±	7.12E-05	4.81E-04	±	1.17E-04
297.2	70.52	5.16E-03	±	8.35E-04	1.03E-02	±	2.42E-03	9.86E-03	±	8.82E-04	9.70E-03	±	2.01E-03
281.2	70.62	3.69E-04	±	1.39E-04	6.56E-04	±	3.29E-04	1.38E-03	±	2.48E-04	1.23E-03	±	3.14E-04
332.2	70.75	1.80E-04	±	3.42E-05	4.20E-04	±	1.04E-04	4.08E-04	±	7.80E-05	3.44E-04	±	9.04E-05
217.1	70.95	1.19E-03	±	2.92E-04	2.34E-03	±	4.66E-04	3.80E-03	±	5.87E-04	3.63E-03	±	1.05E-03
269.1	71.18	4.35E-04	±	8.63E-05	8.02E-04	±	1.91E-04	2.09E-03	±	2.47E-04	2.12E-03	±	5.73E-04
297.2	71.30	2.61E-03	±	5.08E-04	5.78E-03	±	1.16E-03	4.68E-03	±	7.58E-04	4.57E-03	±	1.03E-03
327.1	71.40	3.38E-04	±	6.04E-05	6.97E-04	±	1.33E-04	1.59E-03	±	1.41E-04	1.56E-03	±	3.56E-04
345.2	71.52	4.32E-04	±	1.39E-04	1.25E-03	±	3.47E-04	1.26E-03	±	2.43E-04	1.36E-03	±	4.09E-04
345.2	71.82	5.58E-04	±	1.77E-04	1.51E-03	±	4.10E-04	2.72E-03	±	3.12E-04	2.51E-03	±	5.41E-04
204.1	72.05	2.27E-02	±	3.92E-03	3.72E-02	±	8.55E-03	3.38E-02	±	5.00E-03	3.66E-02	±	1.10E-02
307.2	67.32	2.56E-03	±	2.24E-04	3.30E-03	±	5.23E-04	2.72E-03	±	4.74E-04	3.06E-03	±	6.29E-04

(cont.) Table S 2. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in leaves of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI.

m/z	RT (min)	Leaves											
		S			SI			R			RI		
327.1	72.10	2.72E-04	±	8.38E-05	6.23E-04	±	3.12E-04	2.24E-04	±	1.06E-04	4.10E-04	±	1.27E-04
327.2	72.18	2.70E-03	±	5.15E-04	5.43E-03	±	1.11E-03	4.89E-03	±	4.82E-04	4.93E-03	±	1.01E-03
255.1	72.92	2.16E-04	±	4.63E-05	4.32E-04	±	1.26E-04	4.99E-04	±	8.82E-05	4.84E-04	±	2.27E-04
345.2	73.20	3.54E-02	±	8.13E-03	8.00E-02	±	2.23E-02	1.09E-01	±	9.89E-03	1.06E-01	±	2.14E-02
325.1	73.27	1.92E-04	±	4.35E-05	2.78E-04	±	3.98E-05	2.93E-04	±	6.42E-05	2.83E-04	±	9.25E-05
204.1	73.53	2.44E-02	±	4.32E-03	3.31E-02	±	7.55E-03	2.40E-02	±	4.13E-03	2.33E-02	±	9.12E-03
309.2	73.67	2.52E-04	±	6.94E-05	4.25E-04	±	1.97E-04	9.16E-04	±	1.66E-04	2.46E-04	±	8.92E-05
307.1	73.83	1.94E-03	±	6.75E-04	4.44E-03	±	1.42E-03	8.34E-03	±	1.46E-03	6.87E-03	±	2.27E-03
249.1	73.85	2.17E-03	±	6.67E-04	7.23E-03	±	2.09E-03	3.03E-03	±	4.99E-04	2.66E-03	±	9.15E-04
345.2	73.98	5.08E-03	±	1.40E-03	1.24E-02	±	3.20E-03	1.94E-02	±	1.76E-03	1.71E-02	±	4.60E-03
307.2	75.33	1.68E-03	±	4.69E-04	3.32E-03	±	9.87E-04	1.47E-03	±	4.21E-04	1.19E-03	±	3.41E-04
361.2	76.95	8.13E-03	±	1.88E-03	1.55E-02	±	3.46E-03	3.62E-02	±	4.32E-03	4.07E-02	±	1.38E-02
647.7	79.15	2.10E-03	±	1.10E-03	2.81E-03	±	1.37E-03	3.47E-03	±	2.75E-04	4.99E-04	±	1.16E-04
207.1	79.27	3.29E-03	±	4.19E-04	6.85E-03	±	2.22E-03	6.12E-03	±	1.80E-03	4.41E-03	±	7.51E-04

Table S 3. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in xylem sap of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI. (except for S and R, n=3).

m/z	RT (min)	Xylem sap											
		S			SI			R			RI		
207.1	12.40	5.03E-02	±	6.49E-03	5.14E-02	±	3.60E-03	5.42E-02	±	5.76E-03	5.62E-02	±	4.55E-03
235.2	13.13	1.63E-01	±	1.10E-02	1.45E-01	±	9.63E-03	1.42E-01	±	1.93E-02	1.39E-01	±	1.67E-02
73	13.15	8.19E-01	±	1.15E-01	8.55E-01	±	9.82E-02	7.86E-01	±	1.16E-01	9.33E-01	±	1.14E-01
190.2	13.17	1.69E-02	±	8.56E-03	2.72E-02	±	9.82E-03	5.49E-03	±	3.81E-04	8.59E-03	±	2.36E-03
118.2	13.18	2.14E-02	±	1.38E-02	2.97E-02	±	9.57E-03	8.13E-03	±	9.40E-04	1.67E-02	±	4.45E-03
207.1	13.25	4.67E-02	±	9.98E-03	6.47E-02	±	3.97E-03	1.01E-01	±	3.38E-02	1.42E-01	±	1.46E-02
159.2	13.32	3.58E-01	±	8.16E-03	6.15E-01	±	9.53E-02	4.27E-01	±	8.73E-02	6.00E-01	±	6.05E-02
100.1	14.08	3.97E-02	±	3.02E-03	4.15E-02	±	5.80E-03	6.39E-02	±	7.59E-03	7.51E-02	±	2.08E-03
77	14.37	3.47E-02	±	2.31E-03	3.82E-02	±	3.47E-03	3.98E-02	±	3.44E-03	4.17E-02	±	2.76E-03
131.1	14.63	4.90E-02	±	1.42E-02	5.54E-02	±	5.33E-03	5.61E-02	±	6.29E-03	5.74E-02	±	1.58E-03
204.1	15.08	1.60E-02	±	7.35E-03	8.97E-03	±	8.38E-04	1.04E-02	±	1.57E-03	1.33E-02	±	8.71E-04
116.1	15.12	9.85E-03	±	2.14E-03	1.36E-02	±	1.06E-03	1.40E-02	±	2.67E-03	1.78E-02	±	5.59E-03
97.1	15.57	7.22E-02	±	6.24E-03	6.31E-02	±	2.60E-03	7.29E-02	±	5.69E-03	7.89E-02	±	6.79E-03
73	15.62	2.02E+00	±	1.78E-01	1.86E+00	±	7.55E-02	2.01E+00	±	3.07E-01	2.28E+00	±	2.47E-01
97.1	15.87	6.16E-02	±	3.80E-03	5.52E-02	±	1.31E-03	6.48E-02	±	6.45E-03	6.94E-02	±	6.32E-03
103.1	15.88	6.94E-02	±	9.81E-03	5.49E-02	±	3.14E-03	6.08E-02	±	2.13E-02	7.65E-02	±	1.72E-02
155.1	16.00	3.79E-02	±	5.53E-03	3.28E-02	±	3.23E-03	2.76E-02	±	4.19E-03	2.50E-02	±	2.45E-03
73	16.90	3.97E-01	±	7.90E-02	4.21E-01	±	5.66E-02	4.78E-01	±	4.76E-02	6.28E-01	±	4.44E-02
100	16.92	3.70E-02	±	7.13E-03	3.91E-02	±	5.45E-03	4.53E-02	±	4.30E-03	5.94E-02	±	4.20E-03
89	16.98	4.45E-02	±	2.90E-03	5.11E-02	±	4.42E-03	5.39E-02	±	5.65E-03	6.98E-02	±	5.80E-03
73	18.27	2.07E-01	±	4.55E-02	2.52E-01	±	3.52E-02	5.26E-01	±	5.36E-02	7.87E-01	±	9.84E-02
77	18.65	1.78E-02	±	4.88E-03	2.71E-02	±	1.08E-02	2.04E-02	±	9.55E-03	3.95E-02	±	8.09E-03
281.1	18.72	7.16E-02	±	4.01E-02	4.28E-02	±	7.25E-03	4.16E-02	±	1.07E-02	2.97E-02	±	1.92E-03
132.1	19.03	3.07E-02	±	8.32E-04	2.06E-02	±	3.42E-03	3.78E-02	±	1.07E-02	4.12E-02	±	3.99E-03
69	19.37	1.38E-01	±	1.68E-02	8.57E-02	±	2.57E-02	1.28E-01	±	5.60E-03	1.17E-01	±	2.97E-02

(cont.) Table S 3. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in xylem sap of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI. (except for S and R, n=3).

m/z	RT (min)	Sap											
		S			SI			R			RI		
139.1	19.38	7.76E-02	±	1.33E-02	5.00E-02	±	1.17E-02	6.94E-02	±	2.46E-03	6.83E-02	±	9.68E-03
171.1	21.28	1.14E-02	±	7.23E-03	1.39E-02	±	5.63E-03	2.87E-03	±	9.65E-04	2.21E-03	±	1.25E-04
117.1	21.88	3.00E-02	±	6.21E-04	3.31E-02	±	8.01E-04	3.32E-02	±	2.88E-03	3.54E-02	±	1.14E-03
299.1	23.08	2.05E-02	±	9.41E-03	2.00E-02	±	1.10E-02	3.06E-02	±	2.76E-02	2.20E-02	±	1.39E-02
131.1	23.12	3.21E-02	±	4.01E-03	3.62E-02	±	2.73E-03	3.65E-02	±	3.14E-03	3.57E-02	±	4.80E-04
301.1	23.20	4.99E-03	±	1.27E-03	6.25E-03	±	1.98E-03	1.96E-02	±	1.04E-02	5.11E-02	±	3.64E-02
193	23.22	1.80E-03	±	5.61E-04	2.95E-03	±	1.37E-03	1.11E-02	±	6.40E-03	2.52E-02	±	1.65E-02
198.1	23.97	8.61E-03	±	1.70E-03	3.79E-02	±	2.45E-02	9.68E-03	±	5.45E-03	1.78E-02	±	5.12E-03
140.1	24.47	1.11E-02	±	1.24E-03	1.45E-02	±	2.47E-03	3.62E-02	±	3.87E-03	2.01E-02	±	3.76E-03
70	24.48	2.45E-02	±	1.40E-03	2.78E-02	±	2.08E-03	4.13E-02	±	9.17E-04	3.39E-02	±	1.64E-03
174.1	24.55	4.21E-03	±	1.32E-03	8.07E-03	±	1.17E-03	2.70E-02	±	2.14E-02	7.21E-03	±	2.34E-03
86	24.57	7.39E-03	±	4.74E-04	7.11E-03	±	8.07E-04	1.50E-02	±	4.64E-03	8.14E-03	±	9.68E-04
191.1	24.93	3.70E-02	±	1.82E-03	5.20E-02	±	6.40E-03	3.84E-02	±	5.84E-03	4.28E-02	±	2.05E-03
285.2	25.67	1.24E-02	±	5.50E-03	3.21E-02	±	3.43E-03	3.23E-02	±	5.96E-03	4.55E-02	±	4.99E-03
134.1	25.68	5.72E-02	±	4.28E-03	1.04E-01	±	1.03E-02	1.08E-01	±	1.88E-02	1.63E-01	±	1.84E-02
57	26.55	6.05E-01	±	3.19E-02	5.46E-01	±	2.07E-02	5.80E-01	±	5.25E-02	5.97E-01	±	4.13E-02
69	26.57	1.28E-01	±	1.69E-02	9.49E-02	±	3.42E-03	1.11E-01	±	9.55E-03	1.22E-01	±	7.64E-03
77	26.70	6.07E-02	±	1.35E-02	5.11E-02	±	3.01E-03	4.01E-02	±	1.20E-02	6.23E-02	±	9.10E-03
129.1	29.93	2.59E-03	±	1.43E-03	3.61E-03	±	2.93E-03	3.58E-02	±	5.11E-03	1.89E-02	±	4.64E-03
155.1	29.95	8.17E-03	±	3.12E-04	6.57E-03	±	4.22E-03	5.79E-02	±	9.34E-03	3.10E-02	±	7.06E-03
156.1	30.02	2.01E-02	±	4.13E-04	1.61E-02	±	7.66E-03	4.93E-02	±	3.71E-02	6.02E-02	±	1.25E-02
91	31.20	2.20E-02	±	8.53E-03	1.76E-02	±	8.88E-03	1.36E-02	±	3.11E-03	2.22E-02	±	4.92E-03
350.2	31.25	2.18E-02	±	6.94E-03	2.84E-02	±	9.33E-03	2.22E-02	±	2.29E-03	2.67E-02	±	3.06E-03
57	33.10	5.71E-02	±	4.87E-03	5.25E-02	±	3.31E-03	5.60E-02	±	6.89E-03	5.96E-02	±	3.70E-03
156.1	33.63	8.83E-03	±	1.24E-03	1.15E-02	±	1.26E-03	2.17E-02	±	1.51E-02	2.31E-02	±	1.06E-02

(cont.) Table S 3. Mass/charge ratio (m/z), retention time (RT) and average relative abundance ± standard error (n=4) of metabolites detected by untargeted metabolomics in xylem sap of a susceptible (S) or resistant (R) sugarcane variety mock-inoculated or inoculated with Lxx (I) at 120 DAI. (except for S and R, n=3).

m/z	RT (min)	Sap											
		S			SI			R			RI		
75	35.23	1.91E-01	±	6.70E-02	1.54E-01	±	6.22E-02	8.64E-02	±	1.80E-02	1.59E-01	±	4.17E-02
55	39.80	1.17E-01	±	1.43E-02	8.32E-02	±	6.82E-03	1.28E-01	±	4.89E-02	1.05E-01	±	1.81E-02
217.2	39.88	3.15E-02	±	4.16E-03	5.20E-02	±	1.08E-02	9.07E-02	±	2.31E-02	8.02E-02	±	1.35E-02
86.1	42.35	2.84E-02	±	9.86E-03	3.88E-02	±	1.06E-02	1.59E-02	±	9.75E-03	3.15E-02	±	8.50E-03
103.1	47.37	2.76E-02	±	2.59E-03	2.36E-02	±	4.71E-03	1.01E+00	±	6.38E-01	3.07E-01	±	1.32E-01
172.2	47.38	4.90E-04	±	1.05E-04	6.09E-04	±	1.30E-04	3.08E-02	±	1.89E-02	8.98E-03	±	3.97E-03
103.1	47.73	1.65E-02	±	1.37E-03	1.35E-02	±	2.49E-03	5.71E-01	±	3.70E-01	1.77E-01	±	7.58E-02
319.2	48.22	4.51E-03	±	1.74E-03	3.43E-03	±	8.92E-04	6.06E-02	±	4.87E-02	1.45E-02	±	6.92E-03
205.2	48.23	3.15E-03	±	1.65E-03	2.24E-03	±	5.41E-04	4.99E-02	±	3.99E-02	1.30E-02	±	5.91E-03
73	51.75	2.45E-01	±	1.72E-02	2.49E-01	±	1.91E-02	2.54E-01	±	5.53E-02	3.96E-01	±	3.08E-02
74	51.77	1.25E-02	±	1.02E-03	1.39E-02	±	1.85E-03	1.59E-02	±	2.84E-03	2.35E-02	±	1.84E-03
117	57.60	1.02E-01	±	5.34E-03	1.02E-01	±	4.96E-03	9.64E-02	±	2.20E-02	1.75E-01	±	1.61E-02
361.2	67.27	1.15E-02	±	3.81E-03	8.50E-03	±	1.46E-03	1.39E-02	±	2.84E-03	7.58E-02	±	6.47E-02
207.1	80.08	7.44E-02	±	1.23E-02	6.74E-02	±	6.94E-03	9.34E-02	±	2.35E-02	9.00E-02	±	1.90E-02

Table S 4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Amino acids and derivatives	Alanine	5768.80	±	941.19	1940.42	±	231.69	5341.05	±	628.31	2720.51	±	140.75
	Arginine	93.49	±	11.57	94.66	±	13.23	117.63	±	3.57	104.68	±	17.75
	Asparagine	834.73	±	476.51	242.53	±	93.36	378.02	±	186.40	229.99	±	90.14
	Aspartate	1884.66	±	373.20	833.87	±	126.79	2172.30	±	326.09	1014.14	±	110.94
	Cysteine	8.72	±	1.05	8.27	±	0.77	7.29	±	0.25	5.46	±	0.41
	Glutamate	4066.55	±	368.86	1870.08	±	195.52	4183.46	±	428.83	2240.20	±	194.96
	Glycine	655.08	±	56.66	395.45	±	47.33	689.07	±	106.97	654.86	±	99.51
	Histidine	91.42	±	8.24	65.86	±	11.07	117.71	±	22.62	65.82	±	3.95
	Methionine	16.23	±	7.06	8.42	±	2.63	7.78	±	3.67	6.47	±	3.55
	Proline	369.18	±	34.30	177.03	±	7.90	413.69	±	51.26	250.85	±	29.55
	Serine	879.74	±	133.63	453.16	±	51.99	805.98	±	106.45	562.96	±	18.28
	Tyrosine	187.05	±	15.05	110.16	±	13.86	146.01	±	8.55	115.83	±	2.30
	Valine	730.23	±	69.16	427.43	±	34.12	782.60	±	54.06	571.27	±	36.12
	Citrulline	58.48	±	17.64	27.72	±	3.01	46.71	±	9.83	34.87	±	3.94
	GABA	176.04	±	48.46	27.28	±	6.42	100.85	±	16.09	50.15	±	14.54
	Glutamine	661.26	±	228.53	428.32	±	33.43	519.60	±	96.28	572.76	±	122.41
	Homoserine	288.69	±	30.12	163.66	±	12.49	282.50	±	46.38	252.81	±	11.25
	Threonine	677.29	±	104.15	282.14	±	32.88	678.33	±	69.55	401.71	±	33.52
	Lysine	99.18	±	9.53	72.82	±	10.05	92.16	±	5.61	74.12	±	6.44
	OHProline	21.67	±	1.44	10.43	±	1.32	16.31	±	1.94	15.18	±	1.60
	Leucine	134.29	±	8.94	107.89	±	9.07	143.94	±	14.18	140.46	±	6.72
	Isoleucine	134.29	±	8.94	107.89	±	9.07	143.94	±	14.18	140.46	±	6.72

(cont.) Table S 4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	120 DAI											
		S			R			SI			RI		
Amino acids and derivatives	Alanine	1708.08	±	143.274	808.844	±	63.2656	1618.43	±	56.5067	600.298	±	61.4039
	Arginine	76.4666	±	6.21716	55.2519	±	5.72204	69.6381	±	3.34215	57.368	±	6.82276
	Asparagine	48.1179	±	5.69793	42.118	±	4.54585	44.5465	±	2.7457	37.9445	±	1.71428
	Aspartate	501.006	±	107.023	254.549	±	25.9892	494.341	±	70.002	194.476	±	38.3194
	Cysteine	1.99651	±	0.46548	3.1764	±	0.33693	5.3987	±	0.43542	6.96671	±	0.44678
	Glutamate	1147.07	±	48.9069	705.587	±	11.9002	1282.05	±	45.4284	595.136	±	27.8511
	Glycine	217.517	±	16.2518	197.423	±	32.0315	172.616	±	10.6439	124.872	±	17.2577
	Histidine	52.0384	±	1.82021	43.5679	±	2.14515	53.564	±	2.68011	35.8006	±	2.96798
	Methionine	2.68746	±	0.25815	2.18112	±	0.10962	2.61427	±	0.35917	2.10056	±	0.387
	Proline	390.241	±	21.2542	199.51	±	4.2436	286.727	±	13.6843	188.453	±	9.86765
	Serine	314.491	±	30.6007	271.979	±	28.7253	325.822	±	24.1144	241.608	±	16.6409
	Tyrosine	85.8335	±	13.8917	66.1431	±	3.50379	70.6217	±	5.7422	65.3533	±	3.95762
	Valine	396.849	±	21.7236	284.639	±	28.9135	375.273	±	18.8189	260.749	±	27.7864
	Citrulline	9.13869	±	0.58623	7.84818	±	0.04131	10.0894	±	0.77044	6.84521	±	0.2952
	GABA	45.1481	±	7.1958	17.182	±	1.99118	47.5846	±	3.29731	23.9201	±	3.51684
	Glutamine	137.4	±	11.4103	176.613	±	9.66216	153.47	±	4.77813	167.567	±	8.48856
	Homoserine	262.087	±	24.4315	187.331	±	10.2186	254.136	±	21.3775	144.173	±	4.44526
	Threonine bis	240.247	±	21.8116	188.441	±	11.0908	240.523	±	14.206	139.321	±	12.116
	Lysine	44.5038	±	2.1899	36.4403	±	2.44389	52.6015	±	2.40271	39.5861	±	3.92805
	OHProline	17.4715	±	1.57332	17.8182	±	1.65887	16.6351	±	0.94118	16.6404	±	0.6265
	Leucine	53.6546	±	5.22622	52.6708	±	3.73447	49.0771	±	4.01056	49.509	±	5.28743
	Isoleucine	70.3251	±	9.42235	64.576	±	4.31277	62.771	±	5.77831	58.5954	±	7.33312

(cont.) Table S 4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Sugars and sugar alcohols	Arabitol	3096.03	±	418.696	3487.7	±	354.473	2543.08	±	427.106	4482.73	±	328.567
	Maltose	6031.78	±	1662.11	3245.17	±	362.503	1232.22	±	164.252	1550.6	±	322.02
	Trehalose	989.017	±	219.671	93.1801	±	29.3456	611.679	±	165.259	97.2725	±	34.7449
	Fructose	17041.7	±	5228.97	14472.4	±	7804.57	19644.2	±	6260.39	23299.8	±	7467.67
	Glucose	20987.2	±	6007.45	18630.7	±	8556.57	23577	±	6997.26	28671.2	±	8206.9
	Sucrose	65806.9	±	4307.64	53342.6	±	5618.87	59797.6	±	2166.32	63541	±	1681.65
	Inositol	1557.96	±	221.634	1229.06	±	123.55	2060.47	±	473.934	1731.28	±	125.43
	Sorbitol	76.9057	±	8.98079	75.8829	±	15.6206	73.3323	±	10.1887	87.1978	±	6.88985
	Glycerol	26105.5	±	8983.22	10654.8	±	5243.01	27381.5	±	9355.47	28813.3	±	10556.5
	Ribose	1271.24	±	291.842	1087.09	±	105.336	781.019	±	89.1781	1256.45	±	38.4405
	Erythritol/Threitol	94.6281	±	23.1841	55.4011	±	10.8818	94.5284	±	22.9912	75.0152	±	12.3241

Class	Metabolite	120 DAI											
		S			R			SI			RI		
Sugars and sugar alcohols	Arabitol	3408.33	±	153.859	4431.5	±	122.716	3695.09	±	187.872	3654.98	±	134.906
	Maltose	1796.07	±	879.448	525.59	±	167.231	2146.32	±	384.881	135.131	±	42.9748
	Trehalose	1421.87	±	102.799	504.581	±	16.6946	1547.65	±	168.357	545.795	±	64.2238
	Fructose	8654.23	±	767.013	6355.71	±	808.51	10218.2	±	789.132	5672.42	±	1234.06
	Glucose	11300.7	±	1025.99	11250.7	±	976.426	13634	±	698.314	9518.12	±	1365.85
	Sucrose	46914.5	±	5876.63	40078.2	±	1628.8	48202.9	±	1600.35	45085	±	4235.01
	Inositol	1268.07	±	53.3576	1023.26	±	101.665	1278.89	±	76.5889	782.376	±	27.1764
	Sorbitol	78.0649	±	4.95427	94.7091	±	7.31855	91.5873	±	7.70595	83.7198	±	3.86298
	Glycerol	16280.9	±	2289.35	13066.9	±	1930.72	15346.4	±	2332.13	11557.5	±	1281.61
	Ribose	1174.64	±	197.994	845.453	±	36.0036	988.703	±	48.5894	772.86	±	89.0622
	Erythritol/Threitol	88.906	±	15.3375	63.861	±	11.6328	102.052	±	5.42903	58.7572	±	5.17052

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Organic acids and Phosphorylated compounds	Ribose 1P	6.44352	±	2.4346	7.75096	±	2.45799	6.65194	±	2.26753	5.73778	±	3.22948
	6 Phosphogluconate	215.953	±	75.4978	242.262	±	64.1387	99.1878	±	28.2073	114.438	±	17.7826
	UMP	339.944	±	78.9387	275.191	±	48.9799	297.88	±	47.773	230.48	±	71.9793
	Trehalose 6P	10.5822	±	1.02851	7.72422	±	1.51547	9.33344	±	1.05499	9.34305	±	0.70639
	ADP	239.514	±	26.0719	230.771	±	52.2577	298.638	±	13.6893	307.002	±	37.6332
	CTP	13.1278	±	4.7912	13.6662	±	5.70574	13.5784	±	7.3599	12.6571	±	8.68091
	UDP glucose	126.262	±	43.5765	109.679	±	21.1313	117.007	±	29.2961	89.817	±	36.2688
	Phosphoenolpyruvate	215.035	±	81.2054	484.303	±	187.464	219.75	±	136.935	254.009	±	190.622
	Erythrose 4P	694.622	±	61.2747	990.512	±	328.295	849.905	±	198.591	1129.09	±	333.639
	CMP	194.577	±	21.3893	148.842	±	31.8852	154.775	±	26.6306	136.396	±	41.134
	AMP	153.201	±	13.5766	132.543	±	20.5236	158.781	±	17.2248	138.044	±	35.2378
	IMP	27.6268	±	10.7766	34.282	±	6.75891	13.0941	±	2.8525	12.25	±	2.35319
	GMP	224.372	±	26.3123	162.903	±	26.1176	205.103	±	37.1716	162.388	±	47.6104
	UDP	36.2715	±	5.89729	42.9646	±	9.0564	48.3934	±	6.13239	39.6707	±	9.73869
	GDP	8.35832	±	1.04892	13.2209	±	3.75938	17.3075	±	3.40532	12.4702	±	3.09541
	UTP	73.4904	±	25.9569	60.2205	±	22.7443	56.5758	±	26.8474	42.7444	±	25.3253
	ATP	188.242	±	64.5081	188.06	±	76.4595	199.234	±	94.886	192.554	±	121.393
	GTP	16.7335	±	5.2301	20.3087	±	7.14918	22.4327	±	9.66116	16.6158	±	9.35556
	2 Ketoglutarate	384.05	±	137.43	409.387	±	99.6153	269.305	±	138.37	238.167	±	118.072
	Malate	9540.16	±	813.087	6165.14	±	326.92	8653.58	±	871.521	7491.24	±	221.655
	Succinate	1189.61	±	212.58	839.214	±	115.157	1250.13	±	177.666	1184.95	±	136.441
	CDP	63.036	±	11.4044	61.2058	±	12.7607	65.5503	±	6.25316	67.3517	±	14.9252
	Fructose 1 6bisP	156.888	±	23.1395	221.088	±	76.4184	196.676	±	41.2696	255.91	±	69.9562

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Organic acids and Phosphorylated compounds	Shikimate	314.299	±	53.1812	179.764	±	24.5676	208.113	±	45.0976	259.397	±	56.434
	Sedoheptulose 7P	318.688	±	70.8542	516.719	±	129.976	311.709	±	74.101	542.339	±	133.752
	2 3 Phosphoglycerate	179.519	±	44.9098	347.488	±	122.559	201.451	±	66.3499	287.557	±	126.911
	Isocitrate	8108.74	±	1468.72	4443.52	±	350.501	8372.35	±	1442.91	5639.5	±	869.257
	Mevalonate	5.74163	±	0.51896	4.6655	±	0.42404	5.11622	±	0.09371	5.31673	±	0.62311
	Galactose 1P	401.005	±	100.3	256.605	±	59.3984	505.087	±	157.175	495.104	±	98.6788
	Trans Aconitate	7166.58	±	589.363	5394.36	±	767.913	6141.8	±	1278.78	6611.28	±	416.099
	Gluconate	693.867	±	55.3899	628.786	±	71.2415	767.013	±	78.1752	798.933	±	98.2887
	Glutaric acid	54.5637	±	10.9605	34.6699	±	4.54217	48.8692	±	7.62746	47.1801	±	6.39335
	Sebacic	6.32995	±	0.96212	5.69065	±	0.56194	7.45888	±	1.13109	7.07926	±	0.61744
	Adipic acid	19.6793	±	2.00417	24.1961	±	8.92093	19.8914	±	3.60639	16.3318	±	1.65968
	Malonic acid	764.256	±	81.3996	666.074	±	23.6662	882.164	±	52.1074	878.543	±	38.0791
	Quinic acid	2376.43	±	417.618	1422.42	±	141.012	1802.37	±	432.249	2794.8	±	800.074
	UDP N Galactosamine	12.8804	±	1.97996	7.1164	±	1.27442	9.99508	±	1.23381	7.20441	±	0.97689
	Pentose Ps	428.139	±	135.131	445.23	±	115.473	260.974	±	109.228	229.121	±	118.992
	Ribulose 1 5bisP	222.012	±	88.8608	257.407	±	113.223	244.886	±	127.563	264.615	±	183.458
	Sucrose 6P	91.1771	±	18.8474	53.42	±	16.1911	105.409	±	29.7924	107.809	±	24.5095
	Cis Aconitate	7632.64	±	1511.14	4638.74	±	518.635	6522.87	±	1386.36	8873.52	±	1766.82

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	120 DAI			
		S	R	SI	RI
Organic acids and Phosphorylated compounds	Ribose 1P	4.71319 ± 0.46833	8.25569 ± 0.61945	5.16556 ± 0.64515	6.76511 ± 1.09529
	6 Phosphogluconate	101.424 ± 11.4608	133.173 ± 15.6534	83.5796 ± 10.3714	114.509 ± 9.41856
	UMP	130.32 ± 5.38346	91.2421 ± 6.48502	125.753 ± 3.92715	90.9972 ± 2.96862
	Trehalose 6P	7.03167 ± 0.50113	7.41292 ± 0.15264	6.65929 ± 0.35133	8.78651 ± 1.31251
	ADP	142.673 ± 8.61412	139.475 ± 6.32193	156.642 ± 5.14081	109.733 ± 6.32554
	CTP	1.67977 ± 0.06989	1.77456 ± 0.27896	2.1239 ± 0.14042	1.87992 ± 0.16121
	UDP glucose	20.8063 ± 1.55229	14.3255 ± 1.10287	23.809 ± 1.40272	15.9821 ± 1.46613
	Phosphoenolpyruvate	32.9397 ± 5.10326	42.5888 ± 14.2705	27.0057 ± 4.91188	25.8104 ± 1.94815
	Erythrose 4P	701.124 ± 113.546	906.31 ± 159.06	567.534 ± 70.1653	625.575 ± 75.1567
	CMP	63.7942 ± 6.30889	30.616 ± 5.57847	50.4994 ± 5.88873	35.4294 ± 2.72063
	AMP	86.2234 ± 6.42303	63.6739 ± 1.80539	74.5528 ± 5.67031	61.1527 ± 4.36775
	IMP	43.2236 ± 11.4041	58.3189 ± 5.7976	30.9261 ± 3.49016	63.4824 ± 12.9074
	GMP	86.462 ± 7.5182	46.8999 ± 7.14532	71.2346 ± 7.34227	49.9163 ± 2.7777
	UDP	34.6673 ± 2.45165	35.2501 ± 1.67792	46.4641 ± 2.72069	32.0245 ± 3.22101
	GDP	9.47522 ± 1.604	12.462 ± 2.48726	13.7717 ± 1.53468	11.7268 ± 1.05162
	UTP	9.06534 ± 0.37866	10.1524 ± 0.78846	14.3641 ± 0.99978	10.9039 ± 1.21981
	ATP	24.2137 ± 2.24021	24.0458 ± 2.76792	33.6608 ± 2.97568	26.8083 ± 1.74568
	GTP	5.10543 ± 0.09072	4.63112 ± 0.21761	7.001 ± 0.69883	6.23469 ± 0.89387
	2 Ketoglutarate	435.777 ± 46.8754	346.153 ± 44.1157	361.035 ± 71.2252	189.759 ± 45.3708
	Malate	6225.7 ± 507.391	3917.49 ± 201.202	7127.69 ± 275.147	3169.32 ± 278.356
	Succinate	980.203 ± 55.4076	842.447 ± 29.8468	1072.68 ± 84.9034	827.709 ± 64.867
	CDP	33.0411 ± 0.46759	41.2299 ± 1.46638	40.2601 ± 1.39165	31.4037 ± 2.94003
	Fructose 1 6bisP	195.292 ± 84.4649	213.17 ± 67.0798	184.634 ± 8.33575	150.941 ± 12.0236

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	120 DAI											
		S			R			SI			RI		
Organic acids and Phosphorylated compounds	Shikimate	225.077	±	12.9976	298.139	±	94.5015	286.301	±	22.1835	279.195	±	28.5036
	Sedoheptulose 7P	155.514	±	23.1325	295.082	±	23.4446	146.628	±	23.1249	242.981	±	21.9533
	2 3 Phosphoglycerate	74.9957	±	7.07488	106.635	±	16.4553	50.1858	±	8.17865	66.715	±	17.5205
	Isocitrate	8381.29	±	355.342	7003.76	±	1046.34	9569.9	±	486.529	5732.44	±	341.355
	Mevalonate	3.34898	±	0.14241	3.03558	±	0.46445	3.31031	±	0.63289	3.41359	±	0.49593
	Galactose 1P	685.212	±	35.8112	628.196	±	44.111	694.013	±	34.2708	550.988	±	43.2846
	Trans Aconitate	2403.76	±	197.191	2272.24	±	43.6275	3717.42	±	85.6427	3133.35	±	978.863
	Gluconate	413.922	±	18.1771	377.736	±	9.60082	415.298	±	17.1198	375.527	±	26.2456
	Glutaric acid	43.5236	±	2.13051	40.055	±	3.64737	45.3071	±	1.77164	35.3151	±	1.18825
	Sebacic	4.68117	±	0.34975	4.26525	±	0.50903	4.73641	±	0.45554	4.3604	±	0.36334
	Adipic acid	22.9018	±	4.50691	17.317	±	3.64068	18.3024	±	0.89776	13.8152	±	0.98744
	Malonic acid	646.957	±	16.4445	630.801	±	23.3815	747.323	±	6.51851	630.557	±	25.9289
	Quinic acid	1034.09	±	67.2445	1309.69	±	290.484	1447.05	±	165.352	1307.92	±	238.491
	UDP N Galactosamine	3.78069	±	0.31437	2.32209	±	0.18237	4.73951	±	0.25783	2.50422	±	0.19788
	Pentose Ps	117.673	±	10.9277	153.588	±	19.0021	126.445	±	9.76859	143.634	±	9.53245
	Ribulose 1 5bisP	100.122	±	43.3682	106.225	±	44.8154	88.4116	±	7.81519	46.5863	±	7.39988
	Sucrose 6P	53.4237	±	4.17792	45.4209	±	4.71754	58.7725	±	3.1675	50.0888	±	6.48934
	Cis Aconitate	3167.21	±	299.635	2699.45	±	229.567	4388.84	±	172.43	3845.46	±	1291.08

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Phenolic compounds, Flavonoids and Hormones	Benzoic acid	2.77951	±	0.63315	4.06931	±	0.44399	4.04805	±	0.48158	3.23455	±	0.6638
	Caffeic acid	5.09747	±	1.9281	11.1717	±	1.89319	9.46562	±	1.76339	4.76274	±	1.74755
	Coniferaldehyde	0.71611	±	0.2262	0.75631	±	0.149	0.65037	±	0.05568	0.50565	±	0.14206
	Coumaric acid	6.90037	±	2.58482	13.0177	±	2.10789	9.7427	±	1.04805	8.02363	±	2.77825
	Ferulic acid	0.96817	±	0.39696	2.12137	±	0.42872	1.69309	±	0.23673	1.01486	±	0.4067
	Sinapaldehyde	0.42267	±	0.14596	0.53713	±	0.14259	0.42159	±	0.0494	0.33366	±	0.1008
	Sinapic acid	0.89407	±	0.21204	1.5288	±	0.39045	1.10113	±	0.22955	0.7316	±	0.29133
	Syringic acid	1.34367	±	0.50284	3.11733	±	0.5096	2.27904	±	0.56061	1.69968	±	0.80054
	Vanillin	3.17833	±	0.69302	3.83275	±	0.24424	3.77253	±	0.13739	2.61212	±	0.46931
	Vanillic acid	1.87057	±	0.86473	3.13925	±	0.67429	3.16013	±	0.53156	1.66118	±	0.84938
	Naringenin	0.0997	±	0.01517	0.11294	±	0.01841	0.10822	±	0.01632	0.07677	±	0.02853
	Luteolin	0.14848	±	0.01968	0.24073	±	0.05225	0.12148	±	0.00741	0.22191	±	0.02475
	Maysin	69.0543	±	14.9188	67.3471	±	20.722	48.8663	±	3.40635	54.1049	±	12.39
	Rhamisoorientin	12.2582	±	0.73512	26.2704	±	4.54828	14.2387	±	2.36995	26.9874	±	4.98153
	Eriodictyol	0.03247	±	0.00257	0.16662	±	0.03916	0.04995	±	0.01202	0.10894	±	0.04396
	Isovitexin Vitexin	24.6648	±	1.56949	26.8394	±	2.43346	23.6393	±	1.51258	26.1032	±	0.98997
	Apigenin 7O glucoside	0.05734	±	0.01869	0.06539	±	0.0139	0.09112	±	0.01117	0.05274	±	0.01931
	Luteolin-7-O-glucoside	0.26271	±	0.00899	2.12259	±	0.45945	0.50477	±	0.06993	2.06283	±	0.39476
	4 Hydroxybenzoic	1.27799	±	0.52536	13.6032	±	3.74791	2.70743	±	0.44509	8.01502	±	4.61348
	5 Chlorogenic acid	81.186	±	11.6382	224.524	±	24.8359	59.9803	±	6.77398	155.363	±	10.3451
	4 Chlorogenic acid	16.688	±	1.0112	41.7443	±	3.46306	16.0062	±	1.82921	34.2683	±	2.77077
	3 Chlorogenic acid	58.749	±	3.27629	242.065	±	30.5748	74.2152	±	8.50159	171.982	±	21.121

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	30 DAI											
		S			R			SI			RI		
Phenolic compounds, Flavonoids and Hormones	Salicylic acid	2.03521	±	0.94159	10.4825	±	2.51878	4.20513	±	0.67464	3.32271	±	1.27103
	Isoorientin	83.1843	±	1.83059	221.459	±	22.7227	102.81	±	13.283	226.194	±	20.3479
	Abscisic acid	0.00622	±	0.00097	0.01114	±	0.0028	0.01279	±	0.00327	0.00838	±	0.00196
	Orientin	83.1843	±	1.83059	221.459	±	22.7227	102.81	±	13.283	226.194	±	20.3479
	FQA 1	2.35458	±	0.23235	2.95966	±	0.30486	3.43744	±	0.65857	3.22227	±	0.53817
	FQA 2	7.2262	±	0.9984	6.23178	±	0.52893	7.56321	±	1.54576	10.4213	±	2.50088
	FQA 3	8.13552	±	0.3845	20.1569	±	2.71108	10.8244	±	1.03061	18.3567	±	3.25976
	FQA 4	4.86209	±	1.11034	7.33474	±	0.72622	4.50542	±	0.6037	10.0694	±	2.48672
	Caffeine	0.02247	±	0.0058	0.02131	±	0.00359	0.02279	±	0.00205	0.0173	±	0.00256
	pCoQA 2	11.0361	±	1.66888	45.9473	±	8.05607	9.79566	±	2.29314	36.9786	±	9.36213
	pCoQA 1	4.4341	±	0.41614	23.6493	±	5.16591	4.65718	±	1.14045	15.378	±	3.43066
	ConiferylOH H2O	156.837	±	32.9032	190.015	±	19.5191	159.977	±	12.3137	143.087	±	7.48999
	3 4 Caffeoylquinic acid	1.66372	±	0.38736	1.05862	±	0.20295	0.54787	±	0.11013	0.87505	±	0.15612
	Apigenin	0.90312	±	0.26973	0.36008	±	0.05253	0.48062	±	0.11024	0.30356	±	0.05993

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	120 DAI											
		S			R			SI			RI		
Phenolic compounds, Flavonoids and Hormones	Benzoic acid	2.04432	±	0.12053	2.12623	±	0.05894	1.88545	±	0.17169	1.91115	±	0.22394
	Caffeic acid	14.762	±	0.44541	10.7119	±	0.63085	15.7047	±	0.4382	11.5201	±	0.8986
	Coniferaldehyde	1.01922	±	0.07737	0.86169	±	0.06395	1.16066	±	0.06321	0.97321	±	0.10475
	Coumaric acid	10.8733	±	0.29336	11.7942	±	1.50483	11.9043	±	0.42636	12.9148	±	1.08054
	Ferulic acid	1.51368	±	0.0899	1.56011	±	0.03909	1.63309	±	0.08626	1.70183	±	0.22197
	Sinapaldehyde	0.59901	±	0.03895	0.67944	±	0.16138	0.6491	±	0.03994	0.74132	±	0.11443
	Sinapic acid	1.28922	±	0.17533	1.52962	±	0.36359	1.07743	±	0.10188	2.08707	±	0.39928
	Syringic acid	0.72625	±	0.0918	1.06037	±	0.09613	0.76708	±	0.01187	1.13997	±	0.05203
	Vanillin	3.72615	±	0.1604	3.27969	±	0.0899	3.92244	±	0.16327	3.47141	±	0.2608
	Vanillic acid	1.46754	±	0.15777	1.39632	±	0.16866	1.6389	±	0.0742	1.54438	±	0.0699
	Naringenin	0.06851	±	0.0032	0.04337	±	0.01215	0.06159	±	0.00649	0.04757	±	0.00543
	Luteolin	0.12754	±	0.02174	0.15634	±	0.045	0.08923	±	0.00813	0.17573	±	0.01938
	Maysin	57.1181	±	6.50922	37.7705	±	5.35499	56.3726	±	7.21547	48.6762	±	5.31904
	Rhamisoorientin	17.7485	±	1.14736	28.0244	±	2.60665	15.8252	±	1.01042	29.0731	±	3.22194
	Eriodictyol	0.04319	±	0.00513	0.04284	±	0.01154	0.03746	±	0.00116	0.03904	±	0.00182
	Isovitexin Vitexin	26.8937	±	2.95347	28.9373	±	2.29571	23.6424	±	1.47757	31.4228	±	1.94309
	Apigenin 7O glucoside	0.04892	±	0.00187	0.03183	±	0.00892	0.06594	±	0.00168	0.03612	±	0.00463
	Luteolin-7-O-glucoside	1.29747	±	0.20881	3.6533	±	0.28996	1.18204	±	0.05224	4.15418	±	0.51497
	4 Hydroxybenzoic	1.75744	±	0.12949	5.57894	±	0.81173	2.01646	±	0.17478	5.44817	±	0.8513
	5 Chlorogenic acid	198.078	±	14.9713	282.395	±	19.5843	209.759	±	4.83438	286.352	±	13.9167
	4 Chlorogenic acid	57.3311	±	5.53077	101.204	±	17.7136	70.1235	±	2.99919	96.719	±	3.31515
	3 Chlorogenic acid	339.008	±	29.7713	496.919	±	38.6253	419.747	±	14.8736	526.645	±	29.6722

(cont.) Table S4. Metabolites quantified in sugarcane leaves mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/mg of dry weight) ± standard error, n=4 (except for R, n =3).

Class	Metabolite	120 DAI											
		S			R			SI			RI		
Phenolic compounds, Flavonoids and Hormones	Salicylic acid	5.33224	±	0.71411	7.23845	±	1.43364	7.39821	±	0.84725	7.42312	±	0.41217
	Isoorientin	175.917	±	18.6732	277.824	±	18.1017	163.994	±	8.61441	292.096	±	18.3727
	Abscisic acid	0.00692	±	0.00099	0.00733	±	0.00089	0.00571	±	0.00035	0.00891	±	0.00045
	Orientin	175.917	±	18.6732	277.824	±	18.1017	163.994	±	8.61441	292.096	±	18.3727
	FQA 1	6.32493	±	1.12852	4.97023	±	0.85951	9.39034	±	0.58983	4.7419	±	0.3239
	FQA 2	12.5178	±	2.2778	8.91187	±	1.58209	19.9398	±	1.22291	8.49915	±	0.5836
	FQA 3	38.9912	±	3.68541	53.8907	±	8.82026	51.7091	±	3.71416	60.816	±	12.9257
	FQA 4	13.5047	±	1.34271	16.9299	±	3.27372	16.5948	±	1.07509	17.1217	±	3.34086
	Caffeine	0.02537	±	0.01299	0.0126	±	0.00187	0.01279	±	0.00248	0.01299	±	0.00049
	pCoQA 2	42.1437	±	4.80482	77.2767	±	8.26977	41.134	±	1.69459	95.7402	±	13.0886
	pCoQA 1	29.3925	±	3.08383	57.8558	±	10.5137	29.6797	±	1.26985	81.537	±	13.6711
	ConiferylOH H2O	298.519	±	14.8627	480.451	±	92.967	268.526	±	38.6458	554.342	±	30.231
	3 4 Caffeoylquinic acid	2.63412	±	0.15407	3.48	±	1.00057	3.00606	±	0.13557	3.01617	±	0.30913
	Apigenin	0.46016	±	0.03884	0.32852	±	0.12235	0.43244	±	0.05594	0.36762	±	0.09557

Table S 5. Metabolites detected in sugarcane sap from plants mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/uL of sap) ± standard error, n=4.

Class	Metabolite	30 DAI											
		S			SI			R			RI		
Sugars and Sugar alcohols	Maltose	1.92835	±	0.45879	3.02646	±	0.50846	3.13221	±	1.10359	3.03245	±	1.28918
	Ribose	22.5957	±	6.78465	14.2166	±	2.70218	14.3993	±	3.57081	10.2994	±	1.50705
	Erythritol Threitol	2.81092	±	0.38041	2.07293	±	0.22237	1.68357	±	0.30093	1.43926	±	0.28968
	Glycerol	12.3628	±	2.03272	8.48278	±	0.79473	8.45233	±	0.55633	8.20767	±	2.88884
	Sorbitol	6.79642	±	1.41589	5.42033	±	2.17461	4.13794	±	0.79131	4.25516	±	0.97344
	Inositol	1.98001	±	0.34095	1.79392	±	0.25073	2.22316	±	0.53102	1.62359	±	0.30544
	Sucrose	6.08264	±	2.11963	4.0966	±	0.5191	2.24182	±	0.67571	4.11888	±	1.06084
	Glucose	6.70732	±	0.99185	4.56288	±	0.77847	7.99708	±	2.03839	4.80355	±	1.03448
	Fructose	11.8616	±	1.854	8.75298	±	1.13746	16.7545	±	6.16402	9.65518	±	2.73871
	Trehalose	0.24034	±	0.03292	0.19438	±	0.04469	0.28254	±	0.09337	0.20801	±	0.06011
	Arabinose/Xylose	4.78104	±	0.51633	3.96103	±	1.01828	6.27065	±	1.85696	3.37563	±	0.78467
	Pentitols	1.56066	±	0.46925	0.64653	±	0.09274	1.41529	±	0.29109	0.71345	±	0.14752

(cont.) Table S 5. Metabolites detected in sugarcane sap from plants mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/uL of sap) ± standard error, n=4.

Class	Metabolite	120 DAI											
		S			SI			R			RI		
Sugars and Sugar alcohols	Maltose	9.35921	±	2.34202	10.8491	±	3.25967	11.9523	±	3.22948	12.3123	±	5.38884
	Ribose	10.8468	±	3.41181	8.5988	±	2.22743	14.4355	±	2.33421	11.0509	±	1.31261
	Erythritol Threitol	5.1257	±	0.69674	6.24984	±	1.9636	5.62233	±	1.26897	4.45267	±	1.12384
	Glycerol	2.59804	±	0.16803	12.7342	±	9.10258	5.43922	±	0.48328	7.03018	±	2.28278
	Sorbitol	3.17516	±	0.31624	3.23084	±	0.38006	9.92205	±	1.9206	6.32199	±	1.1329
	Inositol	0.63252	±	0.17095	0.46389	±	0.06668	1.12332	±	0.09658	0.9627	±	0.20624
	Sucrose	4.19325	±	1.70593	3.96375	±	1.74978	2.55642	±	0.71333	2.31566	±	0.50967
	Glucose	3.93781	±	1.03502	4.02599	±	1.02545	18.6817	±	10.0138	22.682	±	13.1019
	Fructose	9.80985	±	3.40671	7.40333	±	2.05955	41.51	±	19.3796	64.3611	±	29.5216
	Trehalose	0.78675	±	0.25726	0.8343	±	0.2377	0.88366	±	0.23927	0.90042	±	0.35284
	Arabinose/Xylose	3.56362	±	0.7848	2.86266	±	0.2489	5.2836	±	0.64218	5.66013	±	1.31671
	Pentitols	1.29103	±	0.29478	0.97899	±	0.29499	3.05746	±	0.98289	1.61271	±	0.48288

(cont.) Table S 5. Metabolites detected in sugarcane sap from plants mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/uL of sap) ± standard error, n=4.

Class	Metabolite	30 DAI											
		S			SI			R			RI		
Phenolic compounds, flavonoids and hormones	Coniferaldehyde	0.00247	±	0.00048	0.00162	±	0.00022	0.002	±	0.00035	0.00795	±	0.00594
	Coumaric acid	0.01758	±	0.00038	0.03114	±	0.00585	0.08465	±	0.02777	0.02478	±	0.00537
	Dihydrokaempferol	0.00016	±	7.3E-06	0.00014	±	2.2E-05	0.00018	±	6.8E-05	0.01694	±	0.0168
	Ferulic acid	0.00317	±	0.00039	0.00197	±	0.00061	0.00918	±	0.00483	0.00362	±	0.00171
	Sinapaldehyde	0.00024	±	5.2E-05	0.00017	±	2.3E-05	0.00053	±	5.1E-05	0.00028	±	6.2E-05
	Syringic acid	0.00599	±	0.00181	0.0047	±	0.00129	0.03431	±	0.01102	0.00457	±	0.0013
	ABA	0.00072	±	0.00012	0.00031	±	3.5E-05	0.00076	±	0.00024	0.0003	±	9.9E-05
	SA	0.06214	±	0.01923	0.08143	±	0.00974	0.0974	±	0.0356	0.04795	±	0.00358
	Rhamnosylisoorientin	0.00025	±	7.3E-05	0.00013	±	2.9E-05	0.01308	±	0.00735	0.00035	±	0.00013
	Isovitexin Vitexin	0.00011	±	8.1E-05	7.5E-05	±	5.3E-05	0.00158	±	0.00103	9.1E-05	±	2.4E-05
	4 Hydroxybenzoic acid	0.10916	±	0.05247	0.05892	±	0.02922	0.15699	±	0.02412	0.06532	±	0.01834
	Luteolin 7 O glucoside	0.00049	±	0.00011	0.00019	±	2.3E-05	8.8E-05	±	7.3E-06	9.7E-05	±	2.4E-05
	Orientin	0.00038	±	5.3E-05	0.00033	±	8.1E-05	0.0078	±	0.00427	0.0003	±	0.00014
	Isoorientin	0.00068	±	5E-05	0.00054	±	0.0002	0.05643	±	0.03157	0.00149	±	0.00021
	Caffeine	0.00931	±	0.00283	0.00412	±	0.00041	0.00483	±	0.00074	0.00461	±	0.00058
	Maysin	0.00059	±	3.1E-05	0.00049	±	4.4E-05	0.01876	±	0.0102	0.00099	±	0.00017

(cont.) Table S 5. Metabolites detected in sugarcane sap from plants mock-inoculated susceptible (S), resistant (R) or Lxx-inoculated susceptible (SI) or resistant (RI) to RSD at 30 and 120 DAI by targeted metabolomics, their chemical class, average concentration (pM/uL of sap) ± standard error, n=4.

Class	Metabolite	120 DAI											
		S			SI			R			RI		
Phenolic compounds, flavonoids and hormones	Coniferaldehyde	0.00206	±	0.00029	0.00234	±	0.00049	0.00207	±	0.00031	0.00212	±	0.00037
	Coumaric acid	0.03161	±	0.00304	0.04	±	0.01683	0.04586	±	0.01964	0.0361	±	0.01061
	Dihydrokaempferol	0.0004	±	0.00026	0.00016	±	4.7E-05	0.00033	±	0.0002	0.00019	±	2.8E-05
	Ferulic acid	0.00644	±	0.0005	0.00938	±	0.00097	0.00816	±	0.00122	0.01349	±	0.00182
	Sinapaldehyde	0.00093	±	0.0003	0.0011	±	0.00025	0.00105	±	0.00015	0.00119	±	0.00039
	Syringic acid	0.01703	±	0.00832	0.02088	±	0.00489	0.02205	±	0.00564	0.01581	±	0.00347
	ABA	0.00518	±	0.00151	0.00454	±	0.00085	0.00307	±	0.00099	0.00267	±	0.00029
	SA	0.06333	±	0.01091	0.10063	±	0.06235	0.05545	±	0.01231	0.04202	±	0.01293
	Rhamnosylisoorientin	0.00491	±	0.00207	0.01247	±	0.00743	0.01529	±	0.00704	0.01596	±	0.01378
	Isovitexin Vitexin	0.00061	±	0.00021	0.0007	±	0.0003	0.00177	±	0.00047	0.00125	±	0.0008
	4 Hydroxybenzoic acid	0.0516	±	0.00836	0.04411	±	0.00732	0.04424	±	0.00577	0.03842	±	0.00575
	Luteolin 7 O glucoside	0.0003	±	8.1E-05	0.00035	±	0.0001	0.00045	±	0.00036	0.00134	±	0.00092
	Orientin	0.00162	±	0.00086	0.0029	±	0.00179	0.01643	±	0.01019	0.0284	±	0.02634
	Isoorientin	0.01576	±	0.00774	0.04554	±	0.03344	0.16705	±	0.10376	0.2054	±	0.19054
	Caffeine	0.00354	±	0.00071	0.00609	±	0.00133	0.00439	±	0.00073	0.00994	±	0.00267
	Maysin	0.02013	±	0.00829	0.07252	±	0.04863	0.05647	±	0.02687	0.04942	±	0.04247