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

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

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DISSERTATION

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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

1. INTRODUCTION

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.

1.1. 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.

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. 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.

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. 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.

4. CONCLUSION AND PERSPECTIVES

4.1. 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.

4.2. 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: 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.

4.3. 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. Interestingly, this amino acid was more accumulated later in both susceptible and resistant inoculated plants at 120 DAI. 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. Moreover, inoculation with Lxx was related to more accumulation of apigenin-7-O-glucoside and 2 and 3- ferulyquinic acids in susceptible plants. In order to validate those results, more sugarcane varieties should be tested to establish a set of markers for RSD resistance.

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