

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

Comparative genomics and transcriptomics analyses reveal genetic complexity of soybean anthracnose caused by *Colletotrichum* species

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Thesis presented to obtain the degree of Doctor in Science.

Area: Plant Pathology

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I dedicate this work with love to
my parents Bernardo and Dirce, my brother Eduardo and
my godparents Laércio (in memorian) and Neiva,
which always supported my dreams.

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*“It is not the strongest of the species that survives,
Not the most intelligent that survives.
It is the one that is the most adaptable to change”*

- *Charles Darwin.*

RESUMO

Análises de genômica comparativa e transcriptômica revelam a complexidade genética da antracnose da soja causada por espécies de *Colletotrichum*

O cultivo da soja (*Glycine max*) ocupa em torno de 6% da área plantada em escala mundial. Atualmente, mais de 90 doenças ameaçam a produção dessa cultura, que devido a sua importância global e múltiplos usos, como fonte de alimentação humana e animal, pode implicar na segurança alimentar. Dentre essas doenças está a antracnose, considerada uma doença de etiologia complexa pois pode ser causada por diversas espécies do gênero *Colletotrichum*. Com o advento das tecnologias de sequenciamento de nova geração, o sequenciamento de genomas e transcriptomas se tornou possível. Análises de genômica comparativa podem ser utilizadas para revelar repertórios de candidatos a efetores de importantes patógenos de plantas; por outro lado, o sequenciamento completo dos transcriptomas de plantas e patógenos durante sua interação permite a identificação de genes e vias bioquímicas e metabólicas envolvidas na defesa das plantas contra doenças. Para aprofundar o conhecimento e as informações disponíveis sobre a antracnose da soja e seus agentes causais, foi utilizada uma abordagem combinada entre experimentos biológicos e tecnologias de sequenciamento de nova geração. A revisão de todas as sequências ITS de *Colletotrichum* associadas a soja disponíveis, revelou que mais de 37% delas está associada ao complexo de espécies errado. Ao menos 9 complexos de espécies associados com antracnose da soja foram encontrados, sendo os complexos *C. orchidearum* e *C. truncatum* os mais numerosos e distribuídos no mundo todo. O primeiro relato de *C. musicola*, como agente causal da antracnose da soja foi registrado. Os genomas de *C. sojiae*, *C. plurivorum* e *C. musicola*, três espécies pertencentes ao complexo *C. orchidearum* foram sequenciados e comparados ao genoma de *C. truncatum* e oito espécies de *Colletotrichum* não patogênicas a soja. Análises de genômica comparativa sugerem que as espécies pertencentes ao complexo *C. orchidearum* e *C. truncatum* adquiriram a capacidade de infectar soja separadamente, devido à ausência de genes candidatos a efetores secretados compartilhados somente entre as quatro espécies. O sequenciamento do transcriptoma de quatro combinações entre soja/*C. truncatum* com diferentes níveis de resistência a antracnose revelou que a defesa da soja contra *C. truncatum* depende da ativação de centenas de genes após o reconhecimento do patógeno pela planta. Os resultados do revelaram que em interações com maior nível de resistência, a porcentagem do transcriptoma da planta modulado é maior quando comparado a interações mais suscetíveis, independentemente da cultivar de soja ou do genótipo de *C. truncatum*.

Palavras-chave: *Colletotrichum truncatum*, *Colletotrichum orchidearum*, *Glycine max*, Sequenciamento de RNA, Efetor, Defesa da Planta

ABSTRACT

Comparative genomics and transcriptomics analyses reveal genetic complexity of soybean anthracnose caused by *Colletotrichum* species

Soybean (*Glycine max*) cultivation occupies around 6% of the world's arable land. Currently, there are more than 90 diseases that threaten soybean production, due to its global importance and multiple uses, as a source of human and animal feeds, may imply in food security. Among these diseases is soybean anthracnose, a disease of complex etiology which can be caused by several species of the genus *Colletotrichum*. The advent of next-generation sequencing technologies (NGS) allowed the high throughput sequencing of entire genomes and transcriptomes. Comparative genomic analyses are useful to reveal repertoires of effector candidates of important plant pathogens, potentially involved in plant infection; while the deep sequencing of transcriptomes of plant and pathogens during their interaction can allow the identification of important genes and pathways involved in plant defense. To gain insights into soybean anthracnose and its causal agents, we used a combined approach of biological experiments with NGS technologies. A revision of all public-available *Colletotrichum* ITS sequences associated with soybean revealed that more than 37% of those are assigned to the wrong species complex (s.c) level. We found at least 9 s.c. and one singleton species of *Colletotrichum* associated with soybean anthracnose worldwide, being the *C. orchidearum* s.c. and *C. truncatum* the most common and distributed. We reported for the first time that *C. musicola*, a member of the *C. orchidearum* s.c. was associated with soybean. Draft genomes of *C. sojiae*, *C. plurivorum* and *C. musicola* were produced and compared with *C. truncatum* and eight additional *Colletotrichum* species not pathogenic to soybean. Comparative genomic analyses suggested that species belonging to the *C. orchidearum* s.c. and *C. truncatum* acquired the capability to infect soybean separately, due to the absence of secreted effector candidates (SECs) shared only among these four species. The transcriptomic sequencing of four different combinations of soybean/*C. truncatum* revealed that soybean defense against *C. truncatum* relies on the activation of several defense genes upon the recognition of the pathogen by the plant. Results revealed a higher modulation of the soybean transcriptome in more resistant interactions, when compared with more susceptible ones, independently of soybean cultivar and *C. truncatum* strain.

Keywords: *Colletotrichum truncatum*, *Colletotrichum orchidearum*, *Glycine max*, RNA Sequencing, Effector, Plant Defense.

1. GENERAL INTRODUCTION

The improvement of crop production to attend to the increasing demand for food worldwide became one of the major problems to be solved in the XXI century (Tilman et al., 2011). Among those crops is soybean (*Glycine max*), considered one of the most cultivated crops (Shea et al., 2020), corresponding to around 6% of the world's arable land, being this area bigger than any other major crop (Hartman et al., 2011). World's soybean production reached up to 370 million tons in the 2019/20 season, being 80% of it produced in Brazil, the USA and Argentina (USDA, 2021).

Due to the global importance and multiple uses of soybean, as a rich source of oil and protein, soybean losses due to abiotic and biotic stresses may threaten food security (Hartman et al., 2011). There are currently more than 90 biotic diseases that threaten soybean production, among them soybean anthracnose (Hartman, 2015). This seed-borne disease can appear during all the stages of the culture in the field due to the possibility of long asymptomatic periods in infected plants (Yang and Hartman, 2015). In the early stages, characteristic symptoms are pre- and post-emergence damping-off. Symptoms appear in the aerial part of the plants after periods of high relative humidity, as petiole and veins necrosis as well as leaf rolling, which lead to premature defoliation. Lastly, during the early reproductive stages, dark, depressed and irregular spots can appear in pods, directly affecting seed quality and production (Yang and Hartman, 2015).

Over the years, several species of the hemibiotrophic fungus *Colletotrichum* were described as causal agents of soybean anthracnose, that became a disease of complex etiology (Dias et al., 2018). *Colletotrichum truncatum* is the most common associated species (Sharma et al., 2011), but *C. destructivum* (Manandhar et al., 1986), *C. coccodes* (Riccioni et al., 1998), *C. chlorophyti* (Yang et al., 2012, 2013), *C. gloeosporioides* (Mahmodi et al., 2013), *C. incanum* (Yang et al., 2014), *C. plurivorum* (Barbieri et al., 2017), *C. sojae* (Damm et al., 2019) and *C. brevisporum* (Shi et al., 2020) were also described as causal agents of the disease.

Disease control rely on accurate species identification of the causal agent. *Colletotrichum* species differentiation based on morphology is unreliable (Cai et al., 2009; Jayawardena, 2016), and simple BLAST searches against the available databases can lead to wrong species identification, as it is estimated that around 10% of all deposited *internal transcribed space (ITS)* sequences are assigned to the wrong species (Nilsson et al., 2006). During the past years, with the advent of DNA-based characterizations, the genus *Colletotrichum* underwent many taxonomic revisions (Damm et al., 2009, 2012a, 2012b, 2014,

2019; Cannon et al., 2012; Weir et al., 2012; Liu et al., 2014; Jayawardena, 2016; Marin-Felix et al., 2017), resulting in more than 200 accepted species classified into species complexes (s.c.) or singletons (Marin-Felix et al., 2017; Damm et al., 2019).

Advances in next-generation sequencing technologies (NGS) allowed the high throughput sequencing of entire genomes (Metzker, 2010) and transcriptomes (Ozsolak and Milos, 2011). The improvement of sequencing technologies, and pipelines for genome assembling and annotation, increased the number of high-quality genomes available (Gibriel et al., 2016). During recent years, at least 42 *Colletotrichum* genomes were published (<http://www.colletotrichum.org/genomics/>), and this number is increasing every year. The availability of genome sequences opens the possibility of innumerable studies, such as comparative genomic analyses that lead to the characterization of effector repertoires (Bhadauria et al., 2015; Gibriel et al., 2016; Baroncelli et al., 2017; de Queiroz et al., 2019), that may have roles in host-pathogen interactions and imply directly in management strategies of several diseases (Gibriel et al., 2016). On the other hand, RNA sequencing can provide an overview of the transcriptomic changes that occur during plant-pathogen interactions, allowing the identification of genes and pathways involved in different stages of plant defense (Vargas et al., 2012; Alkan et al., 2015; Bhadauria et al., 2017; Zhang et al., 2018).

Despite the growing importance of soybean anthracnose (Dias et al., 2018; Rogério et al., 2019) responsible for several losses worldwide (Wrather et al., 2010; Dias et al., 2016), advances in the research of this disease are just beginning to appear. So far, it is unclear if the association of new *Colletotrichum* species with the disease is related to emerging species or if it is due to the undergoing changes in the taxonomy of the genus. Multiple studies identified commercial soybean cultivars with different levels of resistance to anthracnose (Costa et al., 2009; Nagaraj et al., 2014; Dias et al., 2019), however, the genetic mechanisms that support soybean resistance, or *Colletotrichum* pathogenicity have not yet been investigated. To gain a better understanding of soybean anthracnose and its causal agents, this work aimed to:

- clarify which *Colletotrichum* species or complexes are associated with the disease and provide a comprehensive review for future studies on soybean anthracnose (**Chapter 2**);
- report for the first time that *Colletotrichum musicola* was found causing soybean anthracnose in Brazil (**Chapter 3**);
- produce draft genomes of three *Colletotrichum* species associated with soybean anthracnose (**Chapter 4**);

- identify through a comparative computational approach, secreted effector candidates of four species of *Colletotrichum* related to soybean anthracnose (**Chapter 5**);
- Identify genes and/or pathways involved in soybean defense against *C. truncatum* (**Chapter 6**).

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2. SOYBEAN ANTHRACNOSE CAUSED BY *COLLETOTRICHUM* SPECIES: CURRENT STATUS AND FUTURE PROSPECTS

ABSTRACT

Soybean (*Glycine max*) is one of the most important cultivated plant worldwide as a source of protein-rich foods and animal feeds. Anthracnose, caused by different lineages of the hemibiotrophic fungus *Colletotrichum*, is one of the main limiting factors to soybean production. Losses due to anthracnose have been neglected, but their impact may threaten up to 50% of the grain production. While *C. truncatum* is considered the main species associated with soybean anthracnose, recently other species have been reported as pathogenic to this host. Until now, it has not been clear whether the association of new *Colletotrichum* species with the disease is related to emerging species or whether it is due to the undergoing changes in the taxonomy of the genus. Typical anthracnose symptoms are pre- and post-emergence damping-off; dark, depressed and irregular spots on cotyledons, stems, petioles and pods, and necrotic laminar veins on leaves that can result in premature defoliation. Symptoms may still evolve to pod rot, immature opening of pods, and premature germination of grains. As accurate species identification of the causal agent is decisive for disease control and prevention, in this work, we review the taxonomic designation of *Colletotrichum* isolated from soybean to understand which lineages are pathogenic to this host. We also present a comprehensive literature review of soybean anthracnose, focusing on distribution, symptomatology, epidemiology, disease management, identification and diagnosis. We also consider the knowledge emerging from population studies and comparative genomics of *Colletotrichum* spp. associated with soybean providing future perspectives in the identification of molecular factors involved in the pathogenicity process.

Keywords: *Glycine max*; *Glomerella*; Fungal pathogens, *Colletotrichum truncatum*, Emerging Diseases.

2.1 INTRODUCTION

The genus *Colletotrichum* constitutes a large monophyletic group of ascomycetes with more than 200 accepted species, classified into at least 14 species complexes (s.c.) and singletons (Marin-Felix *et al.*, 2017; Damm *et al.*, 2019). Considered one of the top 10 plant pathogenic fungi, *Colletotrichum* spp. is the causal agent of anthracnose in more than 3,000 plant species, leading to considerable yield reduction of economically important crops (Cannon *et al.*, 2012; Dean *et al.*, 2012; da Silva *et al.*, 2020). Due to its hemibiotrophic lifestyle and the facility of being manipulated in the laboratory, the genus is considered a model pathogen for biochemical, physiological and genetic studies (Perfect *et al.*, 1999; O'Connell *et al.*, 2012; Baroncelli *et al.*, 2017).

Soybean crop has great importance worldwide as a source of vegetable oil and proteins for human and animal feeds (Hartman *et al.*, 2011; Pagano and Miransari, 2016), contributing with 3.3% of the global human calorie intake (FAOSTAT, 2018). In 2019/20, world soybean

production exceeded 330 million tons, of which approximately 86% were concentrated in Brazil, the United States and Argentina (USDA, 2020). Diseases are among the major factors that can affect soybean production, and anthracnose is becoming a major threat in producing areas around the world (Wrather *et al.*, 2010; Hartman *et al.*, 2015; Dias *et al.*, 2016; Subedi *et al.*, 2016; Nataraj *et al.*, 2020). This disease can reach up to 100% of incidence in the field (Hartman *et al.*, 1999), and incidence as low as 1% can cause yield losses of up to 90kg/ha (Dias *et al.*, 2016).

Soybean anthracnose is currently recognized as a disease of complex etiology (Dias *et al.*, 2018), with *C. truncatum* the most common associated species (Sharma *et al.*, 2011). During the past years, several other species have been reported as causal agents of the disease, such as *C. destructivum* (Manandhar *et al.*, 1986), *C. coccodes* (Riccioni *et al.*, 1998), *C. chlorophyti* (Yang *et al.*, 2012, 2013), *C. gloeosporioides* (Mahmodi *et al.*, 2013), *C. incanum* (Yang *et al.*, 2014), *C. plurivorum* (Barbieri *et al.*, 2017), *C. sojiae* (Damm *et al.*, 2019) and more recently, *C. musicola* (Bouffleur *et al.*, 2020) and *C. brevisporum* (Shi *et al.*, 2020). *Colletotrichum* species can affect soybean in all physiological stages (Sharma *et al.*, 2011). Typical symptoms of anthracnose are pre and post-emergence damping-off, dark, depressed and irregular spots on stems, petioles and pods that can evolve and cause premature defoliation of the plants (Yang *et al.*, 2015). Such infections have the potential to cause severe losses that can reach up to 100% in soybean-producing areas under favorable conditions (Yang and Hartman, 2016).

An accurate species identification of a causal agent is decisive for disease control and prevention. The occurrence of multiple *Colletotrichum* species associated with soybean anthracnose may affect disease management since distinct species might respond differently to different control strategies. Different studies about the efficiency of fungicides in the control of soybean anthracnose showed contradictory results (Dias *et al.*, 2016; Chen *et al.*, 2018; Poti *et al.*, 2020), which could be due to different responses of *Colletotrichum* species to their active compounds.

Few discriminatory morphological characters are available, and the identification of *Colletotrichum* species based exclusively on these features is unreliable (Cai *et al.*, 2009; Jayawardena *et al.*, 2016). Currently, species identification of this genus is performed using a polyphasic approach that combines morphological and cultural characteristics with multilocus phylogenetic analyses of DNA sequences (Cai *et al.*, 2009; Liu *et al.*, 2016). Several species within the *Colletotrichum* genus show a wide genetic variability however, the mechanisms responsible for such diversity are not yet fully understood (da Silva *et al.*, 2020).

Some population genetics studies of *Colletotrichum* species have offered tools for improving prevention and management strategies for plant diseases of important agricultural crops (Ureña-Padilla *et al.*, 2002; Ciampi-Guillardi *et al.*, 2014; Baroncelli *et al.*, 2015; Rogério *et al.*, 2019).

Many unresolved questions about soybean anthracnose remain. Until now, it is not clear whether the association of new *Colletotrichum* species with the disease is related to emerging species or whether it is due to the undergoing changes in the taxonomy of the genus. Most of the studies available for soybean anthracnose are focused on *C. truncatum*, with limited information about the other *Colletotrichum* species infecting this crop, which could reflect on obstacles during the management of the disease in the field. The aim of this work is to gain a better understanding of soybean anthracnose and its causal agents, clarify which *Colletotrichum* species or complexes are associated with the disease and provide a comprehensive review for future studies on soybean anthracnose.

2.8. CONCLUSIONS AND FUTURE PERSPECTIVES

Knowing precisely the diversity of the pathogen is crucial from the taxonomic, biological and ecological standpoints. Indeed, pathogen identity has direct implications for disease management, either by cultural or chemical strategies as well as for disease resistance breeding programs. Furthermore, an effective management of new *Colletotrichum* species requires tools to discriminate between emerging and established fungal populations associated with soybean, aiming to detect the pathogens at the earliest point to monitor and limit their spread.

Colletotrichum truncatum has been considered the most important causal agent of soybean anthracnose. However, our survey showed that at least twelve *Colletotrichum* lineages are associated with soybean, with the *C. truncatum* and *C. orchidearum* s.c. having the greatest impact and the broadest worldwide distribution. Most of the information available on soybean anthracnose until now is limited to *C. truncatum*. Taking into account the numerous *Colletotrichum* species causing the disease, there is a gap in the knowledge of epidemiology, worldwide movement, distribution, identification, control measures, fungicide efficiency and genetic resistance for all of the species.

In agreement with Vieira *et al.*, (2020), more robust genomic sampling is required to improve our understanding of relationships among taxa in the genus *Colletotrichum*, and also our ability to distinguish species within complexes. Genome data is now available for several *Colletotrichum* species associated with anthracnose in soybean, such as *C. truncatum*, *C.*

musicola, *C. plurivorum*, *C. sojae*, *C. chlorophyti* (Rao and Nandineni, 2017; Gan et al., 2017; Rogério et al., 2020), yet a comprehensive phylogenomic study of the genus is still needed. A population genomics approach and comparative genomics investigations can be used for identifying candidate genes involved in pathogenicity, virulence (or aggressiveness), host specialization, fungicide resistance, and adaptation to different environments, with higher precision, contributing to a better understanding of *Colletotrichum* species dynamic in the agroecosystems.

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3. FIRST REPORT OF *COLLETOTRICHUM MUSICOLA* CAUSING SOYBEAN ANTHRACNOSE IN BRAZIL

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important crops worldwide as a source of protein-rich foods and animal feeds. Anthracnose, one of the major limiting factors to soybean production (Dias et al., 2016), is caused by species such as *Colletotrichum truncatum*, *C. sojae*, and *C. plurivorum* (Damm et al., 2009, 2019). In December 2016 and 2017, soybean plants of cultivars Monsoy 8768 and Pioneer y-70 with typical symptoms of anthracnose (necrotic and irregular brown lesions on stems, leaves and pods) were collected in Mato Grosso state, Brazil. Commercial fields sampled showed 10 – 15 % of incidence of anthracnose in each sampled area. In total, 10 different geographic locations were sampled.

CONCLUSION

Until now, *C. musicola* has been reported to be associated with *Musa* sp. (Damm et al., 2019) and *Colocasia esculenta* (Vásquez-López et al., 2019) in Mexico, and with *Phaseolous lunatus* in Brazil (Cavalcante et al., 2019). To our knowledge, this is the first report of *C. musicola* causing anthracnose in soybean producing regions around the world.

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4. GENOME SEQUENCES RESOURCES OF *COLLETOTRICHUM PLURIVORUM*, *C. MUSICOLA* AND *C. SOJAE*: THREE SPECIES OF THE *C. ORCHIDEARUM* SPECIES COMPLEX PATHOGENIC TO SOYBEAN

ABSTRACT

Colletotrichum is a large genus of plant pathogenic fungi comprising more than 200 species. In this work we present the genome sequences of three *Colletotrichum* species pathogenic to soybean: *C. plurivorum*, *C. musicola* and *C. sojae*. These three species belong to the *C. orchidearum* species complex and have been described and associated with soybean only recently. The genome sequences will provide insights into factors that contribute to pathogenicity towards soybean and will be useful for further research into the evolution of *Colletotrichum*.

Keywords: Anthracnose, Comparative Genomics, *Glomerella*, Illumina, Single Molecule Real-Time Cells, Sequencing.

INTRODUCTION

Colletotrichum is a large genus of plant pathogenic fungi, currently considered one of the top 10 most important fungal pathogens by plant pathologists (Dean et al., 2012; Crouch et al., 2014). Several species of *Colletotrichum* have been associated with soybean (*Glycine max*) anthracnose worldwide, with *C. truncatum* globally considered the most prominent (Rogério et al., 2017). In recent years emerging species such as *C. plurivorum*, *C. sojae* (Barbieri et al., 2017; Damm et al., 2019) and *C. musicola* (Bouffleur et al., 2020) were associated with the disease for the first time. Such species belong to the *C. orchidearum* species complex (s.c.) (Damm et al. 2019), a monophyletic clade of species closely related to the *C. orbiculare* s.c. Here, we report the reference genome sequences of three species pathogenic to soybean: *C. sojae*, *C. musicola*, *C. plurivorum* from the *C. orchidearum* species complex.

CONCLUSION

To the best of our knowledge the genome sequences of *C. musicola*, *C. plurivorum* and *C. sojae* represent new resources. The data reported here may provide insights into pathogenicity factors towards soybean and will be a useful resource for further research into comparative genomics and evolutionary studies in *Colletotrichum*.

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5. COMPARATIVE GENOMIC ANALYSIS REVEAL SECRETED EFFECTOR CANDIDATES OF FOUR SPECIES OF *COLLETOTRICHUM* PATHOGENIC TO SOYBEAN

ABSTRACT

Colletotrichum is considered one of the most important genera of fungi by plant pathologists due to its great potential for destruction. *Colletotrichum* spp. have a hemibiotrophic lifestyle and can infect a wide range of hosts, causing losses in essential crops worldwide, such as soybean (*Glycine max*). This crop is one of the most important agricultural commodities. Large areas of soybean cultivation under monoculture affects the intensity of diseases, amongst them, anthracnose. In the past, soybean anthracnose was mainly associated with *C. truncatum*, but during the last decade, other species have emerged from commercial soybean fields, including three species belonging to the *C. orchidearum* species complex (s.c.) (*C. musicola*, *C. plurivorum* and *C. sojiae*), becoming one limiting factor to soybean production in several regions. To better understand the pathogenicity factors of species of *Colletotrichum* that infect soybean we analyzed the repertoire of secreted effector candidates (SECs) of *C. truncatum*, *C. plurivorum*, *C. sojiae* and *C. musicola*, and compared them with 8 additional *Colletotrichum* species not pathogenic to soybean. Through an *in-silico* approach, we tested the hypothesis that all the *Colletotrichum* species that infect soybean have a common ancestor, and therefore share the repertoire of SECs. The repertoire of *C. orchidearum* specific and species-specific SECs were revealed, however, no host-specific SECs were spotted, indicating that the two major species complexes that infect soybean acquired the pathogenicity genes separately. This is the first characterization of effector repertoires of *Colletotrichum* species related to soybean anthracnose. Our results provided a set of candidate genes to be further investigated during functional characterization and could help to understand the role of effector proteins host-pathogen interactions of these species.

Keywords: *Colletotrichum truncatum*, *Colletotrichum musicola*, *Colletotrichum sojiae*, *Colletotrichum plurivorum*, *Glycine max*, Comparative Genomics.

5.1 INTRODUCTION

With more than 200 accepted species classified into species complexes (s.c.) or singletons (Marin-Felix et al., 2017; Damm et al., 2019), the hemibiotrophic fungus *Colletotrichum* is considered among the ten most destructive genera of phytopathogenic fungi (Dean et al., 2012), responsible for several losses in important crops (Vargas et al., 2012; Amil-Ruiz et al., 2016; Dias et al., 2016; Banniza et al., 2018; de Silva et al., 2019). Several species of *Colletotrichum* have been reported as pathogenic to soybean, being the *C. orchidearum* and *C. truncatum* s.c. found as the most distributed complexes associated with symptomatic soybean plants worldwide (Bouffleur et al., 2021). While *C. truncatum* is associated with soybean since 1917 (Nakata and Takimoto, 1934), *C. musicola*, *C. plurivorum* and *C. sojiae*,

members of the *C. orchidearum* s.c. were only described in the past 5 years (Barbieri et al., 2017; Damm et al., 2019; Bouffleur et al., 2020), however this species complex is misidentified at least since 2003 (Bouffleur et al., 2021).

A dispute for survival and adaptation marks the evolutionary battle between plants and pathogens throughout history (Jones and Dangl, 2006; Dong et al., 2014, 2015). This arms race is partially described by the “zig-zag” model (Jones and Dangl, 2006). In this model, the first layer of defense of plants recognizes molecular patterns associated with pathogens (PAMPs) or damage-associated molecular patterns (DAMPs) and active a pattern triggered immune response (PTI) (Jones and Dangl, 2006; Boller and Felix, 2009; Couto and Zipfel, 2016). On the other hand, pathogens can overcome this layer of defense releasing effectors, that are surface-exposed or secreted proteins that cause alterations in structure or processes of the host cell, suppressing the defense responses or enhancing access to nutrients, promoting the colonization of the host by the pathogen (Win et al., 2012). The recognition of effectors or effector targets by resistance (*R*) genes of the host will then trigger the second layer of defense, called effector-triggered immunity (ETI), being a stronger response than PTI that can lead to a hypersensitive reaction (HR) (Win et al., 2012; Dong et al., 2014).

Understanding the pathogenicity mechanisms of *Colletotrichum* and the way that they adapt to their hosts can be a powerful tool in developing sustainable control strategies (Dussert et al., 2019). The evolution through adaptation of pathogens to different hosts can involve sets of effectors, that can specialize and infect a specific host (Ma et al., 2010; Poppe et al., 2015; Hartmann et al., 2017; Petre et al., 2020). Over the past few years, the genomes of at least 42 species of *Colletotrichum* have been sequenced (<http://www.colletotrichum.org/genomics/>), including *C. truncatum*, *C. sojiae*, *C. plurivorum* and *C. musicola* (Rogério et al., 2020). The availability of genome sequences from multiple species of *Colletotrichum* enables unprecedented insights into genome composition (Lo Presti et al., 2015), including the prediction of effector candidates. Identification of fungal genes involved plant infection is becoming a useful tool to improve disease management (Mousavi-Derazmahalleh et al., 2019). The evolutionary trajectory of host-pathogen interactions can help clarify the mechanisms underlying the threat of pathogens to crops (Sánchez-Vallet et al., 2018).

Despite some studies of the sets of effectors in different species of *Colletotrichum* (Bhadauria et al., 2015; Baroncelli et al., 2016; de Queiroz et al., 2019; Lelwala et al., 2019), until now, the total number of effectors of *Colletotrichum* species that infect soybean and how many are unique to each species is unknown. The repertoire of candidate effectors from those species may reveal genes involved in host specificity during the *Colletotrichum*-soybean

interaction. In this work, we predicted the repertoire of secreted effector candidates (SECs) in the proteomes of four species of *Colletotrichum*, pathogenic to soybean and compared these with eight closely related species of *Colletotrichum* non-pathogenic to this host, providing a useful platform for future works regarding soybean anthracnose.

CONCLUSION

The identification of sets of SSECs of the *C. orchidearum* s.c. and *CTRU* open the field to perform evaluations of the functional role of these genes in soybean infection. Besides cultural and chemical control strategies have already been described for soybean anthracnose, recent outbreaks of the disease have been reported by researchers (Rogério et al., 2017, 2019; Dias et al., 2018) and producers (personal communication), suggesting that the control strategies used are not always effective. This may be a consequence of different *Colletotrichum* species present in soybean fields, that allied to the separate evolution of these species, may imply directly in disease management strategies, as the correct identification of the causal agent is crucial to an efficient control (Cai et al., 2009; Jayawardena, 2016).

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6. TRANSCRIPTOME ANALYSES OF DIFFERENT SOYBEAN CULTIVARS PROVIDES NOVEL INSIGHTS INTO THE SOYBEAN RESPONSE TO *COLLETOTRICHUM TRUNCATUM* INFECTION

ABSTRACT

Soybean (*Glycine max*) is one of the major crops worldwide as a source of protein-rich foods and animal feeds. Soybean anthracnose, mainly associated with the hemibiotrophic fungus *Colletotrichum truncatum*, is one of the limiting factors to soybean production. In order to gain a better understanding of the genetic basis of soybean defense against *C. truncatum*, we used a combined approach of pathogenicity assays and deep RNA sequencing. We examined the transcriptional pattern of two soybean commercial cultivars (*Gm1* and *Gm2*) during the development *C. truncatum* infection by two different strains (γ and β). Pathogenicity assays showed that the same soybean cultivar can have contrasting levels of resistance to different strains of *C. truncatum*, and this behavior is confirmed by the sequenced data. We divided the four treatments into two conditions accordingly to the level of resistance to anthracnose. Based on our results, we suggest that upon recognition of the fungus *C. truncatum*, treatments with a higher level of resistance modulate several genes related to plant immune responses, including signaling pathways and extensively transcriptional reprogramming that led to a hypersensitive response related to oxidative burst and activation of genes encoding compounds that enhanced plant resistance to pathogens.

Keywords: Time-course, RNA sequencing, Gene Expression, *Glycine max*, Anthracnose.

6.1 INTRODUCTION

Soybean (*Glycine max*) is one of the most produced crops worldwide, which can be extensively used for human and animal feeds due to its valuable nutritional composition (Hartman et al., 2011; Shea et al., 2020). Its production is constantly threatened by multiple diseases, including anthracnose, caused by several species of the hemibiotrophic fungus *Colletotrichum* (Riccioni et al., 1998; Sharma et al., 2011; Yang et al., 2012, 2014; Barbieri et al., 2017; Damm et al., 2019; Bouffleur et al., 2020; Shi et al., 2020), being *C. truncatum* one of the most distributed species worldwide (Bouffleur et al., 2021). Losses due to this disease can reach up to 100% under favorable climate conditions (Wrather et al., 2010), where 90kg/ha can be lost for each 1% of increment of the disease in the field (Dias et al., 2016).

The plant immune system has multiple layers (Jones and Dangl, 2006a; Poland et al., 2009; Cook et al., 2015; Jones et al., 2016). The resistance of plants against pathogens can be either qualitative, when a plant has a high level or is completely resistant, and is well represented by the zig-zag model, which describes path-triggered immunity (PTI) and

effector-triggered immunity (ETI) (Jones and Dangl, 2006a) or quantitative, that is referred to incomplete or partial levels of resistance phenotypes, and can be explained by the invasion model, which describes plant immunity as a surveillance system that is constantly evolving to trigger plant defense (Cook et al., 2015). However, there is no clear separation among qualitative and QDR genetic mechanisms, as QDR may or may not be a result of variation in components of PTI and ETI (Poland et al., 2009; Corwin and Kliebenstein, 2017).

Even if it is considered the predominant resistance found in nature, genes and mechanisms involved in QDR are poorly understood (Bartoli and Roux, 2017; Delplace et al., 2020), and resistance involving the QDR is more difficult to select due the involvement of several genes (Corwin and Kliebenstein, 2017). Because of the reduced selective pressure on the pathogen and the possibility of providing broad-spectrum resistance for more than one species and/or race of a pathogen, QDR has great potential to be more durable in the field (Krattinger et al., 2009; Corwin and Kliebenstein, 2017; Pilet-Nayel et al., 2017; Nelson et al., 2018).

The coordination of early defense responses to pathogen attack in plants may be required by QDR (Delplace et al., 2020) and New Generation Sequencing (NGS) strategies, such as RNAseq can be a useful tool to understand some components of the complex defense mechanisms involved in QDR, shedding light on genes and pathways involved in plant resistance against pests and pathogens, allowing the design of more resistant genotypes that can be used in agriculture (Nelson et al., 2018; Delplace et al., 2020). Multiple hosts have QDR against *Colletotrichum* species, among them strawberry (*Fragaria x ananassa*) resistance to *C. acutatum* and *C. gloeosporioides* (Amil-Ruiz et al., 2016; Anciro et al., 2018), bean (*Phaseolus vulgaris*) resistance to *C. lindemuthianum* (Geffroy et al., 2000; Mungalu et al., 2020), and potato (*Solanum tuberosum*) resistance against *C. coccodes* (Massana-Codina et al., 2020).

Although chemical control is an efficient method for disease management and allowed farmers to increase production due to the reduction of losses caused by pests and diseases; genetic resistance is a more eco-friendly solution for efficient production systems (Oerke and Dehne, 2004; Nelson et al., 2018), and soybean breeding should be employed as part of a strategy to control soybean anthracnose. Multiple studies aiming the identification of soybean resistant sources were performed around the world (Costa et al., 2009; Nagaraj et al., 2014; Yang and Hartman, 2015; Dias et al., 2019), however, genetic mechanisms that underpin soybean resistance against anthracnose have not yet been investigated, and until now, research progress on this field has been slow. With the advance of biotechnology, mechanisms involved in several pathways underlying plant defense against *Colletotrichum* species have been reported

as a result of the investigation of transcriptomic data (Vargas et al., 2012; Amil-Ruiz et al., 2016; Padder et al., 2016; Bhadauria et al., 2017; Diniz et al., 2017, 2017; Miranda et al., 2017; Mehmood et al., 2021).

In this study, we provide a wide-range transcriptomic analysis of the soybean-*C. truncatum* interaction during the development of anthracnose disease. We sequenced the transcriptome of two soybean cultivars that alters their level of resistance upon inoculation with two different strains of *C. truncatum*. The combined approach between pathogenicity assays and transcriptomic analyses revealed a strong correlation, that will pave the way towards a better understanding of the complex molecular mechanisms underlying the resistance of soybean to *C. truncatum*.

6.5 CONCLUSIONS

Due to the importance of soybean as a source of human and animal feeds, fungal diseases, such as soybean anthracnose may threaten food biosecurity (Hartman et al., 2011). Plants resistance against pathogens relies on their ability to recognize pathogen molecules, transduce the signal to downstream processes and defensively activate several pathways and products to stop the pathogen attack (Andersen et al., 2018). Gene expression studies among susceptible and resistant interactions of plants with pathogens can help to identify genes potentially involved in plant defense and/or susceptibility (Stutts and Vermerris, 2020). This study provided insights into the highly complex network of genes involved in soybean defense against anthracnose; including key-genes activated only at specific time-points and shared among both treatments that have a more resistant response against *C. truncatum*, that may help to explain the resistance mechanisms underlying this pathosystem. Most of these genes encode proteins known to be involved resistance of plants against biotic stresses.

Based on our results, we suggest that upon recognition of the fungus *C. truncatum* by CSRI and IRs encoding genes in soybean, treatments with a higher level of resistance to *C. truncatum* modulate several genes related to plant immune responses. Multiple signaling pathways were activated, and extensively transcriptional control of gene expression led to an HR-related with oxidative burst and activation of genes encoding compounds that enhanced plant resistance to pathogens. Functional studies of putative genes and pathways found in this work are expected to be proved as useful for the improvement of soybean cultivars' resistance to *C. truncatum*.

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