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RAFAEL MONTEIRO DO CARMO

Study of miR156-targeted *SPL/SBP-box* genes in the interaction between the atypical pathogen *Moniliophthora perniciosa* and *Solanum lycopersicum* cv. Micro-tom

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“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Curie - As quoted in Madame Curie: A Biography (1937) by Eve Curie

ABSTRACT

CARMO, R. M. **Study of miR156-targeted *SPL/SBP-box* genes in the interaction between the atypical pathogen *Moniliophthora perniciosa* and *Solanum lycopersicum* cv. Micro-tom**. 2021. 117 p. Dissertation (Master's in Science) – Center of Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, 2021.

Witches' Broom Disease (WBD), caused by the hemibiotrophic basidiomycete *Moniliophthora perniciosa* [syn. *Crinipellis perniciosa* (Stahel) Singer; Marasmiaceae s.l.], is the most significant disease of cacao (*Theobroma cacao*) in Brazil. *M. perniciosa* can infect a variety of hosts, allowing classification into three biotypes, C, L and S. The C-biotype infects cacao and related species. The L-biotype infects lianas (vines) without inducing symptoms. The S-biotype colonizes solanaceous plants such as tomato (*Solanum lycopersicum*). Our group has demonstrated that the tomato cultivar "Micro-Tom" (MT) is a suitable model for studying the biotrophic phase of the interaction with *M. perniciosa*. Mainly because the pathogen induces in MT symptoms of hypertrophy, stem hyperplasia, and uncontrolled branching, forming green brooms (symptoms characteristic of C-biotype infected *T. cacao*). However, the transition to the necrotrophic stage of the disease with the formation of basidiocarps has never been reported in tomato. Additionally, our group showed that cytokinins (CKs) are important for the development and progression of symptoms. Recently, the repression of proteins from the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (*SPL/SBP-box*) family of transcription factors has been shown to be part of the infection strategy of pathogens causing WBD-like diseases. Such transcription factors are plant specific and play essential functions for plant development (*e.g.* branching) in addition to acting in response to abiotic and biotic stresses. Furthermore, some members of this family have been described to interfere negatively with CK signaling. Interestingly, transgenic MT plants with low levels of *SPL/SBP*s, due to the overexpression of microRNA156 (156-OE, which down-regulates *SPL/SBP*s), have some phenotypic characteristics that resemble *M. perniciosa*-infected MT plants. Such as increased branching and number of locules in the fruits. Looking at our group previous mRNA-seq data from inoculated MT plants, we observed that the repression of *SPL/SBP*s is part of the *M. perniciosa* infection strategy. Thus, we investigated whether the repression of such proteins has an impact on susceptibility by inoculating 156-OE plants. Such plants showed not only more symptoms, but also increased severity of symptoms, greater pathogen colonization, and, for the first time in a tomato genotype, we noticed the transition to the necrotrophic stage, producing basidiocarps. Orthologs of the tomato *SlSBP15* gene (*e.g.* arabidopsis *AtSPL9*) participate in the immune response and also in CK signaling. Thus, we inoculated plants that overexpress a

version of this protein resistant to the downregulation by the microRNA156 (rSBP15-OE). These plants developed fewer symptoms, and symptoms developed were milder. Furthermore, they demonstrated less pathogen colonization than MT. Consistently, rSBP15-OE plants show induced defense and CK-degradation genes, which is possibly responsible for the reduction of symptoms. Meanwhile, 156-OE plants have repressed defense genes and induced CK-synthesis genes, which may be favoring the pathogen. Thus, this gene is a potential candidate for further studies involving resistance to *M. perniciosa*, with potential applications in cacao improvement.

Keywords: Witches' Broom Disease, SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE, plant-pathogen, *Theobroma cacao*.

RESUMO

CARMO, R. M. **Estudo dos genes SPL/SBP-box regulados pelo miR156 na interação entre o patógeno atípico *Moniliophthora perniciosa* e *Solanum lycopersicum* cv. Micro-tom**. 2021. 117 p. Dissertação (Mestrado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

A "vassoura-de-bruxa" (VDB), causada pelo basidiomiceto hemibiotrófico *Moniliophthora perniciosa* [syn. *Crinipellis perniciosa* (Stahel) Singer; Marasmiaceae s.l.], é a mais significativa enfermidade do cacaueiro (*Theobroma cacao*) no Brasil. *M. perniciosa* pode infectar uma variedade de hospedeiros, permitindo a classificação em três biótipos, C, L e S. O biótipo-C infecta o cacaueiro e espécies aparentadas. O biótipo-L infecta lianas (cipós) sem induzir sintomas. Já o biótipo-S coloniza solanáceas, como tomateiro (*Solanum lycopersicum*). Nosso grupo ja demonstrou que a cultivar de tomateiro "Micro-Tom" (MT) é um modelo adequado para o estudo da fase biotrófica da interação com *M. perniciosa*. Pois, em MT, o fungo induz sintomas de hipertrofia, hiperplasia caulinar, ramificação descontrolada formando vassouras verdes (sintomas também característicos do cacaueiro infectado pelo biótipo-C). Porém, a transição para o estágio necrotrófico da doença com a formação de basidiocarpos nunca foi reportada em tomateiro. Adicionalmente, nosso grupo mostrou que citocininas (CKs) são importantes para o desenvolvimento e progressão dos sintomas. Recentemente foi comprovado que a repressão de proteínas da família de fatores de transcrição *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL/SBP-box*) é parte da estratégia de infecção de patógenos causadores doenças semelhantes à VDB. Tais fatores de transcrição são específicos de plantas e desempenham funções indispensáveis para o desenvolvimento vegetal (*e.g.* ramificação) além de atuar na resposta à estresses abióticos e bióticos. Ademais, alguns membros dessa família foram descritos por interferir negativamente na sinalização de CK. Curiosamente, plantas transgênicas de MT com baixos níveis de *SPL/SBP*s pela superexpressão do microRNA156 (156-OE, o qual regula negativamente *SPL/SBP*s) apresentam algumas características fenotípicas que se assemelham às plantas de MT infectadas por *M. perniciosa*, como a perda de dominância apical e aumento do número de lóculos nos frutos. Olhando dados prévios do nosso grupo de mRNA-seq, proveniente de plantas de MT inoculadas, observamos que a repressão de *SPL/SBP*s é parte da estratégia de infecção de *M. perniciosa*. Dessa forma, investigamos se a repressão de tais proteínas possuem um impacto na susceptibilidade inoculando plantas com baixos níveis de *SPL/SBP*s. Tais plantas demonstraram não somente mais sintomas, como também maior severidade nos sintomas, maior colonização do patógeno

e primeira vez em um genótipo de tomateiro foi notado a transição para o estágio necrotrófico, produzindo basidiocarpos. Ortólogos do gene *SlSBP15* de tomateiro (AtSPL9 de arabidopsis e OsSPL14 de arroz) participam da resposta imune e também da sinalização de CK. Dessa forma, inoculamos plantas que superexpressam uma versão dessa proteína resistente à regulação negativa do microRNA156 (rSBP15-OE) para avaliar o papel dessa proteína na interação. Essas plantas desenvolveram menos sintomas, e os sintomas desenvolvidos foram mais brandos. Além disso, demonstraram uma menor colonização do patógeno que MT. Consistentemente, plantas rSBP15-OE apresentam genes de defesa e degradação de CK induzidos, o que possivelmente é responsável pela redução dos sintomas. Enquanto isso, plantas 156-OE possuem genes de defesa reprimidos e genes de síntese de CK induzidos, o que pode estar favorecendo o patógeno. Dessa forma, esse gene é um potencial candidato para mais estudos envolvendo resistência a *M. perniciosa*, com potenciais aplicações no melhoramento de cacaueiro.

Palavras-chave: Vassoura-de-bruxa, SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE, planta-patógeno, *Theobroma cacao*.

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1 INTRODUCTION

Plants are sessile organisms that are constantly under attack of pathogens. As a result, the long-term infection pressure from microbes and pests have led plants to develop multiple layers of immune response to detect and defend themselves against pathogenic organisms, such as bacteria, viruses, oomycetes, and fungi (JONES; DANGL, 2006; DANGL; HORVATH; STASKAWICZ, 2013; COOK; MESARICH; THOMMA, 2015; JONES; VANCE; DANGL, 2016). In crop plants, pathogen infections are responsible for significant yield loss worldwide, representing a risk to food production and security (SAVARY et al., 2019).

In this context, the atypical hemibiotrophic basidiomycete *Moniliophthora perniciosa* is known to be the causal agent of Witches' Broom Disease (WBD) of *Theobroma cacao*, the chocolate tree (GRIFFITH; HEDGER, 1994a; PURDY; SCHMIDT, 1996). Together with *Moniliophthora roreri* Frosty Pod Rot and *Phytophthora spp.* Black Pod, the *M. perniciosa* WBD constitute the three most significant diseases of cacao (PURDY; SCHMIDT, 1996; BOWERS et al., 2001). In South America, since the outbreak in Suriname at 1895, this disease has been one of the main limiting factors for cacao management and production (RORER, 1913; BAKER; HOLLIDAY, 1957; PURDY; SCHMIDT, 1996; PINTO; PIRES, 1998).

Because the huge social and economic impact caused by this disease, WBD has been extensively studied. However, since this interaction happens between two non-model organisms and also due to some peculiarities of the disease, it is difficult to relate to model interactions. Since the 1980s, several studies have established the basic knowledge regarding *M. perniciosa* basic biology (*e.g.* classification of strains in biotypes according to their hosts) and WBD (*e.g.* characterization of most disease symptoms in cacao) (EVANS, 1980; CALLE; COOK; FERNANDO, 1982; FRIAS; PURDY; SCHMIDT, 1991; GRIFFITH; HEDGER, 1994a, 1994b).

To increase cacao production, while trying to circumvent challenges imposed by the disease, breading programs started in the 1930s seeking resistant cacao varieties. However, *T. cacao* have a long-generation cycle, and breeding programs suffer with the field size requirement and with abiotic and biotic stressors that are difficult to control. Moreover, the molecular basis of WBD resistance found in some cacao varieties is not known and plant resistance genes and potential fungi avirulance genes still need to be functionally characterized (SILVA et al., 2014b; ROYAERT et al., 2016).

To bypass the limitation of doing functional studies with cacao plants, tomato (*S. lycopersicum*) has been used as a model for this interaction (BASTOS; EVANS, 1985; MARELLI et al., 2009; DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). The use of tomato became possible after was described a *M. perniciosa* isolate (biotype S) that can infect *Solanaceous* hosts and promote WBD (BASTOS; EVANS, 1985; GRIFFITH; HEDGER, 1994a). In tomato, only the biotrophic stage of the disease is present, but by, using tomato, mechanisms of pathogenesis and defense can be further explored during the interaction by applying functional genomics and other approaches (DICKSTEIN; PURDY, 1995; MARELLI et al., 2009; DEGANELLO et al., 2014; SCOTTON et al., 2017; PASCHOAL, 2018; BARSOTTINI et al., 2019; COSTA et al., 2021).

Despite some advances in understanding *M.perniciosa*-host interaction, our knowledge of such complex interaction is still reduced. Thus, the study of the molecular basis of the interaction are needed. For instance, the functional investigation of candidate genes that could be participating in the plant architecture and physiological changes caused by the disease could facilitate the generation of effective strategies to control it. Interestingly, we noticed that MT tomato plants overexpressing the microRNA156 (miR156) have some phenotypic characteristics that are similar to MT plants infected with *M. perniciosa*, such as loss of apical dominance and increased number of locules in the fruits.

The miR156 is known to regulate members of the *SPL/SBP* family. These genes were described to be main players of plant developmental and physiological processes (*e.g.* plant architecture). In addition, they are important as response to biotic stressors (reviewed by JEYAKUMAR et al., 2020). Interestingly, this family was described to be repressed by WBD-like-inducing pathogens, like Aster yellows phytoplasma witches' broom strain (CHANG et al., 2018; HUANG et al., 2021). The similarities between plants overexpressing the miR156 (156-OE) and *M. perniciosa* WBD raised the hypothesis that the repression of *SPL/SBP* genes could be participating in symptom induction by *M. perniciosa*.

Therefore, in this work we carried out the evaluation of the possible contribution of the miR156-targeted tomato *SPL/SBP*s in the interaction between *S. lycopersicum* x *M. perniciosa* (S-biotype), aiming to contribute to the understanding of defense mechanisms or susceptibility to WBD.

2 LITERATURE REVIEW

2.1 *Theobroma cacao* and *Moniliophthora perniciosa* interaction and history

Theobroma cacao, the "chocolate tree", belongs to the family Sterculiaceae, order Malvales. This diploid crop tree has its significance in both past and present. Nowadays, around 4.0 million tons of cocoa beans are produced worldwide, being an important crop (Pipitone, 2016). However, the history of this crop (cocoa) begins in pre-Columbian Central America. The Mayans and Aztecs used its seeds to produce a beverage that was the precursor of what we currently known as the "modern chocolate". Both of these ancient societies used cocoa for culinary and religious purposes and believed that its origin was divine. Theobroma means "food of the gods" (from the Greek, *theo* means gods and *broma* means food) due to the religious beliefs of Mesoamerican civilizations (DILLINGER et al., 2000). The *T. cacao* tree originates from Neotropical rainforests, primarily in the Amazon basin and the Guyana Plateau, and was domesticated around 3600 years ago (CORNEJO et al., 2018).

After Spanish colonization, cocoa started to become popular worldwide. European chefs started to produce what we know as chocolate, which largely increased the *T. cacao* seeds demand (DILLINGER et al., 2000). Thus, *T. cacao* trees started to be cultivated not only in its natural occurrence sites (*e.g.* Amazon region), but it was also introduced into several sites for farming. In Brazil, the cocoa production was initially in the Brazilian Amazon region, like Rondônia, Amazonas, and Pará States. But around 1746, it was also introduced in the south of Bahia state. This region became the second-largest cocoa production site in the world in the 1980s. However, the Witches' Broom Disease (WBD) of cacao was affecting several cacao production farms in South America and after 1989, when it was recorded in Bahia, the Brazilian production of cacao suffered huge losses (PEREIRA; DE ALMEIDA; SANTOS, 1996).

The WBD is caused by the basidiomycete *Moniliophthora perniciosa* [syn. *Crinipellis perniciosa* (Stahel) Singer; Marasmiaceae *s.l.*] (AIME; PHILLIPS-MORA, 2005). This disease stands as one of the three most significant diseases of cacao, together with *Moniliophthora roreri* Frosty Pod Rot, and *Phytophthora spp.* Black Pod (PURDY; SCHMIDT, 1996; BOWERS et al., 2001). Therefore, WBD represents one of the main limiting factors for the management and production of cacao in South America (GRIFFITH; HEDGER, 1994a), being considered a threat to world production (PURDY; SCHMIDT, 1996; MEINHARDT et al., 2008; TEIXEIRA et al., 2014). Both, pathogen and host, are endemic to the Amazonic region, but first record of an outbreak of the Witches' Broom Disease (WBD) in cacao occurred in

Suriname at 1895 (RORER, 1913). Thereafter, most South and Central America cacao-producing countries suffered huge losses on production, and never recovered (BAKER; HOLLIDAY, 1957). Brazil suffered a 50 to 90% drop in production after the disease was recorded in the south of Bahia state at 1989 (ANDEBRHAN; ALMEIDA; NAKAYAMA, 1998). The yield levels never returned to pre-outbreak rates, which led Brazil to move from a typically exporting country to an importer of cocoa (PINTO; PIRES, 1998).

Pathogenic fungi explore three types of strategies for obtaining nutrients from their respective hosts. Such strategies are commonly used to categorize their lifestyle, which classifies them into necrotrophs (*e.g. Sclerotina sclerotiorum*) (AMSELEM et al., 2011), biotrophs (*e.g. Erysiphe graminis sp.*) (CHAURE; GURR; SPANU, 2000)or hemibiotrophs (*e.g. Magnaporthe oryzae*) (WILSON; TALBOT, 2009). Necrotrophic fungi cause the death of host cells to use them as nutrient source, promoting colonization. On the other hand, biotrophs and hemibiotrophs are able to invade and colonize living tissues in order to grow and develop. However, differing from biotrophs, hemibiotrophic fungi goes to a necrotrophic phase at the final stages of infection, killing host cells. But generally, infection by hemibiotrophs implies a transient and asymptomatic biotrophic phase (HAMMOND-KOSACK; JONES, 1997). In addition, some pathogenic fungi and some saprotrophs (which acquire nutrients from decaying biomaterials) can change their feeding strategies according to the available nutrient source (OLSON et al., 2012; SMITH et al., 2017; ZANNE et al., 2020).

M. perniciosa is usually considered a hemibiotrophic fungi (EVANS, 1980). But recently, Hane et al. (2020), by using genome-derived analysis of carbohydrate-active enzyme gene content, had suggested *M. perniciosa* as a saprotroph. Such classification could be because of the possible feeding strategy switch mentioned above. However, further investigation is needed to better classify and assess if such change could happen to this pathogen. Nevertheless, considering *M. perniciosa* as a hemibiotrophic fungi, it is certainly an atypical one. The long biotrophic phase (during up to 90 days) where symptoms are induced and cause significant damage to the host differ this pathogen of other hemibiotrophs (TEIXEIRA et al., 2014). Such symptoms (Figure 1) usually are swelled infected shoots forming new symptomatic branches (green brooms) (Figure 1h), inversion of reproductive tissue into vegetative tissue resulting in swelled flowers similar to green brooms (Figure 1g), formation of parthenocarpic fruits, in addition to the appearance of necrotic spots in developing fruits (Figure 1i) and dried green brooms (dry broom) (Figure 1j) (PURDY; SCHMIDT, 1996; MELNICK et al., 2012).

Figure 1 - *Moniliophthora perniciosa*'s induced symptoms in *Theobroma cacao* stem, flowers and fruits (A) Healthy *T. cacao* plant. (B) Healthy *T. cacao* flower. (C) Healthy *T. cacao* stem. (D) Healthy *T. cacao* fruit. (E) *T. cacao* dry broom, at necrotrophic phase. (F) *T. cacao* naturally infected by *M. perniciosa* with several green brooms. (G) Symptomatic flower of *T. cacao*, showing swelling and broom-like structures. (H) Green broom from a symptomatic stem of *T. cacao*. (I) Symptomatic fruit of *T. cacao*, with rot spot in the middle of the fruit. (J) *M. perniciosa* reproductive structure (basidiocarp) produced in dry broom after wetting and drying cycles.

Infection with *M. perniciosa* occurs usually through the penetration of germ tubes originating from basidiospores into meristematic tissues. The apical and axillary meristems, floral pads or developing fruits. In addition, open injuries and stomata are normally infected by the pathogen (PURDY; SCHMIDT, 1996). Afterward, at the biotrophic stage, *M. perniciosa* is found as monokaryotic mycelium at the extracellular space in the alive plant tissue, where the pathogen induces the formation of symptomatic tissue, such as the swelled green brooms (Figure 1g, h). After 30 to 90 days of infection, the occurrence of necrotic spots in the symptoms starts to develop. These necrotic spots grow in green brooms, resulting in dry brooms (dried green brooms) (Figure 1e) (PURDY; SCHMIDT, 1996). At this stage of infection, *M. perniciosa* dikaryotic mycelia enter the cell, colonizing intracellularly. The dikaryotic mycelia present clamp connections, which are formed by the crossing of hyphae cells that guarantee that two nuclei coexist in each fungal cell. In necrotic tissue exposed to wet and dry periods, basidiocarps (Figure 1j) are formed from necrotrophic hyphae, which release basidiospores, allowing the infection of a new host (CALLE; COOK; FERNANDO, 1982).

For its economic and social impact, WBD has been intensively studied, and the peculiarities of this disease made it harder to relate to model interactions. The 1980s and 1990s studies established the basic knowledge regarding *M. perniciosa* basic biology and WBD (EVANS, 1980; CALLE; COOK; FERNANDO, 1982; FRIAS; PURDY; SCHMIDT, 1991; GRIFFITH; HEDGER, 1994a, 1994b). Breading programs seeking resistant cacao varieties started in the 1930s as an approach to control the disease while the demand for cocoa beans kept increasing. Early breeding programs were mostly carried by expeditions to Amazon regions looking for cacao trees without WBD symptoms. After finding potential resistant trees, efforts have been made to characterize these varieties and to use them in breeding programs. The Scavina 6 (SCA6) clone is one of the most studied sources of resistance to WBD (YAMADA et al., 2009). However, its agronomic traits, like low yield and smaller bean weight, make this genotype not attractive to be used for production. Usually, it has been used as parental and crossed with high yield and large bean weight varieties. Moreover, studies in several locations with SCA6 and its progenies, have shown a shift in the genetic composition of the *M. perniciosa* populations over the years, overcoming the resistance established by SCA6 genotype (GRAMACHO et al., 2012).

Resistant varieties to WBD were shown to be dependent on the pathogen population related to geographical localization. Progenies of the SCA6 clone, for example, provide stable resistance in some locations (*e.g.* Trinidad) while unstable resistance in other locations (*e.g.* Ecuador, Peru, and the Rondônia and the Bahia States of Brazil). This variation in disease susceptibility is assumed to be due to variability and dynamics in the pathogen populations (GRAMACHO et al., 2012). Therefore, novel cacao varieties with resistant traits are essential to expanding WBD resistance resources. Other germplasm groups with some resistance to WBD has been identified. TSH118, CAB0208, CAB0214, and progenies from seven CAB accessions (‘CAB 0064’, ‘CAB 0066’, ‘CAB 0156’, ‘CAB 0194’, ‘CAB 0195’, ‘CAB 0269’ and ‘CAB 0274’) constitute promising varieties to be used in breeding programs (FIGUEIRA; ALBUQUERQUE; LEAL-JR, 2006; PAIM et al., 2006; SERENO et al., 2006; SANTOS et al., 2007; ALBUQUERQUE et al., 2009). Another source of resistance to WBD is the CCN51 variety, developed in the 1960s in Ecuador. However, *T. cacao* breeding goes through a long-generation cycle, field size limitation for large-scale breeding trials, and abiotic and biotic stresses, which, combined with the lack of control of *M. perniciosa* and other major diseases (*e.g.* Black Pod and Frosty pod rot), cause breeding programs to be quite challenging. In addition, the molecular basis of the observed WBD resistance is not known,

and a classic functional characterization of plant resistance genes and fungal avirulence genes remains to be explored (SILVA et al., 2014b; ROYAERT et al., 2016).

The WBD Genome Project revealed gene information of *M. perniciosa* in the mid-2000s through genome sequencing combined with microarray and EST analysis (MONDEGO et al., 2008). This initiative made molecular biology approaches to WBD possible, by identifying a number of fungal genes. Thereafter, gene functions, as well as their roles in WBD started to be the focus of several studies (GARCIA et al., 2007; OLIVEIRA et al., 2012; TEIXEIRA et al., 2012; THOMAZELLA et al., 2012; BARSOTTINI et al., 2013). Likewise, the cacao genome sequencing assisted the search for host genes that may participate in this interaction (ARGOUT et al., 2011; MOTAMAYOR et al., 2013).

Scarpari et al. (2005) were the first to shed some light into the biochemical level of this complex pathosystem by characterizing soluble sugars, amino acids, alkaloids, ethylene, phenolics, tannins, flavonoids, pigments, malondialdehyde (MDA), glycerol, and fatty acids to try and understand the biochemical dynamics of WBD in cacao shoots. Their results demonstrated that coordinated biochemical alterations occurs in infected tissues. The higher glycerol concentration, production of ethylene prior to symptom development followed by a decrease with death of symptomatic tissue, and other alterations (soluble sugars, asparagine, alkaloids, and tannins) were detected in infected cacao. Such data was crucial to shed some light to this complex interaction, providing a better understanding of what the pathogen causes in the host biochemically (SCARPARI et al., 2005).

Other studies explored the potential role of plant sugars and calcium oxalate as signaling molecules for WBD. Both seem to regulate the transition from biotrophic phase to necrotrophic phase in *M. perniciosa* development (CEITA et al., 2007; BARAU et al., 2015). Moreover, fungal proteins were described to participate in the establishment of infection (GARCIA et al., 2007; ZAPAROLI et al., 2011; THOMAZELLA et al., 2012). Among the identified pathogen proteins, the NEPs (Necrosis and Ethylene-inducing Proteins) constitute a very intriguing class of proteins. Some members of this family were described to induce necrosis in plant tissues (*e.g. Fusarium oxysporum* in *Erythroxylum coca*) (BAILEY, 1995). Therefore they are major candidates for being responsible for the death of cacao tissues during this plant-pathogen interaction, once they led to necrosis in cacao and tobacco leaves (GARCIA et al., 2007; ZAPAROLI et al., 2011). Various Nep1-like proteins (NLPs) have been identified in several pathogens, such as bacteria, fungi, and oomycetes (BAILEY, 1995; PEMBERTON; SALMOND, 2004; GIJZEN; NÜRNBERGER, 2006).

From all *M. perniciosa* NEP genes, *MpNEP2* has an expression pattern that overlaps the necrotrophic phase development, being a candidate effector that could be responsible for inducing host tissue necrosis (GARCIA et al., 2007; ZAPAROLI et al., 2011). Such protein is induced in *in vitro* during carbon starvation, and *in vivo* experiments via artificial manipulation of carbon source in the apoplast to modulates the expression of this protein, which affected plant necrosis rate (BARAU et al., 2015). Moreover, WBD seems to increase intracellular sugar accumulation (forming a sugar sink in infected cacao shoots) and up-regulate of carbon starvation markers in infected cacao tissues, which combined with the decrease photosynthetic rates could possibly be responsible for *M. perniciosa*'s change to the necrotrophic stage (BARAU et al., 2015). But further research of this link between sugar, NEP expression and *M. perniciosa* phase change still needed to be better elucidated. Strikingly, *M. perniciosa*'s NEP proteins were probably acquired by horizontal transfer from oomycetes of the genus *Phytophthora*. The fact that many *Phytophthora* species can infect cacao supports the idea that an ancestral *Moniliophthora* species could coexist with these oomycetes, and the transfer of these genes could be determinant to the pathogenic hemibiotrophic lifestyle of *Moniliophthora* species (TIBURCIO et al., 2010).

The mitochondrial alternative oxidase (*MpAOX*) is an important gene for pathogen survival (THOMAZELLA et al., 2012). This protein is key *M. perniciosa* resistance to nitric oxide produced by the host and strobilurin fungicides. Remarkably, there is a clear correlation between *MpAOX*, the functionality of respiratory routes, and the hemibiotrophic lifestyle of *M. perniciosa*. While in the biotrophic stage, the monokaryotic hyphae mostly use the alternative route, in the necrotrophic stage, the dikaryotic hyphae almost exclusively rely on the main respiratory chain (THOMAZELLA et al., 2012). This *M. perniciosa* alternative respiratory chain hinders effective pathogen control, once most fungicides target only the main respiratory chain. Because the lack of effectiveness of traditional fungicides due to the *MpAOX* gene, Barsotinni et al. (2020) purified and characterized MpAOX protein characteristics with tests with activators and inhibitors, to identify important aspects for the development of novel fungicides. The kinetic profile in two environments (aqueous environment and physiologically-relevant proteoliposomes) and structure-activity relationships between the protein and some potential inhibitors were in silico tested based in a structural model and indicated residues for MpAOX-inhibitor interaction (BARSOTTINI et al., 2020). A potential effective strategy of WBD control would be the use of a novel fungicide that combines an inhibitor of the alternative respiratory chain and an inhibitor of the main respiratory chain (*e.g.* strobilurin). However, further application studies aiming to better

understand the MpAOX protein and its inactivation mechanisms in plant are needed for the development of such novel fungicides.

As part of its infection strategy, *M. perniciosa* seems to modulate host hormonal signaling. Transcriptomes from *T. cacao* and *M. perniciosa* during the biotrophic phase of this interaction made it possible to identify genes associated with symptom development, as well as some fungal genes that may act as pathogenicity factors (TEIXEIRA et al., 2014). The hormonal metabolism of auxin, gibberellin, cytokinin, and ethylene was altered in inoculated samples and probably participates in infection establishment. Besides interfering directly with the cacao hormonal-responsive metabolism, plant genes associated with auxin biosynthesis were not differentially expressed, corroborating the study that describes that this fungus produces auxin (KILARU; BAILEY; HASENSTEIN, 2007). Additionally, it was also identified a carbon deprivation in infected plants, which, combined with the reduction of photosynthetic rates leads to a premature senescence process on the ends of infected tissues, the first signs of necrosis during WBD (TEIXEIRA et al., 2014). Amylase-encoding genes, transcripts related to lipases and glyoxylate cycle enzymes were all found to be induced in infected samples (TEIXEIRA et al., 2014), which corroborate the previously described concentration dynamics of soluble sugars, lipids, and glycerol in response to *M. perniciosa* (SCARPARI et al., 2005). Therefore, the economic losses caused by WBD to cacao production could possibly be the combination of carbon deprivation in infected plants by its remobilization to infected brooms, combined with a hormonal unbalance and the direct impact of infecting fruits and flowers.

Recently, Santos et al. (2020) compared the proteomic alterations between two contrasting cacao genotypes (the susceptible Catongo and the resistant TSH1188) to identify proteins related to WBD resistance and susceptibility. Samples were collected at the biotrophic stage (72 hours after inoculation) and also at the necrotrophic stage (45 days after inoculation. Some of the altered proteins identified in the resistant genotype, TSH1188, are players in resistance mechanisms and stress response (*e.g.* Pathogenesis Related proteins and Peroxidases) (SANTOS et al., 2020). Meanwhile, the susceptible genotype exhibits lower levels of such proteins. Each genotype responded differently against the pathogen. Proteins that participate in biological functions, such as stress and defense, photosynthesis, oxidative stress, and carbohydrate metabolism seems to be big players in this interaction. Big data analysis, such as biochemical characterization (SCARPARI et al., 2005), RNA-seq (TEIXEIRA et al., 2014), and the proteomic (SANTOS et al., 2020) are extremally important, once they can drive a number of subsequent studies to characterize novel players of this interaction.

The combination of such data expands the basic knowledge of the disease and how the pathogen possibly could module the transition to necrotrophic stage, unraveling key genes to be functionally characterized and studied as candidates for breeding and/or genetic transformation of *T. cacao*.

Besides all scientific advances in the understanding of this pathosystem, difficulties in genetically transform *T. cacao* hinder functional genomics and profound physiological approaches to further explore WBD in *T. cacao*. Although the transformation of *T. cacao* was proven to be reproducible, the reliable generation of transgenic plants remains a challenge, mainly due to the recalcitrant characteristics of cacao (MAXIMOVA et al., 2003, 2006). Additionally, to date, there is no stable *M. perniciosa* transformation protocol, given de recent advances on using adapted protocols for transforming other fungi, the transformation of *M. perniciosa* is limited to hyphae transformation. The production of transformants basidiospores from such hyphae were not achieved (LIMA et al., 2003; SANTOS et al., 2009). Such limitation precludes functional genomics of *T. cacao* and *M. perniciosa* to understand key genes for the interaction. Thus, to better understand the *M. perniciosa*-host interaction, the use of a model plant became required to circumvent the limitations of this atypical interaction, as described above.

2.2 The use of tomato plants as a model to investigate *M. perniciosa*-host interaction

Moniliophthora perniciosa can infect a variety of hosts, not being restricted only to cacao, and its isolates can be classified into at least three biotypes, C, L, and S (GRIFFITH; HEDGER, 1994a). The C-biotype generally infects cacao and close genera (*e.g. Herrania*). The L-biotype infects lianas, especially from the Bignoniaceae family in the Amazon, without inducing symptoms of the disease. The S-biotype colonizes members of the Solanaceae family, mainly wild ones, such as jurubeba (*Solanum paniculatum*) and lobeira (*S. lycocarpum*), and promotes symptoms similar to the infection of C-biotype in cacao (GRIFFITH; HEDGER, 1994a). Somatic compatibility and molecular analyses have suggested that broom-forming C- and S-biotypes are similar to each other (GRIFFITH; HEDGER, 1994a; MARELLI et al., 2009).

Although WBD is treated as an important disease, the *M. perniciosa*-host interaction is difficult investigate because such interactions occur between two non-model organisms (TEIXEIRA et al., 2014). Thus, to perform functional genomic studies is a challenge. To circumvent such limitation, tomato (*S. lycopersicum*) had been used as a model due to its

susceptibility to isolates of the S-biotype of *M. perniciosa* (BASTOS; EVANS, 1985; MARELLI et al., 2009; DEGANELLO et al., 2014). Therefore, the use of this model plant allowed to explore some mechanisms of pathogenesis and defense during the interaction (DICKSTEIN; PURDY, 1995; MARELLI et al., 2009; DEGANELLO et al., 2014; SCOTTON et al., 2017; PASCHOAL, 2018; BARSOTTINI et al., 2019; COSTA et al., 2021). Similar to C-biotype infection of *T. cacao* plants, the S-biotype infection of tomato plants induces symptoms of stem swelling (between the first and second leaf, which is where tomato plants are inoculated for experiments) (Figure 2b, h), and axillary shoot growth (‘broom-like’ symptom) (Figure 2b, d, j, n), leaf petioles swelling (Figure 2f), lack of full leaf expansion, curling, and chlorosis (Figure 2f), reduction in root growth and fruit production, parthenocarpy on fruits, and increase in the fruit locule number (MARELLI et al., 2009; DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021).

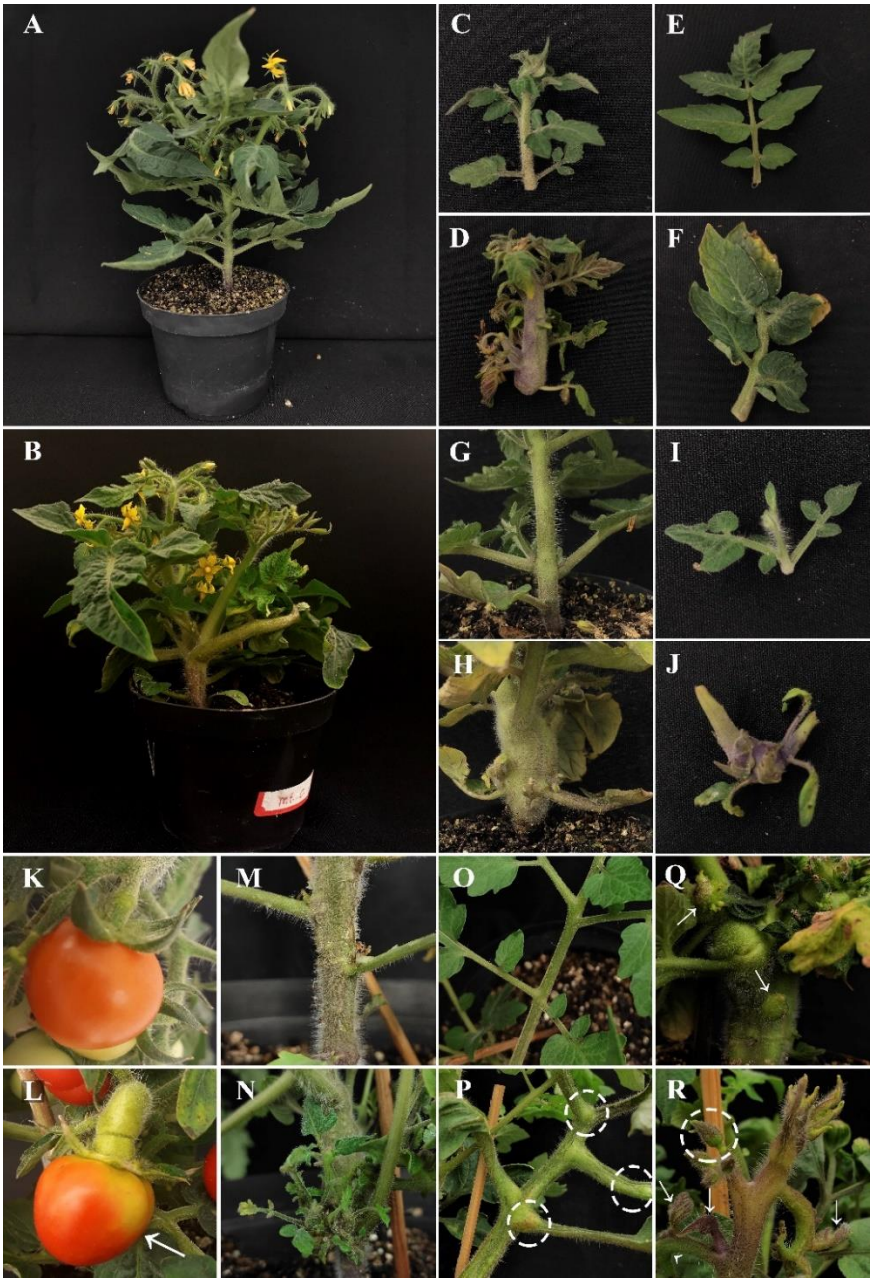

Figure 2 - Witches' Broom Disease symptoms in tomato plants (A) Non-inoculated MT plant (B) MT plant inoculated with S-biotype spores of *M. perniciosa* (C) Control branch from MT non-inoculated plant (D) Symptomatic swelled branch (Broom-like branch) from MT inoculated plant (E) Control leaf from MT non-inoculated plant (F) Symptomatic swelled and curled leaf from MT inoculated plant with chlorosis (G) Control stem (between first and second leaves) from MT non-inoculated plant (H) Symptomatic swelled stem (between first and second leaves) from MT inoculated plant (I) Control young branch from MT non-inoculated plant (J) Symptomatic swelled young branch from MT inoculated plant (K) Control fruit and floral peduncle from MT non-inoculated plant (L) Symptomatic fruit with uneven ripening (arrow) and swelled floral peduncle from MT inoculated plant (M) Control stem from M82 non-inoculated plant (N) Symptomatic stem from M82 inoculated plant with excessive proliferations of shoots and branches (O) Control leaf from M82 non-inoculated plant (P) Symptomatic and swelled leaf from M82 inoculated plant with callus-like structures (dashed circle) (Q) Tuber-like symptom (arrow) in stems of MT inoculated plants (R) Tip of symptomatic swelled branch from M82 inoculated plant with swelled floral buds and floral peduncle (dashed circle), curled apices (arrow), and swelled and curled leaves (arrow head).

The identification of other *M. perniciosa*'s host started at the beginning of the 80's, when wild solanaceous weeds (*Solanum rugosum* and *Solanum lisiantherum*) near Brazilian Amazon cacao production sites (Pará state) were reported to show symptoms similar to the WBD symptoms reported in cacao (BASTOS; EVANS, 1985). The discovery of these new hosts of *M. perniciosa* led to investigation of alternative *Solanum* hosts (*e.g. S. lycopersicum, Capsicum annuum*) by *M. perniciosa* (BASTOS; EVANS, 1985). Although the symptoms described to occur in these hosts are similar to cacao infected, the spores that usually infect solanaceous hosts do not induce any symptoms in *T. cacao.* Similarly, spores that colonize cacao hosts do not induce significative symptoms in tomato and pepper plants, only leading sometimes to loss of apical dominance (BASTOS; EVANS, 1985; DEGANELLO et al., 2014). Because induced symptoms and fungi characteristics are similar, this marked the discovery of a new pathotype of *M. perniciosa*, which later, led to the separation and classification of *M. perniciosa* biotypes (GRIFFITH; HEDGER, 1994a).

With the discovery of this novel biotype that infects alternative *Solanum* hosts and develop symptoms similar to cacao, Dickstein and Purdy (1995) investigated the potential of infection in several wild tomato species searching for resistant species or resistance source. *T. cacao*, *Solanum quitonese*, *S. topiro*, *S. cheesmaniae*, *S. chilense*, *S. chmielewskii*, *S. lycopersicum*, *S. habrochaites*, *S. neoriki*, *S. pennellii*, *S. peruvianum*, and *S. pimpineifolium*, together with several mutants of the cultivated tomato *S. lycopersicum* (*au-6*, *cana*, *diageotropica*, *gib-2*, *gib-3*, *minuta*, *monstrosa*, *procera*, and *torosa*) were inoculated with S-biotype spores (Table 1). The symptom incidence and progression were evaluated. Every species tested, except *T. cacao*, were described as susceptible to S-biotype *M. perniciosa*. However, the authors did not comment which symptoms were induced (*e.g.* green broom). In addition, they did not describe accession number for the species tested, so a new screening with more species and with a more detailed symptom analysis could still be explored as a source of resistance or tolerance against *M. perniciosa*. Regarding the *S. lycopersicum* mutants tested, only the *monstrosa* mutant did not develop any symptoms in response to the infection (DICKSTEIN; PURDY, 1995). However, this mutant is still not much explored and the allele responsible for its phenotype remains unknown. What is known so far, it that the mutation is located at the chromosome 11. As for the phenotype, plants are reduced plant size, unbranched, and leaves and stems strongly epinastic (LA615, details available at: https://tgrc.ucdavis.edu/Data/Acc/GenRepeater.aspx?Gene=mon). In addition, *monstrosa* plants display a high activity of IAA-oxidase, which would affect auxin levels (PALMIERI et al., 1978). The investigation of this mutant, the functional characterization of

the mutated gene and more tests against *M. perniciosa* could be an important step towards unraveling a resistance gene.

Table 1 - List of tomato mutant plants used by Dickstein and Purdy (1995)

Mutant	Route	Description/Phenotype
***au-6* (LA1486)**	Phytochromobilin synthase	Photomorphogenic mutant. Elongated, with reduced anthocyanin and a reduced chlorophyll level, which results in pale-green or yellow looking plant. (TGRC – UC Davis)
***cana* (LA590)**	Chlorophyll deficiency	Cotyledons and leaves gray-green especially on undersides; tiny and unbranched plants. (TGRC – UC Davis)
***gib-2* (LA2894)**	Gibberellin	Gibberellin deficient; greatly reduced germination, and dwarf growth habit; leaves dark green, thick, and wrinkled; application of exogenous GA restores normal phenotype. (TGRC – UC Davis)
***gib-3* (LA2895)**	Gibberellin	Gibberellin deficient; greatly reduced germination, and dwarf growth habit; leaves dark green, thick, and wrinkled; application of exogenous GA restores normal phenotype. (TGRC – UC Davis)
***minuta* (LA614)**	-	Small, upright and almost unbranched plants; shortened leaves prematurely yellowing. (TGRC – UC Davis)
***monstrosa* (LA615)**	High Auxin oxidase activity	Very tiny, upright, and unbranched plants; leaves much reduced, and strongly epinastic; stems slender; heterozygote intermediate for some traits. High IAA oxidase activity. (TGRC – UC Davis)
***torosa* (LA709)**	Branching	Reduced branching. Loss of function of an R2R3 class Myb gene. (TGRC – UC Davis)
***diageotropica* (LA1093)**	Auxin	Reduced auxin sensibility. Cyclophilin defective protein. (TGRC – UC Davis)
***procera* (LA2838)**	Gibberellin	Hypersensitive to gibberellin. Loss of function of DELLA protein repression domain. (TGRC – UC Davis)

As recorded in previous studies, although symptoms induced by S and C-biotype strains are similar, cross infection between biotypes is not commonly observed (C-biotype spores do not infect S-biotype host, and vice-versa) (BASTOS; EVANS, 1985; DICKSTEIN; PURDY, 1995) However, the Cast1 (Castanhal 1, CBS 142685) C-biotype strain of *M. perniciosa* is an exception, because it is the only strain reported so far to induce some symptoms in tomato (3 out of 12 inoculated plants, and seemingly attenuated symptoms of stem swelling and axillary shoot growth) (PIERRE et al., 2017). Such fact raised the question regarding the genetic relation of these biotypes. To verify the phylogenetic relation of different biotype strains, a proteome

analysis from in vitro mycelium of 6 C-biotype, 2 S-biotype, and 1 L-biotype strain resulted in a very similar protein profile between C and S biotypes. They differed from the L-biotype, which could explain the similarities in symptom induction between C and S-biotypes, differing from L-biotype (PIERRE et al., 2017). In addition, phylogenetic analysis with a concatenated 3-loci dataset (Internal transcribed spacer (ITS), *translation elongation factor EF-1alpha* (TEF), and *RNA polymerase II largest subunit* (RBS1)) of 10 C-biotype strains, 8 S-biotype strains, other 12 non-pathogenic (L-biotype) strains were aligned, with several other *Moniliophthora*, *Marasmus* and *Crinipellis* species. The results showed again, a closer relationship between C and S-biotype *M. perniciosa* strains than with all other strains and species analyzed (LISBOA et al., 2020).

Since C- and S-biotypes display similar broom-forming to each other (GRIFFITH; HEDGER, 1994a; MARELLI et al., 2009), the evolutionary proximity can mean that these two biotypes were recently split. Thus, it could be explain the similarities between their morphology and symptoms (LISBOA et al., 2020). Therefore, the investigation of this apparent non-host resistance within the hosts and biotypes of *M. perniciosa,* combined with DNA and RNA sequencing assays of Cast1 strain of the C-biotype, compared to others C and S-biotypes strains could provide some insights about the evolution of this atypical pathogen. Besides, by applying functional genomics (*e.g.* inoculation of transgenic lines) it could unveil potential candidates to be explored as a source of resistance genes, since non-host resistance results in almost no symptom or damage to the non-host infected plant (LEE et al., 2017).

Subsequent studies aimed to further characterize the interaction between *M. perniciosa* and tomato. Symptoms were evaluated in other tomato cultivars (New Yorker, MoneyMaker, Micro-tom, and M82) and the description of physiological and cellular changes started to be explored in tomato plants. These changes encompass hyperplasia and hypertrophy, and also changes in the signaling of cytokinin (CK) in the infected site, which is also reported in cacao (MARELLI et al., 2009; DEGANELLO et al., 2014; COSTA et al., 2021). The first comparative analysis of *T.* cacao infected with C-biotype and tomato infected with S-biotype demonstrated that disease progression is similar on both hosts. The majority of symptoms observed are swelling of the infected region and shoots from that region together with high activation and proliferation of axillary meristems (MARELLI et al., 2009). Despite each biotype having its host specificity, the similar disease-related characteristics indicates that the pathogenicity mechanisms between these two biotypes are conserved (MARELLI et al., 2009).

The pathogen transition to a necrotrophic stage in tomato plants was not confirmed, neither by finding dikaryotic hyphae, intracellular mycelium, or by basidiocarp production. However, since the biotrophic stage is symptomatic in cacao, it is important to stablish a model to the study of this first stage of WBD. Therefore, Deganello et al. (2014) proposed the use of tomato Micro-tom cultivar (MT) as a model to explore the biotrophic phase of the interaction, due to the symptom progression be similar to *T. cacao* infected shoots and other tomato cultivars (*e.g.* MoneyMaker). The MT cultivar harbors characteristics such as small plant size, short life cycle, and is easy to genetically transform it. Besides, combined with availability of several mutant in its background put him as a valuable tool for functional genomics (MEISSNER et al., 1997; PINO et al., 2010; CARVALHO et al., 2011). These characteristics and the fact that the same symptoms are induced in MT give this cultivar advantages compared with other larger size tomato cultivars (*e.g.* MoneyMaker) in the study of this interaction.

In this context, the use of MT as a model system have been provided some interesting and new information regarding the interaction between *M.perniciosa* and host. For instance, symptom development and gene expression of a compatible and an incompatible *M. perniciosa*–host interaction (S-biotype-MT and C-biotype-MT, respectively) was compared. As expected, the C-biotype-MT interaction did not develop symptoms, appearing as a non-host interaction, only decreased plant size was detected, probably due to the activation of defense mechanisms to resist infection that could have a high energetic cost (DEGANELLO et al., 2014). Moreover, the use of MT enabled a better detailing of symptoms, such as leaf petioles swelling, lack of full leaf expansion, leaf curling, broom-like branching from symptomatic stem (swelled and longer branches), and small black dots in symptomatic stem. Even though the occurrence of black dots and death of a few shoot apexes, all infected plants completed their life cycles and the symptomatic region (stem between the first and second leaf) was green and swelled (DEGANELLO et al., 2014; PASCHOAL, 2018).

The establishment of MT as a model to study the biotrophic phase led studies with a different focus involving this newly described pathosystem, like the functional study of *T. cacao* defense gene encoding a putative basal attenuator of programmed cell death by the heterologous transformation of MT (SCOTTON et al., 2017). The cacao *Bax inhibitor-1* (*TcBI-1*) gene when overexpressed in MT appears to decrease the incidence of infection in MT. Moreover, the inoculation of this transgenic plant with necrotrophic tomato pathogens (*Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and *Botrytis cinerea*) resulted in smaller lesions and pathogen growth, probably due to less induced Programmed Cell-Death (PCD, which favors necrotrophic pathogens) (SCOTTON et al., 2017). The use of MT also facilitated the

study of the effectiveness of a novel fungal AOX inhibitor to act against the pathogen, a remarkable accomplishment, once all data regarding such molecules were only *in vitro* generated and screened against the yeast *Pichia pastoris* and *E. coli* transformed with the *MpAOX* gene (BARSOTTINI et al., 2019). One of these potential AOX inhibitors (N-Phenylbenzamide-derived 7j-41) presented promising results when applicated in MT plants simultaneously with the pathogen inoculation. The use of 200 μM of 7j-41 seems to completely prevent spore germination, resulting in no symptom formation in treated MT plants. Once even at 35 days after inoculation, no difference was observed between the stem diameter of non-inoculated plants and inoculated+7j-41-treated plants (BARSOTTINI et al., 2019). Further studies regarding such chemical and similar chemicals are needed for effective chemical control of the disease, once 7j-41 seems to only prevent spore germination hindering the effective application on field.

As aforementioned, the infection of *T. cacao* by C-biotype *M. perniciosa* spores induces symptoms that could be the result of a hormonal imbalance (TEIXEIRA et al., 2014). The similarities with infected cacao biotrophic phase and the handiness of using MT led our group to explore the participation of plant hormones in the S-biotype-MT interaction (COSTA et al., 2021). Five MT mutant lines (*diageotropica*, *entire*, *epinastic*, *Never ripe*, and *procera*, Table 2) and three MT transgenic lines (*35S::AtCKX2*, *35S::PS*, and *35S::nahG*, Table 2) for genes involved in hormone biosynthesis and response were inoculated and the incidence and symptoms progression were evaluated. The *35S::AtCKX2* plants showed the smallest induction of stem swelling symptom (between the first and second leaf) among all inoculated genotypes (including MT). Other genotypes exhibited contrasting responses, with significant reductions or increases in stem diameter, however, regarding disease incidence, inoculated *35S::AtCKX2* plants was the only plants with a significant reduction in incidence compared to MT inoculated plants (COSTA et al., 2021). The *AtCKX2* gene encodes a Cytokinin Oxidase protein, responsible for inactivation of Cytokinin (CK) (WERNER et al., 2001), which indicates that this hormone could be important to the biotrophic stage of this interaction (COSTA et al., 2021).

RNA-seq data from MT inoculated plants, compared with non-inoculated plants at several periods of the interaction (5, 10, 20, and 30 days after inoculation) also highlighted that several CK-related genes were increasingly induced through time (COSTA et al., 2021) and some cacao orthologs of those genes were also highly expressed in *T. cacao* green brooms RNA-seq data (TEIXEIRA et al., 2014). Moreover, a fungal transcript detected in MT inoculated transcriptome encodes for a putative *M. perniciosa* tRNA-ISOPENTENYL-TRANSFERASE (tRNA-IPT), which suggests the production of isopentenyladenine (iP).

Such transcript can also be detected in monokaryotic and dikaryotic mycelium, and in *T. cacao* green brooms (COSTA et al., 2021; TEIXEIRA et al., 2014). The alignment of the putative *M. perniciosa* tRNA-IPT protein with other pathogenic fungi tRNA-IPT proteins (described to produce CKs and interfere in plant defense) suggests that this protein could possibly produce CK as a pathogen strategy to induce symptoms of swelling, which are characteristic of this pathogen (COSTA et al., 2021).

Table 2 - List of tomato mutant and transgenic plants used by Costa et al. (2021)

Mutant	**Route**	**Description/Phenotype**
diageotropica	Auxin	Reduced auxin sensibility. Cyclophilin defective protein.
entire	Auxin	Increased auxin sensibility. *La2922* defective protein, involved in auxin transcription pathway.
epinastic	Ethylene	Increase production of ethylene. Unknown gene function.
Never ripe	Ethylene	Low ethylene sensibility. Ethylene receptor defective protein.
procera	Gibberellin	Hypersensitive to gibberellin. Loss of function of DELLA protein repression domain.
Transgenic lines	**Route**	**Description/Phenotype**
35S::PS	Jasmonic acid	Increased levels of JA. Overexpression of *PROSYSTEMIN*, positive regulator of jasmonic acid signaling pathway.
35S::nahG	Salicylic acid	Low levels of SA. Expression of *NAHG* gene, which catalyzes the conversion of salicylic acid into catechol.
35S::AtCKX2	Cytokinin	Low levels of cytokinin. Overexpression of *AtCKX2* gene.

In addition, CK was quantified in inoculated and control MT plants, and inoculated plants had higher CK concentrations than control plants. Moreover, benzyladenine-treated MT inoculated plants showed increased severity of swelling symptom. Altogether, these results corroborate the potential role of CKs in the formation of the swelling symptom (COSTA et al., 2021). Moreover, the external application on the inoculated sites of PI-55, an inhibitor of CK perception, that acts as a competitor of the CK receptors His kinase (SPÍCHAL et al., 2009), reduced symptom severity, which also highlights the role of this hormone for symptom formation. Altogether, the S-biotype seems to manipulate CK metabolism of infected MT plants and this hormone could possibly be key to the swelling symptom characteristic of the disease (COSTA et al., 2021).

Studies with cacao plants demonstrated that infection by *M. perniciosa* induces some biochemical and transcriptional changes in shoot primary metabolism (SCARPARI et al., 2005; TEIXEIRA et al., 2014; BARAU et al., 2015). But, the limitations of doing physiological and functional genomic experiments with *T. cacao* hinders the further exploration of such changes. In this context, a detailed biochemical and physiological analysis of changes induced in the model MT infected by *M. perniciosa* was described (PASCHOAL, 2018). The analysis of the metabolic content of the swelled portion of stem from MT inoculated plants showed that this symptomatic region acts as a sink of sugar. Sugars seems to be another big player in this interaction. Their accumulation in the symptomatic stem could be inducing the loss of apical dominance of plants and, consequently, increased branching, once sugars can act as signaling molecules (PASCHOAL, 2018).

Lignin content is also higher in the symptomatic stem (PASCHOAL, 2018), and *T. cacao* infected tissue have lignin formation genes upregulated (TEIXEIRA et al., 2014). However, the role of this accumulation still not clear. It could potentially be to provide nutrient source for the necrotrophic phase of the pathogen, or could it be an attempt to limit pathogen colonization. Nevertheless, the lack of the necrotrophic phase of WBD in tomato and difficulties of doing functional studies in cacao hiders the characterization of its role in the disease.

At the same time, some ROS related pathways have been detected by the oxidation of proline, polyamines and ascorbate (PASCHOAL, 2018). The accumulation and response to ROS plays a critical role in plant-pathogen interactions, for acting as signal molecules (CAMEJO; GUZMÁN-CEDEÑO; MORENO, 2016). However, some genes found in *M. perniciosa* genome could produce catalase and thioredoxin, which are ROS-processing enzymes to inactivate ROS (MONDEGO et al., 2008; TEIXEIRA et al., 2014). As a consequence of sink formation and energy cost with defense responses, infection by *M. perniciosa* could potentially causes a delayed flower development, reduction of fruit set and yield in MT (PASCHOAL, 2018). So far, CK seems to be responsible for the characteristic swelling symptom, which promotes the nutrient sink formation (*e.g.* sugars). It would be interesting to test whether CK favors pathogen colonization, once it was shown that it does promote symptoms development (*e.g.* broom-like branching from the swelled stem and decrease in fruit yield). The accumulation of nutrients in the symptomatic region could be used by *M. perniciosa* as nourishment at the necrotrophic phase, however, the identification of key factors responsible for the transition to this stage still unknown.

2.3 Conclusions and perspectives related to tomato application in *M.perniciosa*-host interaction

Although some advances in *M.perniciosa*-host interaction have been made in the past years, our understanding of such complex interaction still limited, specially related to symptom induction and the necrotrophic phase. Since the pathogen genetic transformation it is not possible, the use of tomato to validate pathogen's genes could shed some light on *M. perniciosa*' effector. Some promising necrotrophic phase-related genes like *MpNEP*s still need to be further characterized and the use of transgenic tomato plants could be an important tool for this purpose exploration. Considering that cacao genetic transformation could be time consuming, the use transgenic or mutants identify and validate resistance genes could assure that the time invested engineer cacao plants could have a better chance of success against *M. perniciosa*.

In addition, there is one promising alternative to produce edited plants that were recently described (MAHER et al., 2020) and could potentially generate *T. cacao* mutants. The de novo induction of meristems through transient expression of the CRISPR cassette along with meristem development genes *WUSCHEL* (*WUS*) and *SHOOT MERISTEMLESS* (*STM*) or *WUS* with the cytokinin biosynthesis gene *ISOPENTENYL-TRANSFERASE* (*IPT*) produces edited shoots from non-edited plants without tissue culture (MAHER et al., 2020). Such strategy could be tested in cacao plants, which problems in genetically manipulate are partially due to its recalcitrant problems in tissue culture.

The use of these different strategies, combined with a deeper understanding of the molecular basis of the interaction are needed to try and control this disease in cacao. For instance, studies exploring the participation of genes that might be responsible for the plant architecture and physiological changes induced by the pathogen (*e.g.* master regulators of branching, like *SPL/SBP* family) could help in the understand of *M. perniciosa* and host interaction. In addition, with the application of molecular, it would be able to test genes that could confer susceptibility, resistance or tolerance against this important disease.

2.4 Squamosa-Promoter Binding Protein-like family

The SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (*SPL/SBP*) family comprises several transcription factors known to be specific to plants and play important roles in growth, development, abiotic stress, and have been recently related to biotic stress. The *AmSBP1* and *AmSBP2* were the first *SPL/SBP* genes identified because of their ability to

bind to the promoter of the gene *SQUAMOSA*, which is a gene related to flower development, in *Antirrhinum majus* (HUIJSER et al., 1992; KLEIN; SAEDLER; HUIJSER, 1996). Thereafter, several studies identified *SPL/SBP* members in all green plants, including single-celled green algae, mosses, gymnosperms, and angiosperms (CARDON et al., 1997; ARAZI et al., 2005; RIESE et al., 2008; ZHANG; LING; YI, 2015). The accumulation of *SPL/SBP* genes transcripts seem to be regulated by an age-mediated mechanism (WU; POETHIG, 2006; WU et al., 2009), sugar accumulation (YU et al., 2013; PONNU et al., 2020), and both biotic and abiotic stress (CUI et al., 2014; CHANG et al., 2018).

The model species *Arabidopsis thaliana* have 16 *SPL/SBP* genes, named *SPL1* to *SPL16*, and they were found to bind to the promoter of *APETALA1* (*AP1*, the Arabidopsis orthologous gene of *Antirrhinum majus SQUAMOSA* gene), in their characteristic motif (BIRKENBIHL et al., 2005). Most members of this family were described to be regulated post-transcriptionally by the microRNA156 (miR156), the miR157 (which have only some nucleotide differences, compared to miR156), and, in monocots, the miR529. In arabidopsis, 11 *SPL/SBP* genes contain the regulation site for the miR156 and the miR157, however, miR529 seems to have been lost in dicots, including the models *Arabidopsis thaliana* and *Solanum lycopersicum* (CUPERUS; FAHLGREN; CARRINGTON, 2011; CHÁVEZ MONTES et al., 2014; MOREA et al., 2016). Nevertheless, *SPL/SBP* mRNAs can be regulated by these microRNAs, either by cleavage (*e.g. SPL9*) or by mechanical inhibition of translation (*e.g. SPL3*), constituting the crucial miR156/157/529-*SPL/SBP* module (SCHWAB et al., 2005; GANDIKOTA et al., 2007; CUPERUS; FAHLGREN; CARRINGTON, 2011).

Regarding the binding sites for these miRNAs, while the miR156/157 binding sites can be in coding and in untranslated regions, the miR529 binding sites are predominantly in coding regions (which can overlap miR156/157 sites) (LING; ZHANG, 2012; ZHANG; LING; YI, 2015). Although dicots have lost the *MIR529* gene, and therefore, do not have the mature miR529 to regulate its targets. The *Arabidopsis thaliana SPL/SBP* genes *AtSPL9* and *AtSPL15* still carry the miR529-target sites (MOREA et al., 2016). Interestingly, the overexpression of *MIR529* precursor from rice in *A. thaliana* display similar phenotypes as the *spl9spl15* double mutant. Such downregulation shows that even with the absence of miR529 in arabidopsis, the target sites in both these genes are still functional (MOREA et al., 2016).

The miR156-targeted *SPL/SBP*s can be divided in two groups based on the size of the produced protein and the position of the regulatory site for miR156 (SALINAS et al., 2012). Small *SPL/SBP* genes, such as tomato *SlSBP3*, *4* and *CNR*, have the miR156 recognition site in the 3' UTR region. Meanwhile, large *SPL/SBP* genes, such as tomato *SlSBP10*, *13*, and *15*,

present the miR156 recognition site in its coding region (SALINAS et al., 2012). Such pattern regarding protein size and regulatory site position is conserved in *Arabidopsis thaliana* (RHOADES et al., 2002). As aforementioned, *SPL/SBP* are deeply conserved on plants. For instance, there are 19 *SPL/SBP* genes in rice (*Oryza sativa*) (XIE; WU; XIONG, 2006), 56 in wheat (*Triticum aestivum*) (ZHU et al., 2020), 17 in barley (*Hordeum vulgare*) (TRIPATHI; BREGITZER; SINGH, 2018), 28 in black cottonwood (*Populus trichocarpa*) (LI; LU, 2014), 41 in soybean (*Glycine max*) (TRIPATHI et al., 2017), 15 in tobacco (*Nicotiana tabacum*) (HAN et al., 2016), and 15 in tomato (*Solanum lycopersicum*) (SALINAS et al., 2012). 10 miR156-targets *SPL/SBP* genes have been reported in tomato, although most of them are not functionally characterized (SALINAS et al., 2012).

Members of this family are characterized by the highly conserved SBP domain of approximately 80 amino acids (KLEIN; SAEDLER; HUIJSER, 1996; YANG et al., 2008). The SBP domain is implicated in nuclear import and binding sequence-specific DNA. The SBP-responsive DNA binding site usually contain a central GTAC motif with a gene-specific flanking region (BIRKENBIHL et al., 2005; YAMASAKI et al., 2006; LIANG; NAZARENUS; STONE, 2008). Two Zn-finger-like structures present in SPL/SBP proteins are responsible for the sequence-specific binding of DNA (YAMASAKI et al., 2004; BIRKENBIHL et al., 2005). These structures are formed by two zinc ions coordinated by conserved cysteine and histidine residues. Each one is involved with zinc coordination and are essential for DNA binding (YAMASAKI et al., 2004; BIRKENBIHL et al., 2005). Moreover, at the C-terminal end of the SBP-domain, a putative nuclear localization signal (NLS) sequence is located. This NLS, which could be responsible for the presence of these protein in the nucleus, overlaps with the DNA-binding domain (specifically the second Zn-finger-like structure) (YAMASAKI et al., 2004; BIRKENBIHL et al., 2005). In summary, the SPL/SBP-box family is an important plant-specific family of transcription factors that have been widely studied in the past years. However, our understanding regarding this gene family still limited. Most researchers explored this family in plant development, however, their functions and the mechanisms that they are involved in other situations (*e.g.* biotic stress) still mostly unknown.

2.5 *SPL/SBP* genes functions in plant development and growth

SPL/SBP genes are widely described to participate in several growth and developmental processes, such as sporogenesis (UNTE et al., 2003), male inflorescence size (WU et al., 2016a) and fertility (XING et al., 2010, 2013), plant architecture (STONE et al., 2005; JIAO et al.,

2010), vegetative-to-reproductive phase transition (CARDON et al., 1997; WU; POETHIG, 2006; WANG; CZECH; WEIGEL, 2009), the establishment of lateral meristems and primordia initiation (CHUCK et al., 2010, 2014; PRESTON; HILEMAN, 2010), leaf development (YAMAGUCHI et al., 2009; HOU et al., 2017), shoot maturation (SCHWARZ et al., 2008; SHIKATA et al., 2009), ovary and fruit development (MANNING et al., 2006; SILVA et al., 2014a; CHEN et al., 2015), as well as ear development, yield, and seed development (UNTE et al., 2003; WANG et al., 2005; ZHANG et al., 2014, 2017; WU et al., 2016b; WANG; ZHANG, 2017).

Overexpression of *SPL3*, *SPL4*, and *SPL5* in *Arabidopsis thaliana* promotes vegetative phase change, faster development of adult characteristics compared with wild-type (*e.g.* abaxial trichomes, and an increased number of cells with reduced size in leaves) (WU; POETHIG, 2006; USAMI et al., 2009). Moreover, *AtSPL3*, *AtSPL4*, and *AtSPL5* redundantly promote the reproductive transition, through signals from the photoperiod, age, and gibberellic acid (GA) pathways (Figure 3) (CARDON et al., 1997; GANDIKOTA et al., 2007; WANG; CZECH; WEIGEL, 2009; YAMAGUCHI et al., 2009; JUNG et al., 2012; PORRI et al., 2012; YU et al., 2012).

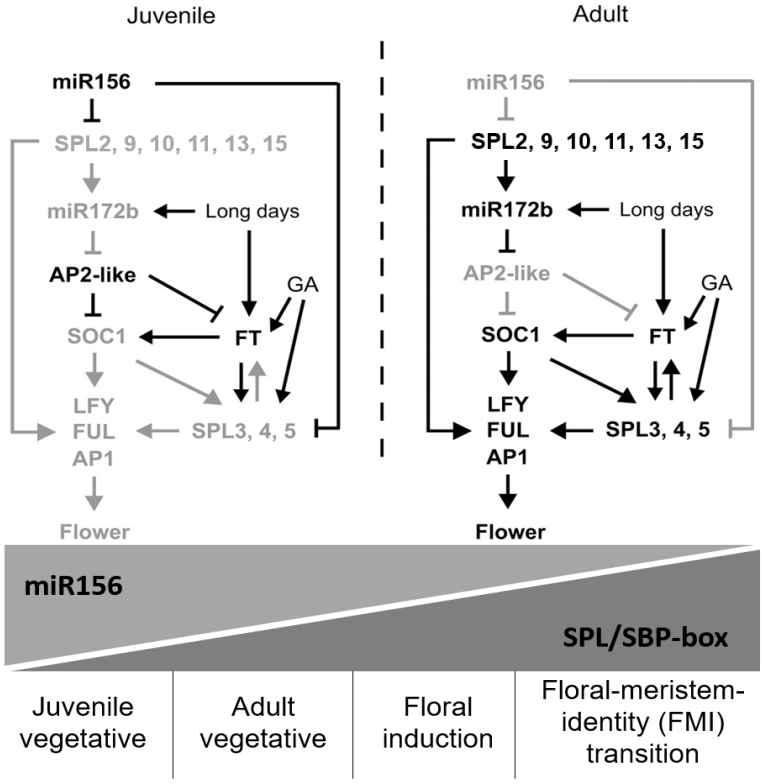

Figure 3 – The miR156-*SPL/SBP* module regulates vegetative and reproductive transition in arabidopsis by interacting with *SOC1*, *LFY*, *FUL*, *AP1*, and GA pathways. Adapted from Poethig (2013) and Xu et al. (2016)

In short-day conditions, these *SPL/SBP* genes are downregulated by the miR156. However, under long-day conditions, *AtSPL3/4/5* are upregulated in leaves as a response to photoperiod signals by *SUPPRESSION OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) through the GA pathway, *FLOWERING LOCUS T* (*FT*), and *FLOWERING LOCUS D* (*FD*) (Figure 3) (JUNG et al., 2011, 2012). These SPL proteins also indirectly activate *FT* expression (Figure 3), probably by inducing *FRUITFULL* (*FUL)*, *APETALA1* (*AP1*), and *LEAFY* (*LFY*) in the shoot apical meristem (CORBESIER; COUPLAND, 2006; CORBESIER et al., 2007; WANG; CZECH; WEIGEL, 2009; YAMAGUCHI et al., 2009).

In tomato, a naturally occurring epigenetic mutation causes the loss-of-function of the *COLORLESS NON-RIPENING* (*SlSPL-CNR*) gene (*AtSPL3/4/5* ortholog) resulting in fruit unripening (MANNING et al., 2006). The *Cnr* epimutant was described to be a spontaneous mutation that leads to hypermethylation in the *SlSPL-CNR* gene promoter (approximately 2.4 kb upstream of the gene). The *SlSPL*-CNR protein seems to interact with the promoter of the ripening-related gene TDR4 (ortholog of the *A. thaliana FUL* gene) (MANNING et al., 2006). This is the only SBP related to fruit ripening. Moreover, other closely related SBP genes (*SlSBP3* and *SlSBP4)* expression data suggest that these homologous genes are not involved in fruit ripening, but points to a function in earlier carpel development (SALINAS et al., 2012, SILVA et al., 2014a).

Recently, other functions have been attributed to the *SlSPL-CNR* gene (LAI et al., 2020). This gene seems to be capable of triggering cell death in plants. This protein has two zinc-finger motifs (ZFM) within the C-terminal SBP domain and one nuclear localization signal (NLS) sequence (LAI et al., 2020). While both ZFM and NLS are important for controlling ripening, only NLS seems to be important to promote cell death in tomato leaves, but further research is still needed to uncover the mechanism. Furthermore, yeast-two-hybrid screening and a co-immunoprecipitation (CoIP) assay showed that *SlSPL-CNR* protein interacts with *SUCROSE NON-FERMENTING-1-RELATED PROTEIN KINASE1* (*SnRK1*) (LAI et al., 2020). The SnRK family act as a global regulator of carbon metabolism and affects several aspects of plant development (JAMSHEER K; KUMAR; SRIVASTAVA, 2021). But the repression of *SlSnRK1* also inhibits fruit ripening in tomato. Possibly the SlSPL-CNR protein are phosphorylated by SlSnRK1 protein in the cytoplasm by physical interactions. Afterward, the phosphorylated SlSPL-CNR protein may be translocated to the nucleus (because of the NLS), and there it controls expression of target genes binding to target-promoter region in a zinc-dependent manner (LAI et al., 2020). Although more studies have been unraveling new

information about this intriguing and different *SPL/SBP* gene, we still have little understanding of its mechanism and functions.

The arabidopsis *SPL7* (*AtSPL7*) is required for regulation of copper homeostasis (YAMASAKI et al., 2009). Copper is an essential micronutrient, and at low levels, growth and production are hindered. Conversely, is toxic at high concentrations. Therefore, the maintenance of a specific copper concentration benefits plant fitness (CLEMENS; SCHROEDER; DEGENKOLB, 2001; PUIG et al., 2007). *AtSPL7* bind to the SBP motif (GTAC) in the *MIR398* promoter, which could explain the downregulation of such miRNA in *atspl7* mutants. This miRNA targets arabidopsis cytosolic *COPPER/ZINC SUPEROXIDE DISMUTASE1* (*CSD1*) (YAMASAKI et al., 2007), the chloroplastic *CSD2* (YAMASAKI et al., 2007), the *MITOCHONDRIAL CYTOCHROME C OXIDASE* (*COX5b-1*) (JONES-RHOADES; BARTEL, 2004; SUNKAR; ZHU, 2004), and the *COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE1* (*CCS1*) (BEAUCLAIR; YU; BOUCHÉ, 2010), which are all related to copper homeostasis. This family of SUPEROXIDE DISMUTASE proteins (SOD family) is known as antioxidant enzymes that reduces ROS levels (MITTLER et al., 2004), which could, therefore, participate in biotic and abiotic stress responses.

Moreover, the *AtSPL7* also modulates the expression of the *MIR397*, which predicted targets are *LACCASE2* (*LAC2*), *LAC4*, and *LAC17* (ABDEL-GHANY; PILON, 2008; YAMASAKI et al., 2009). Another target of *AtSPL7* is the *MIR408*, which targets plantacyanins, *LAC3*, *LAC12*, and *LAC13* (ABDEL-GHANY; PILON, 2008; YAMASAKI et al., 2009), and the *MIR857*, which targets the *LAC7* (YAMASAKI et al., 2009). Although laccases are known as multi-copper glycoprotein oxidases, their precise roles in higher plants remain largely unclear (TURLAPATI et al., 2011). Some members of this family have been described in arabidopsis to participate in seed coat oxidative polymerization of flavonoids (POURCEL et al., 2005) and lignin polymerization (BERTHET et al., 2011). Plantacyanins are in the subfamily of blue copper proteins (RYDÉN; HUNT, 1993) and are involved with oxidative burst that could occur during biotic stresses and/or in lignin formation (NERSISSIAN et al., 1998). In summary, the *AtSPL7* regulated several copper homeostasis-related genes being an important player to both plant growth under different copper concentrations and also potentially to biotic and abiotic stress responses (SUNKAR; KAPOOR; ZHU, 2006; YAMASAKI et al., 2007, 2009; ABDEL-GHANY; PILON, 2008).

Another SBP gene that regulates copper homeostasis is the *SBP2* of *Physcomitrella patens* (NAGAE; NAKATA; TAKAHASHI, 2008). When there is a high concentration of copper, *PpSBP2* represses the *IRON SUPEROXIDE DISMUTASE* (*FeSOD*) gene by binding to

its promoter, which in turn switches to copper/zinc SOD catalysis genes to take over the *FeSOD* function, mitigating the reactive oxygen species (ROS) accumulation. By contrast, when copper and zinc are limited, it is hypothesized that *PpSBP2* expression is low, resulting in the reinstatement of *FeSOD* functioning (MITTLER, 2002; NAGAE; NAKATA; TAKAHASHI, 2008).

The arabidopsis *SPL8* (*AtSPL8*) have a major effect on microsporogenesis and megasporogenesis in the anthers and ovules, respectively (UNTE et al., 2003). Loss-of-function mutants for this gene display shorter stamen filaments, fewer pollen grains, and low seed set. The *AtSPL8* seem to be required for the proper formation of microsporangium in the anther and for the regular entrance of spore mother cells into meiosis (UNTE et al., 2003). Thus, the seed set is greatly affected by *AtSPL8* (UNTE et al., 2003; reviewed by XING; SALINAS; HUIJSER, 2011).

Besides seed set, petal trichome production and root growth are also regulated by *AtSPL8* (ZHANG et al., 2007). Such functions are affected by the positive and negative (flowers and roots, respectively) regulation of gibberellic acid (GA) signaling (ZHANG et al., 2007). Gibberellic acid is a plant hormone that regulates many processes (*e.g.* seed germination, rosette leaf expansion, stem and root elongation, floral induction and anther development) being crucial for plant development (FLEET; SUN, 2005). While the *AtSPL8* loss-of-function mutants have a phenotype of seed germination and root growth similar to constitutive GA-response mutants, the *AtSPL8* gain-of-function mutants are similar to GA-deficient mutants (GA-deficiency results in germination failure and shortened roots, and constitutive GA-response results in the opposite) (ZHANG et al., 2007). Moreover, genes involved in GA biosynthesis and signaling are transcriptionally affected by altered *SPL8* expression. The overexpression of *AtSPL8* elevates *GA5* (which encodes a key GA biosynthetic enzyme, the GA 20-oxidase) transcript levels in inflorescences, while lowering them in seedlings (ZHANG et al., 2007).

The most studied SPL genes are the arabidopsis *AtSPL9* and the close related *AtSPL15* (which are mostly functionally redundant). These genes participate in juvenile-to-adult transition in plants (vegetative phase change) and the plastochron (time for leaf initiation) length (SCHWARZ et al., 2008; WANG et al., 2008; USAMI et al., 2009; WU et al., 2009). Overexpression of *AtSPL9* increased plastochron length and increases leaf size (WANG et al., 2008), and the same characteristics can be observed in a gain-of-function mutant of *AtSPL15* (USAMI et al., 2009). Meanwhile, loss-of-function mutants of either *AtSPL9* or *AtSPL15* seem to have minor effects on development. With a stronger phenotype for the double mutant atspl9/15 (SCHWARZ et al., 2008; WANG et al., 2008; USAMI et al., 2009; WU et al., 2009).

Both *AtSPL9* and *AtSPL15* are regulated by the miR156, which overexpression leads to a similar but stronger phenotype of delayed vegetative phase change and shortened plastochron length of the *spl9spl15* double mutant (SCHWARZ et al., 2008; WANG et al., 2008; USAMI et al., 2009; WU et al., 2009). Therefore, other miR156-targeted *SPL/SBP* genes might also have functions in vegetative phase change. However, when *AtSPL9* is overexpressed in the *hyponastic leaves1* (*hyl1*) mutant plants (which displays defective processing of primary miRNAs into mature miRNAs, and, therefore, reduced miR156 levels) the juvenile phase is not detectable, with the primary leaves showing adult characteristics (LI et al., 2012). Moreover, *AtSPL9* and *AtSPL15* negatively participate in axillary meristem initiation in cauline leaf axils through suppression of *LATERAL SUPPRESSOR (LAS) gene* expression, as the reported for their maize and rice orthologous *tasselsheath4* and *OsSPL14*, respectively (XIE; WU; XIONG, 2006; CHUCK et al., 2010; JIAO et al., 2010; TIAN et al., 2014). Additionally, *AtSPL9* has other reported functions not described for *AtSPL15*, like participating in the activation of *TRICHOMELESS* (*TCL1*) in arabidopsis, which affects petal trichome initiation, and reduce the accumulation of anthocyanin in stems (YU et al., 2010; GOU et al., 2011).

The *FRUITFULL* (*FUL*) expression - which is known to participate in fruit and leaf development - seems to be regulated by the *AtSPL10* and *AtSPL11* (GU et al., 1998; SHIKATA et al., 2009). However, the *spl10* and *spl11* loss-of-function mutant plants seems to have no effect on flower or fruit development, unlike *ful* mutants (SHIKATA et al., 2009), which suggests differential regulation of *FUL* in different tissues. Moreover, *AtSPL10* and *AtSPL11* have been linked to other roles, both in early and late-stage cell differentiation on embryogenesis (NODINE; BARTEL, 2010). The *Dicer-like 1* (*dcl1*) mutant, which has decreased miRNA expression and, therefore, increased miRNA-targets levels, displays precocious differentiation of early embryonic cells. But *dcl1* mutants crossed with *atspl10atspl11* double mutants, partially restore the embryogenesis, demonstrating potential roles for these genes in both early and late-stage cell differentiation (NODINE; BARTEL, 2010).

Additionally, arabidopsis *AtSPL10* is also involved in the repression of lateral root growth and branching, and alterations in leaf morphology (YU et al., 2015; GAO et al., 2018). The *AtSPL10* controls these characteristics by directly inducing the *AGAMOUS-LIKE 79* (*AGL79*) gene (GAO et al., 2018). Downregulation of *AtSPL10* (and thereby *AGL19*) produces an increased number of rosette leaves, lateral branches, and root length, whereas upregulation causes narrow leaf shape, a smaller number of leaves and early flowering time (GAO et al., 2018). Other genes regulated by *AtSPL10* are *WRKY TRANSCRIPTION FACTOR 12*

(WRKY12) and *WRKY13*. *AtSPL10* binds to the promoter to positively regulate *WRKY12* and negatively regulate *WRKY13* (MA et al., 2020). Besides, both *WRKY12* and *WRKY13* proteins seem to physically interact with *AtSPL10* to regulate the miR172b. Interestingly, WRKY12-SPL10 interaction facilitates, while WRKY13-SPL10 interaction inhibits *AtSPL10* function of promoting miR172b expression (MA et al., 2020). Thereby, both of these *WRKY* genes control age-mediated flowering in short-day conditions by binding to *AtSPL10* to co-regulate miR172b (MA et al., 2020).

In arabidopsis, the *AtSPL13* gene controls leaf outgrowth after the emergence of the cotyledons during germination by regulating miR172, which regulates *SCHNARCHZAPFEN* (*SNZ*), an *APETALA2*-like gene. Loss of miR156-mediated regulation of *AtSPL13* by inserting silenced mutation in the miRNA binding site delay the emergence of leaf primordia significantly (MARTIN et al., 2010a, 2010b). However, in tomato plants, a recent study demonstrated that *SlSPL13* is related to inflorescence structure determination and lateral branch production (CUI et al., 2020). Suppression of *SlSPL13* levels results in a phenotype of an increased number of inflorescences on branches, however, these inflorescences have reduced number of flowers, reducing yield. The regulation of inflorescence development by *SlSPL13* is through inducing directly the expression of the *SINGLE FLOWER TRUSS* (*SFT*) gene (CUI et al., 2020). The *SFT* is the tomato ortholog of arabidopsis *FLOWERING LOCUS T* (*FT*). This gene was described to regulate the flower morphology, primary flowering time, and sympodial habit (LIFSCHITZ et al., 2006).

Strikingly, Silva et al. (2019) also demonstrated the interplay of *SPL/SBP* genes and the *SFT* gene. Specifically, by the integration of miR156b-targeted *SPL/SBP* genes (*SlSBP3* and *SlSBP15*), GA response, and the miR319-targeted *LANCEOLATE* (*LA*) (SILVA et al., 2019). The PROCERA/DELLA protein (which is a repressor of GA responses) seem to not only promote the *SINGLE FLOWER TRUSS* (*SFT*) gene in leaves, but also promote *SlSBP3*, *SlSBP15*, and *APETALA1* (*AP1*) at the shoot apex (SILVA et al., 2019). PROCERA/DELLA physically binds to *LANCEOLATE* (*LA*) possibly to promote the derepression of flowering-relate genes. In addition, the *LA* gene positively controls GA concentrations (which represses PROCERA/DELLA activity) and is negatively regulated by the miR319. Therefore, by interplaying with PROCERA/DELLA, the miR156-*SlSBP3* and miR156-*SlSBP15* modules, together with miR319-*LA* module, could be orchestrating transition to flowering in tomato by fine-tuning the GA responses (SILVA et al., 2019).

Another interesting *SPL/SBP* gene is the arabidopsis *SPL14* (*AtSPL14*). This gene was described to be important for maintaining plant growth in the presence of a programmed cell

death (PCD)-inducing fungal toxin FB1. Besides, this gene also has roles in development (STONE et al., 2005). Loss-of-function of *AtSPL14* results in elongated petioles, serrated leaf margins, and a quicker transition to the adult phase, accelerating flowering time (STONE et al., 2005). This *SPL/SBP* gene which was described to participate in resistance to biotic stress also seems to be related to the juvenile to adult transition, like other members of this family (*e.g. AtSPL9* and *OsSPL14*) (STONE et al., 2005; ZOU et al., 2018; LIU et al., 2019; YIN et al., 2019).

Notably, several studies have been conducted aiming a better understanding of the roles of *SPL/SBP* family members, mainly due to their important roles in plant development. Moreover, changes in development caused by the expression of these genes can be crucial for plant and organ growth via modulation of hormone signaling and sensibility (*e.g.* GA and SPL8, and cytokinin and SPL9) (ZHANG et al., 2007, 2015). Within the plant hormones that somehow interact with *SPL/SBP* genes, cytokinin is a hormone that this interplay needs to be further characterized by.

2.6 Interaction between *SPL/SBP* family genes and cytokinin, and the impact on plant immune response

Cytokinin (CK) is an important developmental regulator, and is required in many aspects of plant life cycle. In addition, it is known to be important during response to the environmental cues (*e.g.* biotic and abiotic stresses). This hormone is derived from substitutions at the N6 terminal of the adenine molecule aromatic ring or from the tRNA degradation pattern, being classified as isoprenoids or aromatic cytokinins (ARGUESO; FERREIRA; KIEBER, 2009). Isoprenoid cytokinins are considered the predominant type in plants. They are synthesized by the attachment of an isopentenyl group to the ADP or ATP molecule, which are catalyzed by the enzyme isopentenyl transferase (IPT) (KAKIMOTO, 2003). The resulting product, isopentenyladenine riboside (iP), can be converted to the active form by the lonely guy enzyme (LOG) (KURAKAWA et al., 2007). This active form is called cytokinin riboside 5'-monophosphate phosphoribohydrolase and belong to the group of cytochrome-P450 monooxygenase enzymes (KURAKAWA et al., 2007). After the completion of its function or when there is high CK levels, the endogenous cytokinin can be regulated by cytokinin oxidase/dehydrogenase enzymes (CKXs) (HOUBA-HÉRIN et al., 1999). These proteins degrade cytokinin side chains at position N6 back into adenine or adenosine (HOUBA-HÉRIN et al., 1999).

The cytokinin signal transduction in plants involves a phosphate transfer cascade in a two-component system (KAKIMOTO, 2003; ARGUESO; RAINES; KIEBER, 2010). This system is composed by a kinase-type sensor (histidine kinase - HKs), which senses the stimulus from the environment and acts as a receptor (BIANCO; GIUSTINI; SABATINI, 2013). Cytokinin binds to HK, resulting in autophosphorylation and the signal is transduced to response regulators (RRs) (ARGUESO; RAINES; KIEBER, 2010). Such signaling occurs through histidine-containing phosphotransferase proteins (HPs). RRs propagate the signal, often regulating the transcription of target genes (BIANCO; GIUSTINI; SABATINI, 2013). RRs can be grouped into at least two classes depending on the type of plant species (ARGUESO; FERREIRA; KIEBER, 2009; ARGUESO; RAINES; KIEBER, 2010). While type-A RRs act as a negative feedback that regulates the cytokinin signaling pattern, type-B RRs act as transcription factors activating the expression of cytokinin response genes, including type-A RRs and cytokinin response factors (CRFs) (ARGUESO; FERREIRA; KIEBER, 2009; ARGUESO; RAINES; KIEBER, 2010). CK-related responses are important to diverse processes, including stem-cell control, vascular differentiation, chloroplast biogenesis, seed development, growth and branching of the root, shoot and inflorescence, leaf senescence, nutrient balance, and stress tolerance (MÜLLER; SHEEN, 2007). The roles of CK in plant growth and development have been reviewed extensively (MOK; MOK, 2001; SAKAKIBARA, 2006).

In this context of CK hormonal regulation and *SPL/SBP* genes, the overexpression of *TLOG1* (*TOMATO LONELY GUY 1*), a gene that participates in the final stage of the cytokinin biosynthesis pathway (KURAKAWA et al., 2007), induces complete loss of apical dominance and the formation of aerial mini tubers, small and rounded, sessile from their axillary meristems (EVIATAR-RIBAK et al., 2013). Such tubers are morphologically and metabolically homologous to the tubers presented by potatoes. Interestingly, it was also shown that the *SPL/SBP* pathway regulated by miR156 can interact with *TLOG1* by extending the tuber formation potential to distal axillary buds (EVIATAR-RIBAK et al., 2013). While transgenic tomato plants overexpressing miR156 did not produce tubers, when they are crossed with the transgenic that overexpresses *TLOG1* (*p35S*:*TLOG1* / + *p35S*:*MIR156* / +) (double transgenic) they generated small tubers from axillary buds of the primary and secondary shoots. In addition, these plants have purple coloration and a greater number of branches. These phenotypes were more prominent in the double transgenic than in plants only *p35S*:*TLOG1*. Such results demonstrate that repression of miR156-targeted *SPL/SBP*s act keeping the plant more sensitive to cytokinin (EVIATAR-RIBAK et al., 2013).

In addition, potato (Solanum tuberosum) plants with low levels of miR156-targeted *SPL/SBP* genes (overexpressing the miR156) display high levels of *LOG1* transcripts and CK, which led to aerial tuber formation (BHOGALE et al., 2014). Such alterations could be the result of the *StSPL9*-miR172-*StSP6A* pathway been downregulated, probably by the miR156 modulating *StSPL9*. These results suggest the potentially conserved role of this family across species in cytokinin-related pathways (BHOGALE et al., 2014). Moreover, it has been shown in arabidopsis that the increase in the activity of some SPLs regulated by miR156 decreases plant regeneration capacity (ZHANG et al., 2015). This is a consequence of the interaction of *AtSPL9* with the B-type *ARABIDOPSIS RESPONSE REGULATOR2* (*ARR2*), and such interaction acts by attenuating the response to cytokinin by hindering the promotion of *ARR2*-promoted CK-response genes. Thus, a molecular link was established between the miR156-*SPL/SBP* module, the cytokinin-mediated aerial development, and regeneration time (ZHANG et al., 2015).

Another example of a *SPL/SBP* gene interfering in CK-related response is the maize *UNBRANCHED3* (*UB3*) gene (DU et al., 2017), which is known to repress shoot branching. This gene is the orthologue of rice *IPA1* (*OsSPL14*) and arabidopsis *AtSPL9* and *AtSPL15* (CHUCK et al., 2014; MAO et al., 2016). *UB3* seems to be able to bind and regulate the promoters of *LONELY GUY1* (*LOG1*) and type-A response regulators (Type-A RRs), interrupting cytokinin biosynthesis and signaling (DU et al., 2017), with a similar effect of arabidopsis SPL9 in the CK signaling (ZHANG et al., 2015). Moreover, the heterologous overexpression of *UB3* in rice severely reduced tillering and panicle branching as a result of the decrease of CK synthesis and response, and increase of CK degradation by CKX genes (DU et al., 2017). Consistently, in maize, the *ub3* mutant (which have low *UB3* expression) have up-regulated cytokinin biosynthesis-related genes and down-regulated degradation-related genes (DU et al., 2017).

Although these studies have demonstrated an interaction between the *SPL/SBP* pathway and CK during plant development, little is known if such family could be important for CK signaling in response to biotic stresses. A range of pathogens are known to produce hormones such as auxin, gibberellic acid, ethylene, abscisic acid, and cytokinin (CK) as part of their pathogenesis strategy (CHANCLUD; MOREL, 2016; MA; MA, 2016). Some pathogens also have the ability to manipulate the levels and/or signaling of host cytokinin, which can, together with cytokinin produced by the pathogen, induce changes in the host's physiological responses, such as gall formation and hypertrophy, and also favor host colonization by the pathogen

(ROBINETTE; MATTHYSSE, 1990; CHANCLUD; MOREL, 2016; ALBRECHT; ARGUESO, 2017; COSTA et al., 2021).

However, cytokinin may have a different role in inducing or hindering the host's immune response at different concentrations. For instance, treatment with 1 μM of CK induces the Arabidopsis immune system against the bacterium *Pseudomonas syringae* pv. tomato DC3000 (CHOI et al., 2010). Meanwhile, Argueso et al. (2012) showed that low levels (<1 μM) of cytokinin are sufficient to make Arabidopsis susceptible to the biotrophic oomycete *Hyaloperonospora arabidopsidis*. Such complex relation of CK and immune response still need to be further explored and also is needed to elucidate the potential role of *SPL/SBP* genes in the CK-related response or modulation by plant-pathogens.

2.7 *SPL/SBP* relation with biotic stresses and cell death

The *SPL/SBP* family is widely studied in the context of plant development. But recently, some of these genes are been associated with plant defense against biotic stress. The miR156/157-*SPL/SBP* module seems to play an important role in the plant immune system, tailoring the age-related immunity (ZHENG et al., 2019). Many genes that participate in plant development downstream of *SPL/SBP* genes can also participate in plant defense, such as *LEAFY* (YAMAGUCHI et al., 2009) and *SOC1* (LEE; LEE, 2010), which can partake in PAMP Triggered Immunity (PTI) (WINTER et al., 2011) and Salicylic Acid (SA) signaling (WILSON et al., 2017), respectively (Figure 4).

The core hormones in plant defense responses are SA, Jasmonic Acid (JA), and Ethylene (ET) (MA; MA, 2016). While SA responses promote resistance against biotrophic and hemibiotrophic pathogens, JA/ET responses are associated with the mechanism of resistance against necrotrophic pathogens (ROBERT-SEILANIANTZ; GRANT; JONES, 2011; MA; MA, 2016). Usually the SA and JA/ET responses are antagonistic (the activation of one suppresses activation of the other). The other hormones normally participate in pathogen-host interactions via SA or JA/ET responses (ROBERT-SEILANIANTZ et al., 2007; ROBERT-SEILANIANTZ; GRANT; JONES, 2011; MA; MA, 2016). Auxin and cytokinin promote susceptibility in biotrophic pathogens by inducing JA/ET defense responses. On the other hand, GA induce susceptibility in necrotrophic pathogens through the SA resistance mechanism (ROBERT-SEILANIANTZ et al., 2007). Therefore, for its aforementioned relation with SA, GA and CK, the *SPL/SBP* pathway can be a promising source of potential plant resistance genes associated with greater productivity.

For instance, Liu et al. (2019), demonstrated in rice, that the overexpression of the *Ideal Plant Architecture1* gene (*IPA1*/*OsSPL14*) and *OsSPL7* - two target genes of miR156 - provides greater resistance against bacteriosis caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (LIU et al., 2019). However, overexpression of these genes has a negative impact on productivity because of their participation in plant development, affecting plant's fitness (LIU et al., 2019). In order to circumvent the effects of overexpression, only one of the SPL/SPBs were used in further experiments. The transgenic rice plants were generated expressing *IPA1* under the control of the promoter *OsHEN1*, instead of an overexpression to avoid pleiotropic phenotypes. The *OsHEN1* promoter was chosen because it has a Tal9a binding site, which is a Transcription activator-like effector (TALE) that is secreted by the pathogen and promote the expression of some genes. Therefore, *OsHEN1* is a *Xanthomonas*-infection responsive gene (LIU et al., 2019). These transgenic plants showed resistance to infection and substantially increased productivity. Such phenotype is related to the activity of the *OsHEN1* promoter controlling *IPA1*, which does not maintain its high expression in the absence of the pathogen, which in turn does not affect the plant's fitness. Thus, the *IPA1* was identified as a new regulator between growth, productivity, and defense (LIU et al., 2019).

In addition, it has been shown that transgenic plants with high levels of miR156-targeted *SPL/SBP* genes, by the reduced miR156 activity via target mimicry (MIM), can be resistant to biotic stressors (GE et al., 2018; ZHANG et al., 2020a). MIM consists of altering a nucleotide that interrupts pairing by an incompatible loop at the expected miRNA cleavage site. Such alteration results not in the expected cleavage by the microRNA (FRANCO-ZORRILLA et al., 2007). Thereby, MIM156 plants have reduced miR156 transcripts available to regulate their targets, increasing miR156-targeted *SPL/SBP* genes levels. Rice plants with increased *SPL/SBP* levels by MIM156 approach exhibited resistance to infestation by rice brown leafhopper, *Nilaparvata lugens* (GE et al., 2018). The expression of *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*), *MPK6*, and *WRKY70* (all related to defense or JA signalling) are altered in the MIM156 plants. The bioactive jasmonoyl-isoleucine concentration and the expression of JA-biosynthesis genes are significantly reduced in MIM156 plants, which confers resistance to *N. lugens* (GE et al., 2018). After restoring the level of JA by exogenous application, there was an increase in the number of brown leafhoppers feeding on plants MIM156, reducing its resistance. These data suggests that some miR156-targeted *SPL/SBP* gene is important to positively regulate the resistance to this pest, by reducing the level of JA (Figure 4) (GE et al., 2018).

Furthermore, the expression of a target mimic of miR156fhl-3p (MIM156-3p) enhances the resistance to rice blast disease (caused by *Magnaporthe oryzae*) not affecting yield by promoting the derepression of *IPA1* (*OsSPL14*) (ZHANG et al., 2020). Such derepression lead to higher *IPA1* accumulation, which also increases *WRKY45* levels (WRKY45 induces resistance to blast disease). Consistently, IPA1 repression by miR156 overexpression leads to susceptibility to *M. oryzae* by reducing levels of *WRKY45* (ZHANG et al., 2020). *IPA1* increases productivity by increasing grains per panicle while assisting in plant immunity by inducing *WRKY45* when phosphorylated. The *WRKY45* is important to induce immune response-related genes (*e.g. PR1a* and *PR1b*) (Figure 3) (WANG et al., 2018; ZHANG et al., 2020a). This is another example of the importance of miR156/*IPA1* (*OsSPL14*) in rice immune response, raising the question regarding the function of *IPA1* orthologous *SPL/SBP* genes in other crops (*e.g.* tomato).

Interestingly, two non-miR156-targeted *SPL/SBP* genes of tomato, the *SlSBP8b*, and the *SlSBP12a*, participate in spontaneous cell death (Figure 4) (KESSENS; SORENSEN; KABBAGE, 2018). The overexpression of these genes increases the ROS accumulation and favors the growth of the necrotrophic pathogen *Alternaria alternata*. This new pro-death role for *SlSBP8b* and *SlSBP12a* is an interesting example of an alternative role of SBP genes that should be further explored (KESSENS; SORENSEN; KABBAGE, 2018).

An *SPL/SBP* gene that so far has not been characterized to participate in plant development is the *AtSPL6*. However, this gene is directly related to the plant's immunity against infection by *Pseudomonas syringae* that employs effector avrRps4 (*Pst*::avrRps4) (which triggers RPS4-mediated resistance) (PADMANABHAN et al., 2013). Pathogenicity tests with transgenic plants overexpressing miR156 (low level of all miR156-targeted *SPL/SBP*, including *AtSPL6*), together with *AtSPL6*-RNAi plants (low level of only the *AtSPL6*), have shown that both transgenics are more susceptible to the pathogen (PADMANABHAN et al., 2013). Transcriptome analysis and in silico tests identified more than 300 genes that were responsive during RPS4-mediated resistance (DISEASE RESISTANCE PROTEIN RESISTANT TO P. SYRINGAE 4). Some of those genes that are shown to be responsive, such as *WALL ASSOCIATED KINASE 3* (*WAK3*), *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*), and *NUDIX HYDROLASE 6* (*NUDT6*), among others, are less expressed in *AtSPL6*-RNAi plants. Therefore, *AtSPL6* could positively regulate a subset of defense genes important to Effector-Triggered Immunity (ETI) (Figure 4) (PADMANABHAN et al., 2013).

In contrast, the arabidopsis *AtSPL9* gene has extensively been studied and related to several aspects of plant development. However, it also contributes to the resistance against *Pseudomonas syringae* regulating defense genes and reactive oxygen species (ROS) accumulation (YIN et al., 2019). Such contribution occurs by the miR172, which is promoted by *AtSPL9, AtSPL10*, and *AtSPL15* (WU et al., 2009). This miRNA represses *TARGET OF EAT 1* (*TOE1)* and *TOE2*, which represses the *FLAGELLIN-SENSITIVE 2* (*FLS2)* gene (Figure 3). Thus, the decrease of miR156 in seedlings leads to an increase of its target *AtSPL9* transcripts, which then induces miR172 levels, eliminating TOE1/2 transcripts by 65% and ultimately increasing FLS2 transcripts that can participate in immune response (ZOU et al., 2018). Moreover, *AtSPL9* hinders JA defense responses by stabilizing the JASMONATE-ZIM-DOMAIN PROTEIN 3 (JAZ3) from the ubiquitin-proteasome system (UPS)-mediated degradation in response to herbivory (Figure 4) (GAQUEREL; STITZ, 2017; MAO et al., 2017).

As previously mentioned, another reported role of *AtSPL14* is the participation in the response to the fungal toxin fumonisin B1 (FB1). Fumonisin B1 (FB1) is the most abundant of a series of sphingosine analog mycotoxins and is produced by the fungus *Fusarium moniliforme* (ABBAS et al., 2000). FB1 exhibits a variety of biological activities including phytotoxicity, by inducing cell death, which leads to necrosis and also inhibits growth (ABBAS et al., 2000). Mutations in the *AtSPL14* gene result in plants that fail to respond to FB1 (STONE et al., 2005). Arabidopsis plants germinated in FB1-containing agar media hinder plant development, severely slowing its growth, probably by the activation of PCD on the seedlings. However, the growth of *atspl14* mutants (also named *FB1-resistant6* or *fbr6*) is not affected by FB1 (STONE et al., 2005).

The first example of a pathogen modulating the miR156/SPLs module is in the interaction between arabidopsis and the Aster yellows phytoplasma witches' broom strain (AY-WB) (CHANG et al., 2018; HUANG et al., 2021). Phytoplasmas usually are carried to their host by sap-feeding insects (WEINTRAUB; BEANLAND, 2006). These bacterial plant-pathogens infect most plant species and usually induce plant architecture alterations, such as excessive branching (witches' brooms) and/or inversion of reproductive tissue into vegetative leaf (phyllody) (HOSHI et al., 2009; MACLEAN et al., 2011; SUGIO et al., 2011; MAEJIMA et al., 2014). They usually colonize the cytoplasm of vascular tissue cells and use host's nutrients as nourishment (DOI et al., 1967).

SPL/SBP genes were described to be modulated by the AY-WB SAP11 and SAP05 effectors (Figure 4) (CHANG et al., 2018; HUANG et al., 2021). Arabidopsis plants expressing $SAP11_{AYWB}$ have a phenotype of late flowering and repressed flowering-related genes. These alterations were caused because SAP11 seem to downregulate some SBP genes (*SPL3/SPL4/SPL5*). Such downregulation observed in *p35S*::$SAP11_{AYWB}$ transgenic lines, could be a result of $SAP11_{AYWB}$-mediated destabilization of *TCP4* through *SOC1*. This effector family, specially SAP11, are core virulence factors responsible for the witches' broom symptoms caused by phytoplasmas (CHANG et al., 2018). Another phytoplasma effector which affects *SPL/SBP* functions in plants are the SAP05 (Figure 4). This effector acts by inducing the degradation of several *SPL/SBP* proteins by hijacking the plant ubiquitin receptor RPN10 with or without substrate lysine for ubiquitination, which decreases *SPL/SBP* protein levels, affecting their target expression (HUANG et al., 2021). The modulation of these genes could be resulting in the altered plant architecture caused by these pathogens and similar strategies possibly are applied by other witches' broom-like symptom-inducing pathogens (*e.g. M. perniciosa*).

As mentioned above, some *SPL/SBP* genes have been shown to positively participate in some plant-pathogen interactions (Figure 4), but mechanisms still need to be elucidated.

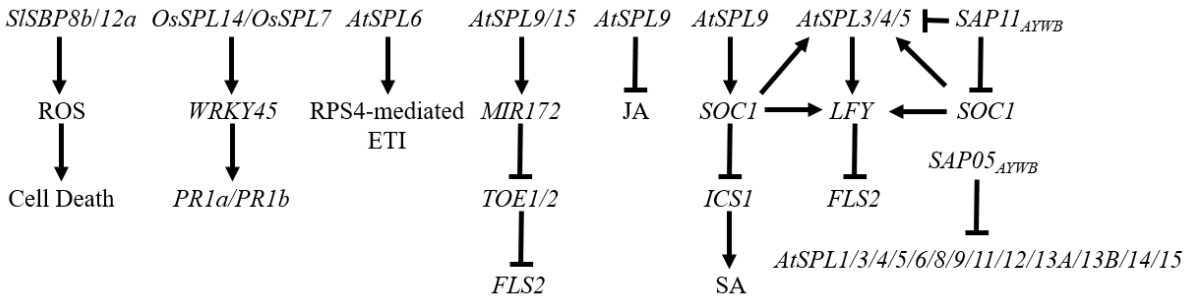

Figure 4 - Summary of *SPL/SBP* genes participation in plant-pathogen interactions and cell death

In summary, *SPL/SBP* genes, that are main players of plant developmental processes, seem to be also important as response to some pathogens. However, overexpression of these genes commonly results in reduced production, mainly for affecting plant's fitness (LIU et al., 2019). A viable strategy to circumvent such problem would be these *SPL/SBP* genes driven by specific promoters that are responsive to the biotic stressor of interest, as previously and successfully done (LIU et al., 2019). But to do that, pathogen-responsive promoters need to be characterized and, therefore, new studies in this context are needed.

Another interesting topic that needs to be investigated is the occurrence of the repression of this family by pathogen proteins, like phytoplasma SAP effectors (CHANG et al., 2018; HUANG et al., 2021). Altogether, these studies raise the hypothesis of the possible participation of other *SPL/SBP* genes in the interaction with other pathogens and also if the repression this family could be by other pathogen effectors. Thus, this is a field that needs to be further explored.

Altogether, our main goal was to investigate the role of miR156-targeted *SPL/SBP-box* genes of tomato during the interaction between *M. perniciosa-host.* Specifically, we aimed to check rather any *SPL/SBP-box* genes were participating in CK-mediated symptom induction by *M. perniciosa* while searching for potential sources of resistance.

3 MATERIAL AND METHODS

3.1 *M. perniciosa* grown and spore production

Basidiocarps of *M. perniciosa* of the biotype-S were obtained from dried brooms of naturally infected lobeira (*S. lycocarpum*) branches collected on site (21°11′9.4″S, 44°19′15.8″W) in Tiradentes, MG, Brazil (DEGANELLO et al., 2014). The dry brooms were exposed to an alternating cycle of wet and dry (12 hours each) to induce the formation of basidiocarps. The basidiocarps were harvested regularly, and had their stipes removed and were fixed by the cap with silicone gel in the lid of Petri dishes to release basidiospores over storage buffer (16% glycerol; 0.01 MES, pH 6.1; 0.01% Tween 20). After 16 hours, the spore suspension was collected and stored in cryogenic tubes in liquid nitrogen. The basidiospore concentration was estimated in a Neubauer chamber and an Axiovert 35 microscope (Zeiss, Germany) and diluted and standardized for use in the experiments.

3.2 Plant material

Micro-Tom and transgenic lines seeds were germinated in 250 mL pots and M82 and crossed lines were germinated in 2,5 L pots. Seeds from all genotypes were germinated in a 1:1 mixture of substrate: expanded vermiculite (CARVALHO et al., 2011). Transgenic lines overexpressing *AtMIR156* (*p35S::AthMIR156b*) (156-OE) (SILVA et al., 2014a) and versions resistant to regulation by the same miRNA of the *SlSBP3* (rSBP3-OE) and *SlSBP15* (rSBP15-OE) genes (SILVA et al., 2019) were kindly provided by professor Fábio T. S. Nogueira from ESALQ / USP. Plants were grown under controlled conditions in a growth chamber set at 26ºC, 14 h photoperiod and 80% humidity.

3.3 Inoculation and evaluation of response against *M. perniciosa*

The shoot apex and first two axillary buds of tomato seedlings of all genotypes were inoculated each with 25 µL 10^6 of the basidiospore suspension in 0.3% agar-water (w/v) at 15 days after sowing. Afterwards, all plants were kept in a humid chamber for 48 hours. The control plants were treated with 0.3% agar-water. The inoculations were completely randomized and the treatments consisted of 40 inoculated plants and 40 non-inoculated control plants of each genotype. Symptom's development was assessed at 5, 10, 20, 30, 40, 50, 70 and

90 days after inoculation (dai). The stem diameter was measured between the 1st and 2nd leaves (the site of inoculation) with a digital pachymeter. The biomass measurement (dry weight) was taken at 30 dai. For the fruit evaluations, we collected fruits from inoculated and control plants at 45 dai, and we assessed the °brix, mass, number of seeds and number of locule per fruit.

3.4 Statistical analyses

All data were analyzed in the R Studio software with ExpDes.pt package (FERREIRA; CAVALCANTI; NOGUEIRA, 2014). The experiment of inoculation of MT and 156-OE plants was conducted in a completely randomized design in a 2 x 2 factorial scheme (2 genotypes x 2 inoculation conditions; n = 40), the data were subjected to two-way ANOVA, and the means compared by the Tukey test at 5% significance. The SPL screening experiment was conducted in a completely randomized design in a 5 x 2 factorial scheme (5 genotypes x 2 inoculation conditions; n = 40), the data were subjected to two-way ANOVA, and the means compared by the Tukey test at 5% significance. The M82 and hybrids inoculation experiment was conducted in a completely randomized design in a 3 x 2 factorial scheme (3 genotypes x 2 inoculation conditions; n = 40), the data were subjected to two-way ANOVA, and the means compared by the Tukey test at 5% significance.

3.5 RNA extraction and Real-Time Quantitative PCR

Stem tissue and apex meristem of inoculated and control plants were collected at 6, 12, 24 and 48 hours after inoculation (HAI), and a section of the stem, between the first and the second leaves, once we inoculated the first and second auxiliary buds, of inoculated and control plants were collected at 10, 20, 45 and 70 dai. After, total RNA was extracted using Trizol reagent (Invitrogen), and treated with DNase I (Invitrogen), according to the manufacturer's instructions. DNase I-treated RNA (2.0 lg) was reverse-transcribed to generate first-strand cDNA using superscript III (Invitrogen) according to the manufacturer's instructions. SYBR Green PCR was performed using a Qiagen real-time PCR system. Briefly, 1µl of 1:10 v/v cDNA dilutions were added to 12.5µl of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 3 pmol of each primer, and ddH2O to a final volume of 25 µl. The reactions were amplified for 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The tomato *ACTIN IV* gene (Solyc04g011500.2.1) was used as an internal control (GUTIERREZ et al., 2008; ANWAR et al., 2019). PCR products for each primer set

were subjected to melt-curve analysis, confirming the presence of only one peak on thermal dissociation generated by the thermal denaturing protocol and also the primer amplification efficiency was assessed to each primer designed in this work by using the Real-time PCR Miner program (ZHAO; FERNALD, 2005). Two technical replicates were analyzed for each of five biological samples, together with template-free reactions as negative controls. The threshold cycle (CT) was determined automatically by the instrument, and fold changes for each gene were calculated using the equation $2^{-\Delta\Delta CT}$ (LIVAK; SCHMITTGEN, 2001). Primer sequences are listed below in Table 3.

3.6 Stem-loop pulsed RT-qPCR

To detect the expression pattern of small mature RNAs (21nt) in plants the pulsed stem-loop methodology was used (VARKONYI-GASIC et al., 2007). Along with the specific primer for the small mature RNA (Table 4), OligodT was also added for further normalization of the amount of cDNA used in each RT-qPCR reaction. The RT-qPCR methodology was the same as described above.

3.7 Pathogen quantification

For relative *M. perniciosa* genomic DNA analysis, four samples of minced symptomatic stem (delimited between the first and second leaves) of each inoculated genotype were used for genomic DNA (gDNA) extraction, using the CTAB method followed by chloroform extraction and isopropanol precipitation (DOYLE; DOYLE, 1990). The isolated DNAs were quantified in 3.0 Qubit Fluorometric Quantitation with the Broad-range DNA quantification kit, and diluted to a concentration of 5 ng μl^{-1}. Afterwards, gDNA was quantified by qPCR on a qPCR cycler (Qiagen Rotor-Gene Q) using SYBR Green (Invitrogen, Thermo Fischer Scientific) using species-specific reference gene primers (Table 3). Relative DNA content was calculated using equation $2^{-\Delta\Delta CT}$ (LIVAK; SCHMITTGEN, 2001), and MT inoculated samples were used as reference (set to 1.0).

Table 3 - Primer sequences used in this work for RT-qPCR

Gene name	Primer sequences	Average Primer Efficiency (Miner)	ID	From
SlACT4	TGGTCGTACCACCGGTATTGTG AATGGCATGTGGAAGGGCATAC	89,53%	Solyc04g011500	ANWAR et al., 2019
SlChi-II	GGGAGTGCAATAGGTGTGAATC GTTGTGCTGTCATCCAGAACC	85,63%	Solyc02g061770	DEGANELLO et al., 2014
SlCKX2	TGGTCCAAAATGGGATGTTT AGGAGCAAGCATGGCTAAAG	82,12%	Solyc01g088160	COSTA, 2017
SlEIX1	TCTCCCACTTCTCTAGACTTGTCAC CAGCTAGACTTGTCATGTTCATCC	89,90%	Solyc07g008620	DEGANELLO et al., 2014
SlERF1a	ATTGGAGTTAGAAAGAGGCCAT CTCATTGATAATGCGGCTTG	89,21%	Solyc05g051200	JIA et al., 2013
SlIPT3	TTTCGCGGTGAAAAACTTCT TGCAAGAAGGAAGTTGACGA	87,19%	Solyc01g080150	COSTA, 2017
TLOG1	TGGCAAAGAATTGGTGTCAA GCAACAGCCTTCACTTCTCC	82,83%	Solyc10g084150	COSTA, 2017
SlLOG5	CATGTTGCTCCCCATGAAA TGGAGACTGCTCCTTTGGAT	83,70%	Solyc08g062820	COSTA, 2017
SlNPR1	GGGAAAGATAGCAGCACG GTCCACACAAACACACACATC	87,24%	Solyc07g040690	EL-OIRDI et al., 2011
SlPerox	GTGCTCACACTATTGGAGTCTCTCT TTCGCTGTCTAGAGATGGATCTTG	87,62%	Solyc05g046010	DEGANELLO et al., 2014
SlPR1a	TATACTCAAGTAGTCTGGCGCAAC CCTACAGGATCGTAGTTGCAAGA	88,40%	Solyc09g007010	DEGANELLO et al., 2014
SlPR1b	TATACTCAAGTAGTCTGGCGCAAC GCCTACAGGATCATAGTTGCAAGA	87,53%	Solyc00g174340	DEGANELLO et al., 2014
SlPTi5	GAGAGTATGGCTAGGTACGTTCG TAAGTAGTGCCTTAGCACCTCGC	89,25%	Solyc02g077370	DEGANELLO et al., 2014
SlRbohA	GAGAGTAGGATTCAGCGGT GCCTCTTTTCGAGCTTGCT	86,43%	Solyc01g099620	LI et al., 2015
SlRbohB	AGGGAATGATAGAGCGTCG CATCGTCATTGGACTTGGC	85,07%	Solyc03g117980	LI et al., 2015
SlRR1	TGAACAAGAGAAAGTTGCCAG AGAGGTCAAGTGATGAGGGTG	86,30%	Solyc05g006420	HE et al., 2016
SlRR9	GCATGGTATCGCAAAGGC GCATCCCACAAACAGCAC	83,57%	Solyc05g054390	HE et al., 2016
SlSBP15	GGTTCAGCTACCAGGACCAG TGTGAACTTGGCTGTTGACC	88,20%	Solyc10g078700	SILVA et al., 2014a
MpACT	CCCTTCTATCGTCGGTCGT AGGATACCACGCTTGGATTG	85,86%	MP12536	TEIXEIRA et al., 2014

Table 4 - Primer sequences used in this work for Stem-loop method

Primer name	Sequence	ID	From
mature miR156	CCTGAGTGACAGAAGAGAGTG	MIMAT0000167	ZANCA et al., 2010
RT 156	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTGCTCT	MIMAT0000167	ZANCA et al., 2010
mature miR157	GTGCTCTCTATCTTCTGTCAA	MIMAT0000172	ZHANG et al., 2011
RT 157	CCTTCACCTCTTTCGTAAAAATATTTAAATCT	MIMAT0000172	ZHANG et al., 2011
Reverse universal	GTGCAGGGTCCGAGG	-	SILVA et al., 2014

3.8 RNA-sequencing analysis

For the RNA-seq data, we used our lab RNA-seq data (COSTA, 2017; COSTA et al., 2021). This data was generated from MT plants inoculated at the first and second axillary shoots compared with non-inoculated controls collected at different times after inoculation. The section of the stems delimitated from inoculated sites described above was collected, total RNA was extracted, libraries were prepared and sequenced using a HiSeq 4000 sequencer (Illumina). Sequencing data quality was cleaned using Trimmomatic (BOLGER; LOHSE; USADEL, 2014). The cleaned reads were mapped and counted using tomato genome ITAG3.1 (SATO et al., 2012) as reference with the program Salmon (PATRO et al., 2017). Quantified transcripts data in TPM (Transcripts Per Kilobase Million) was exported to R and heatmaps were produced using the pheatmap R package (KOLDE, 2019).

4 RESULTS AND DISCUSSION

4.1 MT infected by *M. perniciosa* and transgenic plants overexpressing the miR156 presents similar patterns of axillary outgrowth and fruit morphology

Our group had previously characterized the infection of MT by an isolate of the S-biotype of *M. perniciosa*. Besides the stem swelling between the first and second leaf (inoculation sites), we described that MT inoculated with *M. perniciosa* presented excessive axillary shoot outgrowth (green broom-like branch) among other described symptoms in response to inoculation (*e.g.* leaf curling and chlorosis, Figure 2). In addition, we have shown a change in the fruit morphology due to a substantial increase in the locule number in fruits of MT plants inoculated with S-biotype basidiospores (DEGANELLO et al., 2014; COSTA et al., 2021). Curiously, the overexpression of *Arabidopsis thaliana*'s microRNA156 (*AtMIR156b*) in MT (156-OE) induces vigorous development of axillary shoots, and interferes with fruit development by promoting a higher and variable number of locule in fruits (ZHANG et al., 2011; SILVA et al., 2014a). In this work, we observed that these phenotypes are similar to MT infected by *M. perniciosa* (Figure 5) (DEGANELLO et al., 2014; COSTA et al., 2021).

The microRNA156 (miR156) targets most members of the plant-specific transcription factors SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL or SBP-box) family (SCHWAB et al., 2005; SALINAS et al., 2012). Members of this family were described to participate in diverse aspects of plant development (*e.g.* leaf formation, branching, and transition to reproductive stage) (SCHWARZ et al., 2008; WANG et al., 2008; USAMI et al., 2009; WU et al., 2009; SILVA et al., 2019; CUI et al., 2020). Moreover, some *SPL/SBP-box* genes have been related to abiotic stresses and plant-pathogen interactions (*e.g.* salt stress, bacterial attack, and fungi attack) (WU et al., 2009; CUI et al., 2014; CHANG et al., 2018; LIU et al., 2019; YIN et al., 2019; ZHANG et al., 2020). Recently, a bacterial pathogen (*Phytoplasma spp.*) that causes a type of witches' broom disease in arabidopsis was described to produce two SAP effector proteins (SAP5 and 11). These proteins act repressing *SPL/SBP-box* gene expression (SAP5) and promoting SPL proteins-degradation (SAP11) as part of its infection strategy (CHANG et al., 2018; HUANG et al., 2021). Curiously, the symptoms caused by this pathogen resemble symptoms caused in MT by *M. perniciosa* (*e.g.* uncontrolled branching) (Figure 2) (CHANG et al., 2018; HUANG et al., 2021), and those resemble 156-OE plants phenotype.

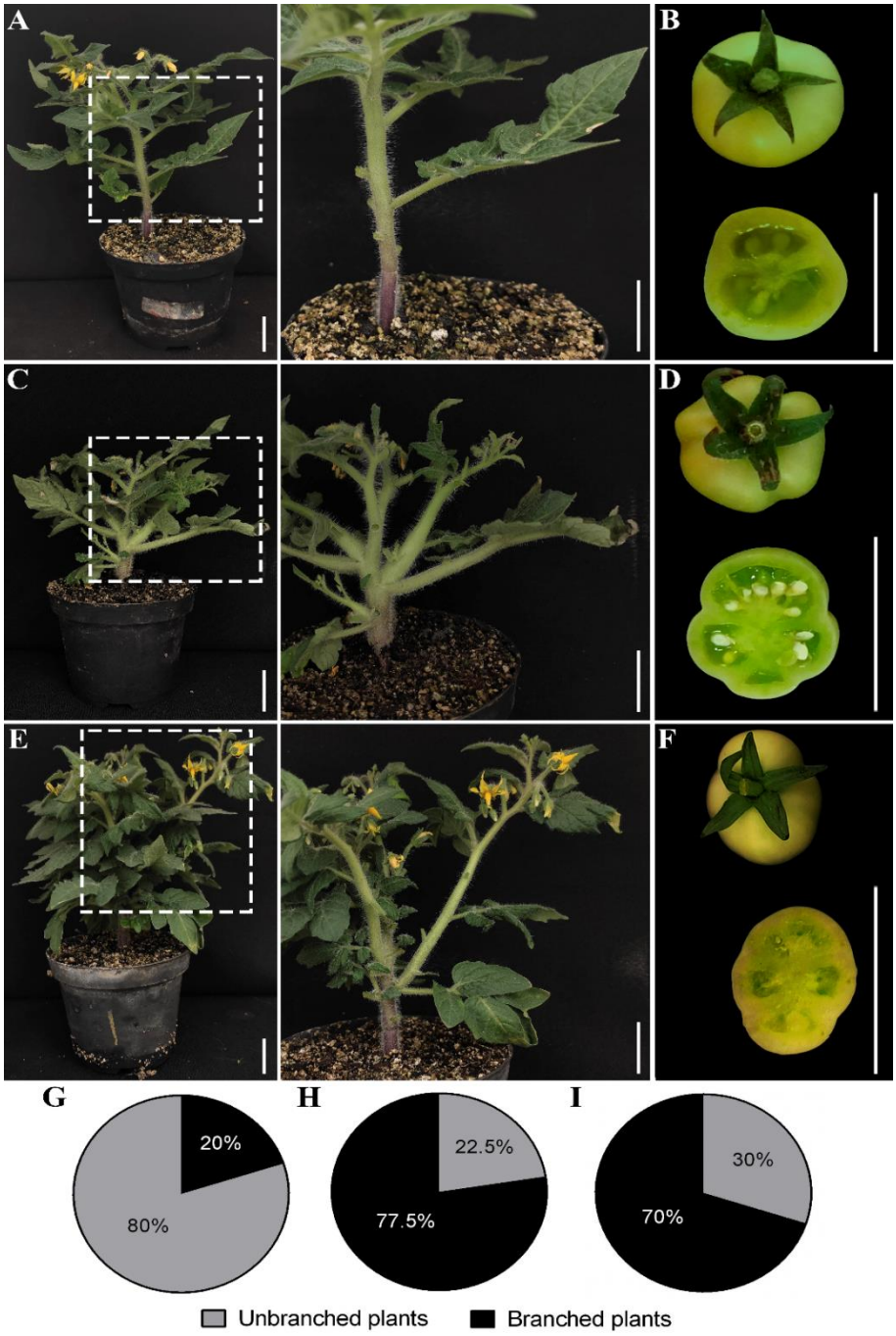

Figure 5 - Axillary growth and increased fruit locule number symptoms induced by *M. perniciosa* S-biotype in MT plants mimic 156-OE plants phenotype (A) MT non-inoculated plants at 20 days after inoculation (dai) (equivalent to 35 days after germination). (B) Fruits from MT non-inoculated plants. (C) MT inoculated plant at 20 dai. (D) Fruits from MT inoculated plants. (E) 156-OE non-inoculated plants at 20 dai. (F) Fruits from 156-OE non-inoculated plants. (G) Percentage of branched and unbranched of MT non-inoculated plants at 20 dai (H) Percentage of branched and unbranched of MT inoculated plants at 20 dai (I) Percentage of branched and unbranched of 156-OE non-inoculated plants at 20 dai. Scale bar = 4cm (A, C, and E) or 2cm (B, D, F, and zoomed sections of A, C, and E).

Based in our observations, we decided to inoculate the apices and the first two axillary buds of *T. cacao* with an isolate of the C-biotype of *M. perniciosa*. At 20 days after infection (dai), we observed that the inoculated axillary shoots also started to grow in response to the infection (Figure 6). Thus, like the S-biotype in tomato, the C-biotype induces axillary shoot

outgrowth in cacao to form “green brooms” (Figures 5, 6) (EVANS, 1980; DEGANELLO et al., 2014, COSTA et al., 2021). Since, tomato 156-OE plants also show the axillary shoot outgrowth and fruit development, similar to what is typically observed in MT plants infected by S-biotype *M. perniciosa* strain (Figure 5) (DEGANELLO et al., 2014; COSTA et al., 2021), we hypothesized that miR156-targeted *SPL/SBP-box* genes could potentially play a role in symptom development by *M. perniciosa* in hosts.

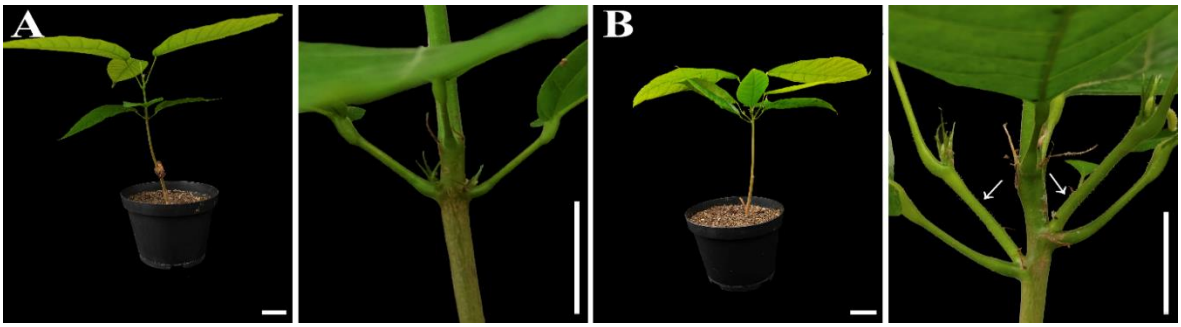

Figure 6 - *M. perniciosa* C-biotype spores induces axillary growth in *T. cacao* (A) *T. cacao* non-inoculated plant and zoomed inoculation site at 20 dai. (B) *T. cacao* inoculated with C-biotype spores’ plant and zoomed inoculation site at 20 dai. Arrows point to *M. perniciosa*’s infection-induced branching. Scale bar = 4cm and 2cm (zoomed sections).

4.2 *SPL/SBP* genes are differentially expressed in response to *M. perniciosa* infection independently of miR156/157 induction

In tomato, the *SPL/SBP* family is composed of 15 members, organized in eight groups based on orthologous relationships of *SPL/SBP-box* genes-domain with other plants (*e.g.* Arabidopsis) (SALINAS et al., 2012). From the 15 members, 10 *SlSBP* genes carry the miR156- and miR157-regulation sites. The miR157 shares 18 nucleotides with the miR156, (SALINAS et al., 2012). Besides, their similarities include an extra 5’ uracil and two internal nucleotide substitutions in at positions 12 and 15, which discriminate miR156 and miR157 sequences in tomato and Arabidopsis. However, they both can modulate the expression of some members of the *SPL/SBP* family (MOXON et al., 2008; SILVA et al., 2014a). Some *SPL/SBP* members are known to control various aspects of plants development (*e.g.* branching, fruit), and are important to response to biotic and abiotic response (JIAO et al., 2010; PADMANABHAN et al., 2013; CUI et al., 2014; SILVA et al., 2014a; DU et al., 2017; LIU et al., 2019; YIN et al., 2019).

To initially investigate our hypothesis, we examined the expression profile of *SPL/SBP-box* genes in our previous mRNA-seq data from MT inoculated in comparison with non-inoculated (COSTA et al., 2021). We found that all *SlSBP* genes are differentially

expressed at different points after infection (Figure 7). The infection of MT by *M. perniciosa* caused the repression of 14 *SlSBP* genes (only *SBP8b* and *SBP13* was not repressed) at 12 hours after infection (hai) (Figure 7a). Meanwhile, at 24 hai, 12 *SlSBP* genes were found to be repressed in inoculated samples (*CNR*, *SlSBP2*, *3*, *6a*, *6b*, *6c*, *7*, *10*, *12a*, *12b*, *15*, and a putative *SBP*), *SlSBP4* and *SlSBP13* levels did not change by the inoculation, and *SBP8a* seem to be mildly induced (Figure 7a). In addition, 14 *SlSBP* genes were mildly repressed by the pathogen at 48 hai, in which only *SlSBP2* and *SlSBP6b* levels did not change (Figure 7a). After 10 dai, the *SlSBP3*, *4*, *6a*, *6c*, *12a*, *12b*, and *15* are still downregulated in inoculated samples, while *SlSBP2*, *6b*, *8a* and *8b* did not change by the inoculation, the putative *SBP* gene was found to be induced, and *CNR*, *SlSBP7*, *10*, and *13* was mildly repressed (Figure 7b). These changes in *SlSBP* gene expression as result of the *M. perniciosa* infection in tomato supports our initial hypothesis that this pathogen could be repressing *SPL/SBP-box* genes. More specifically, the pathogen could be inducing the repression of *SlSBP*s to promote the symptoms of increased branching and perhaps the changes observed in fruits, which are both similar to 156-OE phenotype.

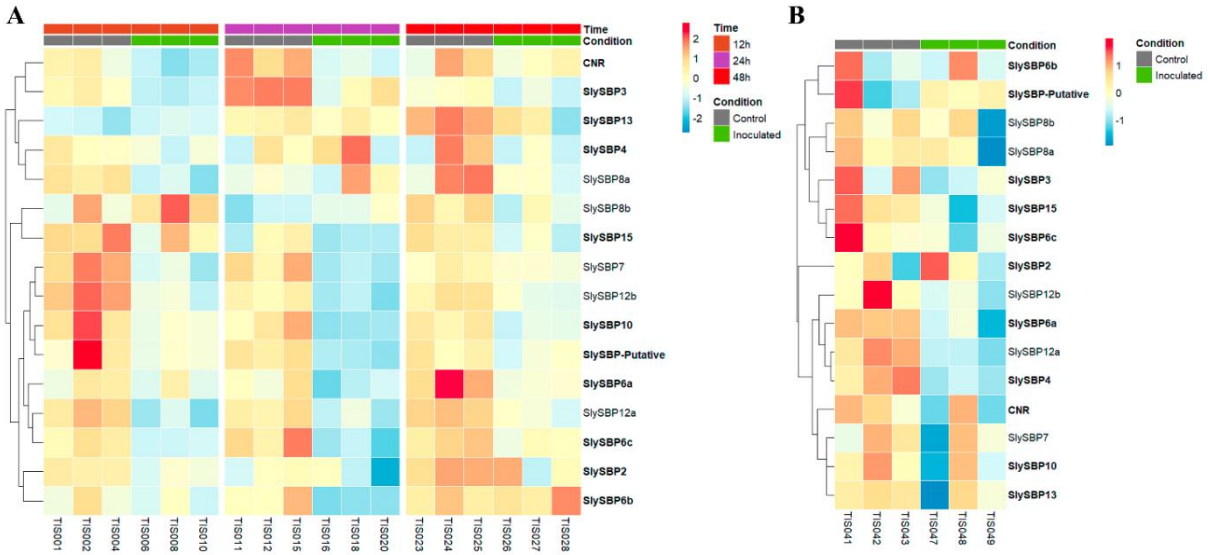

Figure 7 - Expression profile of *SPL/SBP* genes in response to *M. perniciosa* infection (A) mRNA-seq expression profile of MT *SPL/SBP* genes at 12, 24 and 48 hours after infection. (B) mRNA-seq expression profile of MT *SPL/SBP* genes at 10 days after infection. The scale bar represents normalized transcript per million (TPM) (A, B). miR156-targeted SPL/SBP-box genes are highlighted in bold letters (A, B). n = 3.

Since *SPL/SBP* genes are conserved among land plants (ZHANG; LING; YI, 2015), we identified all cacao *SPL/SBP* orthologs (*TcSPL*) (Figure 8a) and predicted all miR156-targeted *TcSPLs* (Figure 8b). After, we accessed public mRNA-seq data from *T. cacao* control and inoculated plants at 30 dai (TEIXEIRA et al., 2014) and evaluated the expression profile of

TcSPL (Figure 8c). We found that several *SPL/SBP* family members are also been differentially expressed in response to inoculation by the C-biotype of *M. perniciosa* (Figure 8c). The *TcSPL1.t2*, *2*, *3*, *3.t2*, *4*, *8*, *9*, and *14* were repressed by the pathogen (Figure 8c). Meanwhile, *TcSPL6*, *6.t2*, *7*, *10*, *12*, and *TcTGA1* were induced, and *TcSPL12.t2*, *13*, and *16* did not seem to be changed by the inoculation (Figure 8c). Such findings reinforce the idea that the repression of this gene family could be important for this pathogenesis, possibly in relation to the formation of new symptomatic branches (Green brooms) that are characteristic to this disease.

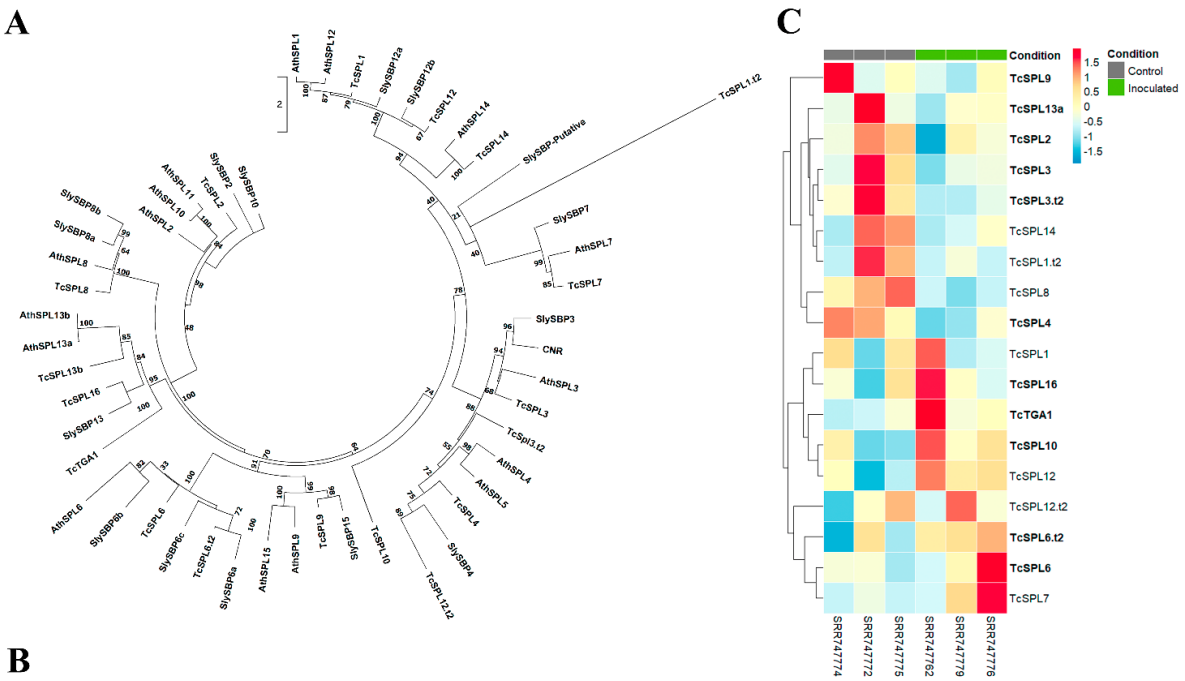

Figure 8 - *Theobroma cacao SPL/SBP-box* genes expression profile in response to *M. perniciosa* infection (A) Phylogenetic analysis of *AtSPL*, *SlSBP*, and *TcSPL* proteins. We used the conserved SBP domain sequences encoded by arabidopsis (*Arabidopsis thaliana*), cacao (*Theobroma cacao*), and tomato (*Solanum lycopersicum*). SBP-box genes were aligned using MUSCLE. The phylogram was constructed using the neighbor-joining algorithm in IQ-TREE 2 (Minh et al., 2020). Bootstrap values are indicated. Bar indicates phylogenetic distance. (B) miR156 binding-sites for *TcSPL*s were predicted using the psRNATarget software (DAI; ZHUANG; ZHAO, 2018). (C) mRNA-seq expression profile of *T. cacao SPL/SBP* genes at 30 days after infection by C-biotype *M. perniciosa* spores (disponible at: http://bioinfo08.ibi.unicamp.br/wbdatlas/, TEIXEIRA et al., 2014). The scale bar represents normalized reads per kilobase of transcript per million mapped reads (RPKM). miR156-targeted *SPL/SBP-box genes* are highlighted in bold letters. n = 3.

Together, these results support our initial hypothesis by showing that the infection by *M. perniciosa* promotes a downregulation of *SPL/SBP* genes. Such repression is also induced by other witches' broom disease causing pathogens, like Aster yellows phytoplasma witches' broom strain (CHANG et al., 2018; HUANG et al., 2021). The repression of this gene family could possibly be, as previously mentioned, favoring the pathogen growth in the host plant and/or be responsible for some of the disease symptoms (*e.g.* increased branching), since some arabidopsis orthologs of *SlSBP* genes and some *SlSBP* genes were described to participate in these pathways (SCHWARZ et al., 2008; WANG et al., 2008; USAMI et al., 2009; WU et al., 2009; SILVA et al., 2019; CUI et al., 2020). We do not dismiss the possibility that other *SlSBP*s non-targeted by microRNAs could potentially be relevant to this pathosystem. However, the repression of miR156-targeted *SPL/SBP* could explain the similarities between MT inoculated and non-inoculated 156-OE plants and suggest that these genes are important for the interaction between *M. perniciosa* and both hosts. The interference of miRNA-related pathways was already been described to be part of an infection strategy by pathogens (*e.g. Magnaporthe oryzae*) (*e.g.* promotion of specific microRNAs expression and the hijack of host's microRNAs machinery) (NAVARRO et al., 2006; ZHANG et al., 2018).

Thus, we asked ourselves whether *M. perniciosa* could potentially, by some pathway (*e.g.* effector), induce the expression of the miR156 or miR157 to repress some SPLs as part of its infection strategy. We evaluated the expression of miR156 and miR157 in symptomatic stem tissue of MT at 12, 24 and 48 hai. Despite the repression of several miR156-targeted *SlSBP* genes in inoculated plants, miR156 and the miR157 levels did not change in response to inoculation (Figure 9a-d). Even at 10 dai the miR156 and miR157 did not changed the expression (Figure 9d). In addition, we observed that not all miR156-tageted *TcSPL* and *SlSBP* genes were repressed in inoculated samples at the same time (Figures 7, 8). Moreover, other non-miR156-targets were also modulated by the inoculation (Figures 7, 8). Therefore, the *M. perniciosa*-induced *SPL/SBP-box* gene repression is miR156-independent, and could possibly be mediated by a potential pathogen effector protein, as occurs in arabidopsis witches' broom disease caused by *Phytoplasma spp*. (CHANG et al., 2018; HUANG et al., 2021).

The SAP family of phytoplasma effectors has been described to transcriptionally repress *AtSPL* gene expression and also promotes de degradation of SPL proteins (CHANG et al., 2018; HUANG et al., 2021). Thus, *M. perniciosa* could potentially have some type of effector protein that could act in a similar way. Furthermore, the decreased SPL proteins-related function led to the characteristic increased branching symptom, which is similar to *M. perniciosa*-induced branching symptom. Therefore, we hypothesized that *TcSPL* and *SlSBP* gene repression could

also be responsible to the symptom formation, especially for the formation of broom-like branches and increased locule number in the fruits. Since the inoculation by *M. perniciosa* mimics the increased axillary outgrown and locule number phenotypes of 156-OE plants (Figure 5), we concluded that some miR156-targeted *SPL/SBP*s have a role during this interaction.

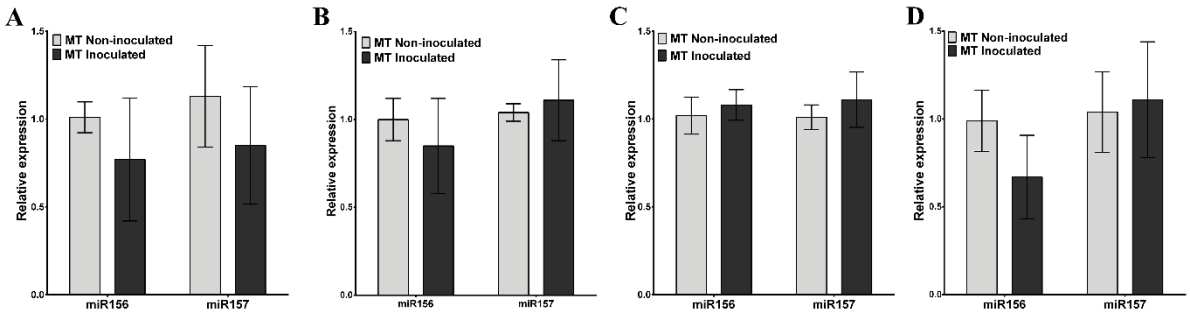

Figure 9 - Detection of miR156/miR157 transcripts by Stem-loop RT-qPCR in response to *M. perniciosa* infection (A) Comparative abundance of miR156 and miR157 in MT control and inoculated plants at 12 hours after inoculation. (B) Comparative abundance of miR156 and miR157 in MT control and inoculated plants at 24 hours after inoculation. (C) Comparative abundance of miR156 and miR157 in MT control and inoculated plants at 48 hours after inoculation. (D) Comparative abundance of miR156 and miR157 in MT control and inoculated plants at 10 days after inoculation. The quantitative RT–PCR experiments used non-inoculated MT as the reference sample (set to 1.0). Error bars indicate standard error of five biological samples.

4.3 miR156-targeted *SlSBP* genes repression increased colonization and the symptoms severity in response to *M. perniciosa* infection

Based in our findings, the tomato 156-OE transgenic plant became an important tool to functionally investigate the role of miR156-targeted *SlSBP* genes during the infection by *M. perniciosa*. Therefore, we inoculated shoot apex and the first two axillary meristems of MT and 156-OE plants with basidiospores of *M. perniciosa* (S-biotype) and monitored the symptoms emergence and progression through time.

Interestingly, 156-OE inoculated plants displayed more severity of symptom development with a higher incidence of symptomatic organs (*e.g.* broom-like branching, swelled and/or curled leaves; Figure 2), and enhanced stem swelling between the first and second leaf than inoculated MT plants (Figure 10). Moreover, we noticed that more 156-OE inoculated plants displayed symptoms at 20 dai, compared to MT (Figure 10g, h), suggesting a higher disease incidence in this genotype. No change in the stem diameter or any organ from both inoculated genotypes were observed at 5 dai. After 10 dai, we notice 156-OE inoculated plants started to display a slightly, but not significantly increased of stem diameter (Figure 10e).

However, from 20 dai 156-OE inoculated plants presented a significative difference compared with MT (Figure 10e). Such difference was enhanced during the time of the interaction. After 90 dai, the steam swelling ceased in both genotypes, but MT did not exhibit the same severity of 156-OE (Figure 10e). The severity of stem swelling at 90 dai (when the swelling has stabilized) in 156-OE inoculated plants increased the stem by 347% comparing to control plants, while MT inoculated plants increased by 218% (Figure 10e).

The incidence of symptomatic organs was quantified in parallel with the stem diameter measures. From 10 dai, the change of stem diameter was mild in 156-OE inoculated, but we already could observe the development of a greater number of symptomatic organs (Figure 10a-d, f). Throughout the interaction, we noticed that the symptoms of curled leaves, petioles, and the emergence of broom-like branches occur more in 156-OE inoculated plants than in MT inoculated plants (Figure 10f). At 50 dai, the emergence of symptomatic organs ceased. However, MT did not present the same number of symptoms displayed by 156-OE from 10 to 70 dai (Figure 10f).

Our results suggests that the strong repression of miR156-targeted *SPL/SBP*s by miR156 in our plants (156-OE), added to the *SPL/SBP-box* genes repression caused by *M. perniciosa* infection result in increased susceptibility to the pathogen, developing more symptoms that are more severe (Figures 7, 10). Therefore, we quantified the *M. perniciosa* DNA content at specific points after inoculation in both MT and 156-OE. At 10 dai, the pathogen DNA content was slightly, but not significantly increased in 156-OE compared with MT (Figure 10i). Nevertheless, at 20 dai a higher and significative pathogen DNA content in 156-OE (Figure 10j), and after 70 dai, 156-OE inoculated plants still displayed more *M. perniciosa* genomic DNA (Figure 10k). Altogether, our data allow us to conclude that *M. perniciosa* induces the repression of miR156-targeted *SlSBP* to facilitate infection (Figures 7-10). Besides, the strong repression of *SlSBP* genes in 156-OE enhances the susceptibility to *M. perniciosa* infection, promoting colonization (Figure 10i-k).

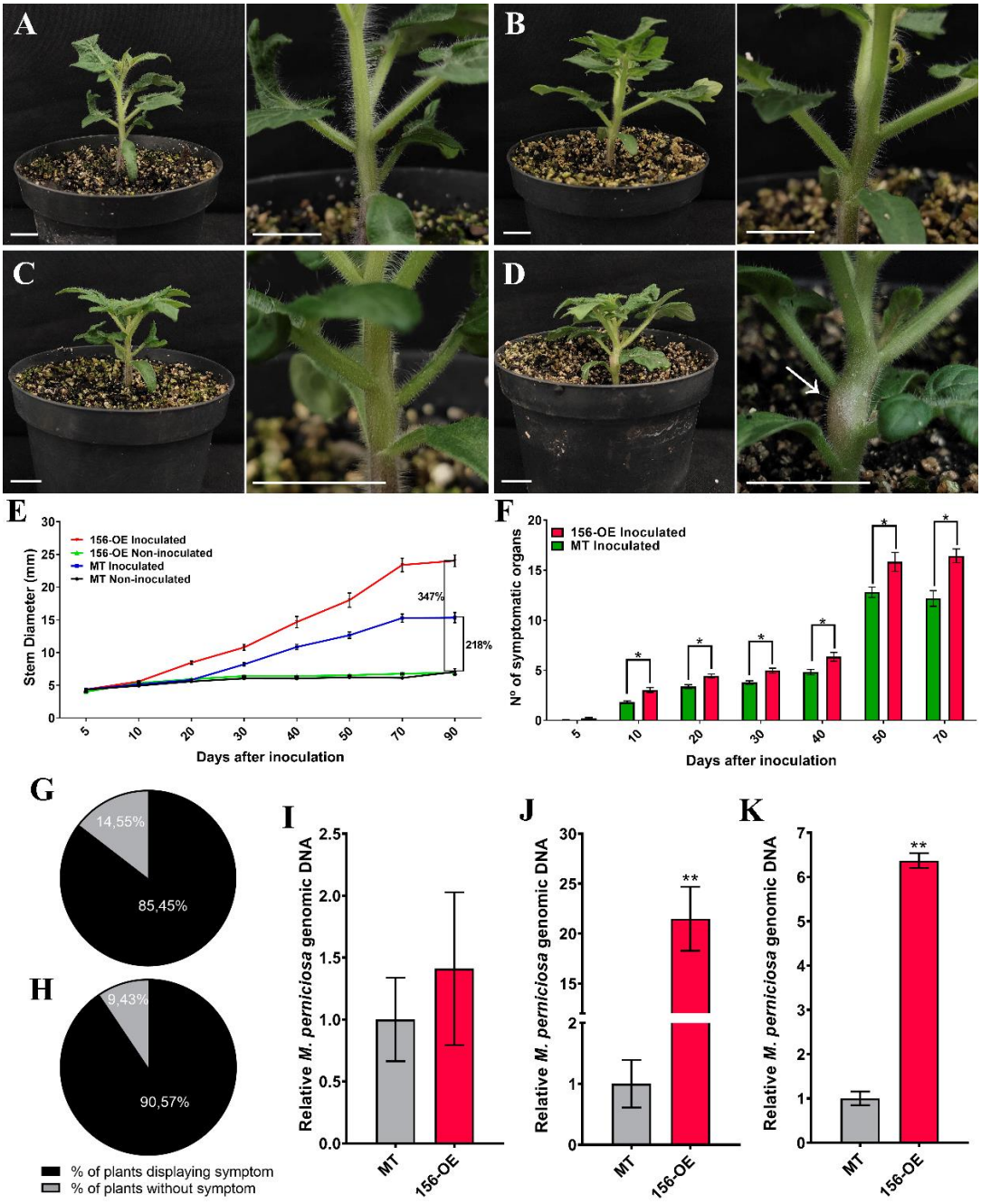

Figure 10 - Characterization of symptoms of MT plants and 156-OE infected with S–biotype from *M. perniciosa* (A) MT non-inoculated plant and zoomed inoculation site at 10 dai. (B) MT inoculated plant and zoomed inoculation site at 10 dai. (C) 156-OE plant non-inoculated plant and zoomed inoculation site at 10 dai. (D) 156-OE inoculated plant and zoomed inoculation site at 10 dai. (E) Mean of the stem diameter showing that 156-OE displayed more severe symptoms from 20dai. (F) Mean of symptomatic organs per plant showing that, from 10 dai, 156-OE inoculated plants displayed more symptomatic organs than MT inoculated. (G) Percentage of MT inoculated plants that displayed symptoms at 20 dai. (H) Percentage of 156-OE inoculated plants that displayed symptoms at 20 dai. (I) *M. perniciosa* genomic DNA was quantified in 156-OE and MT inoculated plants by qPCR at 10 dai relative to plant genomic DNA. (J) *M. perniciosa* genomic DNA was quantified in 156-OE and MT inoculated plants by qPCR at 20 dai relative to plant genomic DNA. (K) *M. perniciosa* genomic DNA was quantified in 156-OE and MT inoculated plants by qPCR at 70 dai relative to plant genomic DNA. The quantitative RT–PCR experiments used inoculated MT as the reference sample (set to 1.0). Error bars indicate standard error. Asterisks indicate results statistically different according to the t-test (* = 5% of significance, ** = 1% of significance). n = 40 (E-H) or n = 5 (I-K). Scale bar = 1 cm. Arrow points to stem swelling symptom.

Plants with low levels of miR156-targeted *SPL/SBP*-box genes have been shown to be more susceptible to other pathogens (YIN et al., 2019; ZHANG et al., 2020a). *Arabidopsis thaliana* transgenic plants overexpressing the miR156 (which have lower miR156-targeted *AtSPL* gene levels) showed increased susceptibility to bacterial infection by *Pseudomonas syringae*, including higher pathogen colonization compared to control plants (YIN et al., 2019). In addition, these plants have less accumulation of reactive oxygen species (ROS) and fewer transcripts of SA-related immune response genes (YIN et al., 2019). The reduced SA-related response and ROS accumulation is due to the downregulation of *AtSPL9*. Therefore, *AtSPL9* seem to be important to defense against biotrophic and hemibiotrophic pathogens (YIN et al., 2019). Moreover, rice plants with low levels of miR156-targeted *SPL/SBP*-box (*e.g.* by overexpressing the miR156) have been described to be more susceptible and favor colonization of the hemibiotrophic fungi *Magnaporthe oryzae* (ZHANG et al., 2020). Such increased susceptibility is due to OsSPL14 repression, which is known to participate in defense against this pathogen and against *Xanthomonas oryzae* (WANG et al., 2018; LIU et al., 2019; ZHANG et al., 2020a). Both *AtSPL9* and *OsSPL14* are *SlSBP15* orthologs (SALINAS et al., 2012), which is *TcSPL9* ortholog (Figure 8a), and could potentially have similar functions. Curiously, *M. perniciosa* seem to repress *SlSBP15* (Figure 7a, b) and *TcSPL9* (Figure 8c) as part of its infection strategy, and the increased susceptibility found in miR156-OE tomato plants could be result of *SlSBP15* gene repression.

4.4 miR156-targeted *SlSBP* genes repression allows *M. perniciosa* to complete its life-cycle in tomato

In tomato, the inoculation with the S-biotype of *M. perniciosa* led to similar biotrophic symptoms of *T. cacao* inoculated with *M. perniciosa* C-biotype (PURDY; SCHMIDT, 1996; DEGANELLO et al., 2014; TEIXEIRA et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). Our group had previously established that in MT infected plants, *M. perniciosa* is not able to induce senescence in symptomatic organs (*e.g.* stem) to make the transition of the necrotrophic phase, and complete its life cycle (Figures 10c, 11, 12) (DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). In this work, from 45 dai, 156-OE inoculated stem and leaf started to present the first signs of necrosis (Figure 10e, f), which resemble lesions found in infected *T. cacao* at necrotrophic stage (EVANS, 1980; PURDY; SCHMIDT, 1996; TEIXEIRA et al., 2014). As expected, those signs were not present in MT inoculated plants (Figure 11b, c). Dry brooms are known to be the main characteristic of the necrotrophic phase of infection, but were never demonstrated in any infected tomato genotype (PURDY;

SCHMIDT, 1996; DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). Only the occurrence of some black dots and death of a few shoot apexes in MT inoculated plants as reported (DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). Infected MT plants complete their life cycle while the symptomatic region (stem between the first and second leaf) stay green and swelled even after 90 dai, differing from *T. cacao* brooms in the necrotrophic stage (DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). However, we hypothesize that the pathogen could possibly promote tissue senescence in 156-OE plants due to higher pathogen colonization and its increased susceptibility (Figure 10), which it could potentially represent the transition of S-biotype *M. perniciosa* to the necrotrophic stage.

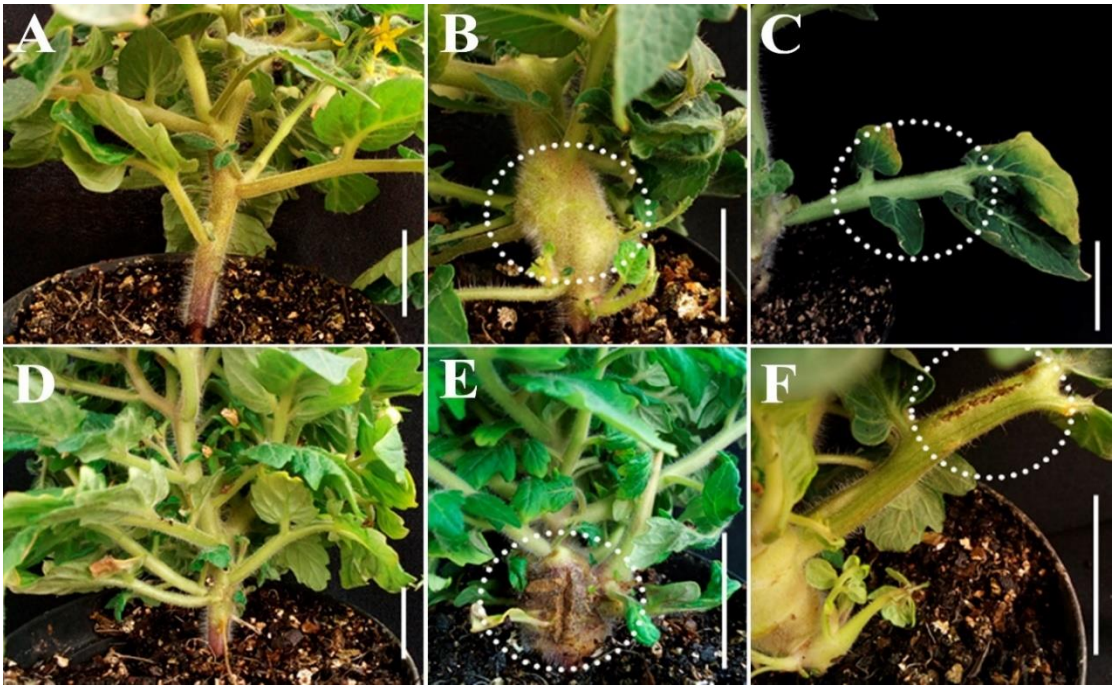

Figure 11 - 156-OE plants inoculated with *M. perniciosa* S-biotype spores produced brown lesions (A) Non-inoculated MT plant at 45 dai. (B) Symptomatic stem of MT inoculated plant at 45 dai with no lesions. (C) Symptomatic leaf of MT inoculated plant at 45 dai with no lesions. (D) Non-inoculated 156-OE plant at 45 dai. (E) Symptomatic stem of 156-OE inoculated plant at 45 dai showing a brown lesion that resemble tissue death. (F) Symptomatic leaf of 156-OE inoculated plant at 45 dai showing a brown lesion that resemble tissue death. Dashed circle shows possible tissue death compared with MT plants inoculated plants. Scale bar = 2cm

We noticed that the senescence area in 156-OE plants kept developing and growing. Therefore, we kept monitoring MT inoculated plants (Figure 11a-e) and 156-OE inoculated plants (Figure 11g-k). At 90 dai, while 156-OE symptomatic stem was completely dried (Figure 11h), MT inoculated plants remained with the symptomatic steam non-senescent corroborating previous results from our group (PASCHOAL, 2018) (Figure 11b). After, we kept the plants for more 30 days to evaluate whether MT would display the same characteristics of 156-OE

(Figure 11c, i). However, MT did not display the same appearance of dried tissue, as 156-OE in response to inoculations. Thus, due to the resemblance with necrotrophic phase lesions in cacao (Figure 1e, j), our results could be result of the transition to a necrotrophic phase of infection in 156-OE inoculated plants, but further confirmation were needed still.

For *M. perniciosa* to complete its life cycle, dry brooms should be exposed to an interchange of wet and dry periods to trigger the development of the fruitification body (basidiocarps) (PURDY; SCHMIDT, 1996), which is known to be the reproductive structure of *M. perniciosa*. Thus, we collected 10 156-OE, and 10 MT inoculated plants at 180 dai. After, they were exposed to consecutive cycles of wet and dry to simulate the natural condition for the pathogen form its reproductive structure (PURDY; SCHMIDT, 1996). Although the MT plants did not present such necrotic tissue symptoms, they were also tested. As controls, we put together 10 dry brooms from cacao naturally infected with the C-biotypes and 10 from *S. lycocarpum* plants naturally infected with the S-biotypes. Remarkably, after 30 days of exposure (210 dai), 156-OE plants started to get purple while MT plants stayed unchanged (Figure 12d, j). But, from 60 days of exposure, basidiocarps started to grow from symptomatic stems of 156-OE inoculated plants (240 dai) (Figure 12k). As expected, we could not observe basidiocarps from MT inoculated in any moment (Figure 12e). Besides, all the control brooms produced basidiocarps normally, which confirmed that the conditions were adequate (Figure 12f, l). Although it has been speculated that *M. perniciosa* could develop to the necrotrophic phase in tomato (BASTOS; EVANS, 1985; MARELLI et al., 2009), compellent evidence it has never been provided for any tomato genotype. Therefore, our results showed for the first time a tomato genotype in which *M. perniciosa* can change the necrotrophic stage of infection and complete its life cycle. In addition, our results link the strong repression of miR156-targeted *SPL/SBP*-box to favor the pathogen complete its development.

To check the viability and virulence of spores from basidiocarps produced in 156-OE inoculated plants, we inoculated MT with those spores and compared with MT inoculated with spores from basidiocarps produced in naturally infected *Solanum lycocarpum*, and used non-inoculated MT as control (Figure 12m-o). No difference was observed between both spores regarding the swelling of the stem and also in number of symptoms formed (Figure 12p, q). This data shows that basidiocarps produced in 156-OE plants are as viable as the naturally occurring ones. Thus, 156-OE plants could be an important asset to not only study symptom severity and the pathogen phase change, but also is an alternative to produce basidiocarps and spores of *M. perniciosa* S-biotype strains.

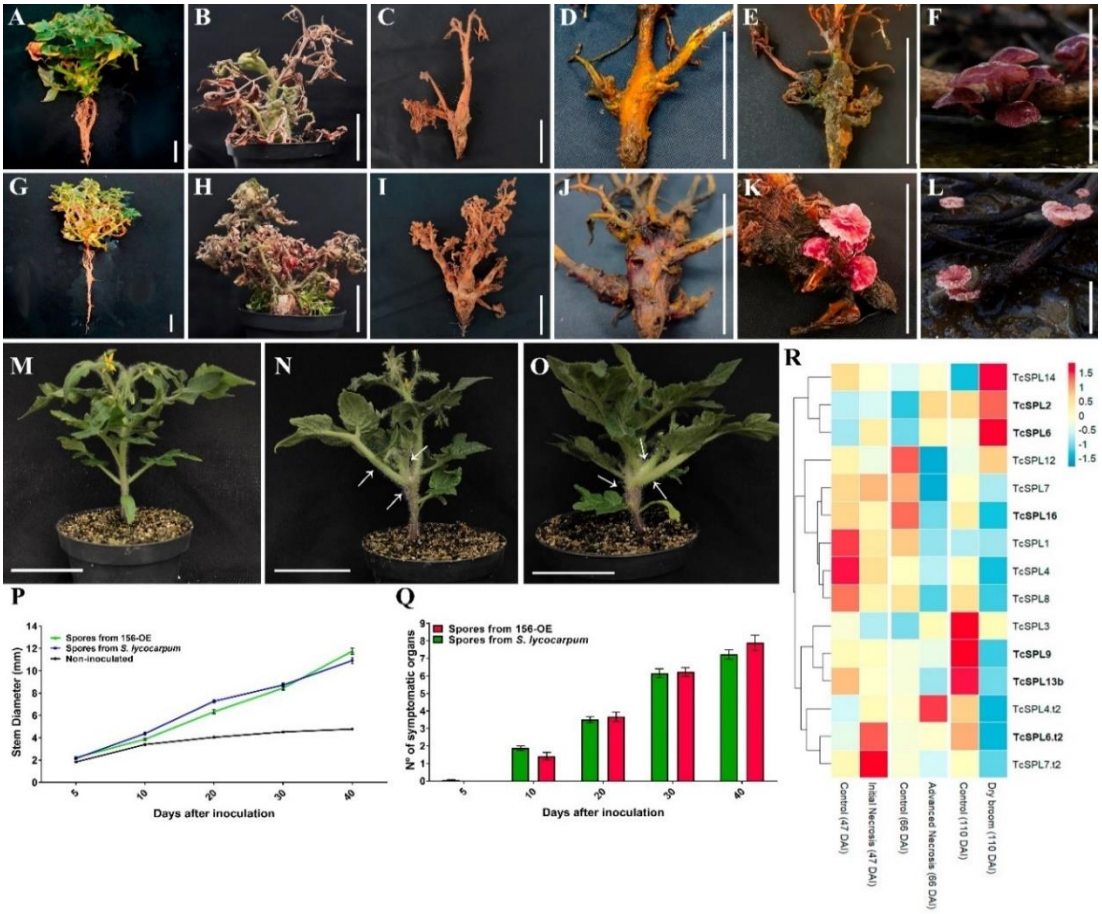

Figure 12 - The strong repression of miR156-targeted *SPL/SBP-box* genes facilitate *M. perniciosa* life cycle completion (A) MT Inoculated plant at 70 dai. (B) MT inoculated plant at 90 dai showing green symptomatic tissue while the rest of the plant organs were with a dead aspect due to the end of plant life cycle. (C) MT inoculated plant at 110 dai. (D) MT inoculated plant at 210 dai. (E) MT inoculated plant at 240 dai. (F) Naturally infected branch of *Solanum lycocarpum* producing basidiocarps after 60 days of wet and dry interchange treatment. (G) 156-OE inoculated plant at 70 dai starting to show more brownish tissue. (H) 156-OE inoculated plant at 90 dai showing expansion of necrotrophic lesions. (I) 156-OE inoculated plant at 110 dai. (J) 156-OE inoculated plant at 210 dai with purple colored stem. (K) 156-OE inoculated plant at 240 dai producing basidiocarps. (L) Naturally infected *T. cacao* branch producing basidiocarps after 60 days of wet and dry interchange treatment. (M) MT control plant at 20 dai. (N) MT plant infected with *S. lycocarpum*-derived spores at 20 dai. (O) MT plant inoculated with 156-OE-derived spores at 20 dai. (P) Mean of the stem diameter showing no difference between MT plants infected with naturally occurring spores from *S. lycocarpum* and spores from basidiocarps produced in 156-OE infected plants. (Q) Mean of symptomatic organs per plant showing no difference between MT plants infected with naturally occurring spores from *S. lycocarpum* and spores from basidiocarps produced in 156-OE infected plants. (R) Heatmap of mRNA-seq analysis of *TcSPL* genes in response to *M. perniciosa* infection in *T. cacao* plants. Upregulated and downregulated genes in infected plants are shown in red and blue, respectively. The scale bar represents normalized reads per kilobase of transcript, per million mapped reads (RPKM). Data from WBD Transcriptome Atlas (www.lge.ibi.unicamp.br/wbdatlas, TEIXEIRA et al., 2014). miR156-targeted SPL gene family members are highlighted in bold letters. n = 20 (P, Q). Scalebar = 4cm. Arrows point to symptomatic stem, leaf, and broom-like branch.

Moreover, as aforementioned, *M. perniciosa* induces the downregulation of miR156-targeted *SPL/SBP-box* genes during the infection, which led to similarities between non-inoculated 156-OE and MT inoculated plants (Figures 5, 7); since 156-OE have the same miR156-target SBPs repressed. Thus, we concluded that the strong repression of miR156-target SBPs presented by 156-OE promotes a better environment to *M. perniciosa* colonization

(Figure 10). We do not have any expression data about SBP genes in MT at the necrotrophic stage of infection, since tomato does not normally present this stage. However, we went back to the public mRNA-seq data of infected *T. cacao* (TEIXEIRA et al., 2014). Interestingly, we found that some miR156-targeted *SPL/SBP* genes repressed from 47 dai samples (annotated as initial necrosis stage) and several *SPL/SBP* genes repressed at advanced necrosis (66 dai) and in the dry broom (110 dai) (Figure 12r). At 45 dai, *T. cacao* infected plants had *TcSPL1*, *3*, *4*, *8*, *12*, *13b*, and *16* repressed, while *TcSPL4.t2, 6, 6.t2*, and *7.t2* were induced by the infection, and *TcSPL2* and *9* did not change (Figure 12r). Meanwhile, at 66 dai, *TcSPL1*, *4*, *7*, *7.t2*, *8*, *9*, *12*, *13b*, and *16* was found to be repressed in inoculated samples, *TcSPL2*, *3*, *4.t2*, *6*, and *14* were induced, and *TcSPl6.t2* levels were not different between inoculated and control samples (Figure 12r). However, at 110 dai, most *TcSPL* genes were repressed, only *TcSPL1* levels was the same between inoculated and control plants, and *TcSPL2*, *6*, and *14* were induced (Figure 12r).

This expression pattern reinforces our hypothesis that *SPL/SBP-box* genes repression could be important for *M. perniciosa.* One hypothesis is that the sugar and nutrient concentration in the symptomatic region is determinant to *M. perniciosa* phase change (TEIXEIRA et al., 2014; BARAU et al., 2015). Therefore, the *SPL/SBP-box* genes repression could be assisting the pathogen phase transition for developing bigger swelled symptomatic tissues that could accumulate more nutrients and trigger the transition and in the necrotrophic phase. Another hypothesis is that the pathogen could need the repression of *SPL/SBP-box* genes to hinder plant defense response and induce pathogen growth (Figure 10), and these could be crucial for the switch to the necrotrophic phase. Both hypotheses above could be true for the *M. perniciosa*-host interaction. However, as for most hemibiotrophic fungus (CHOWDHURY; BASU; KUNDU, 2017), still not clear what is determinant for *M. perniciosa* to switch to the necrotrophic stage. Nevertheless, since only in 156-OE presented the basidiocarp grown, our results suggest that the repression of miR156-target *SPL/SBP*s not only favors susceptibility, but is required for the pathogen transition to the necrotrophic stage of infection in tomato.

4.5 Increased susceptibility and transition to necrotrophic phase observed in MT overexpressing the miR156 is not influenced by the MT background

MT has been a tomato cultivar used in research, mainly because of its compact habit and relatively short generation time. Nevertheless, it retains four independent mutations (MARTÍ et al., 2006; ARIE et al., 2007) that may affect plant development and potentially affect

stress response. We ask ourselves whether our results could be related to MT background in any matter. Thus, we crossed wild-type MT plants and 156-OE transgenic MT plants with plants of the cultivar M82 (M82xMT and M82x156-OE, respectively), which is broadly used as a model system in plant development and microbe interaction. The F1 offspring of those crossings (which have MT recessive mutations in heterozygosis) were inoculated and symptoms were evaluated (Figure 13).

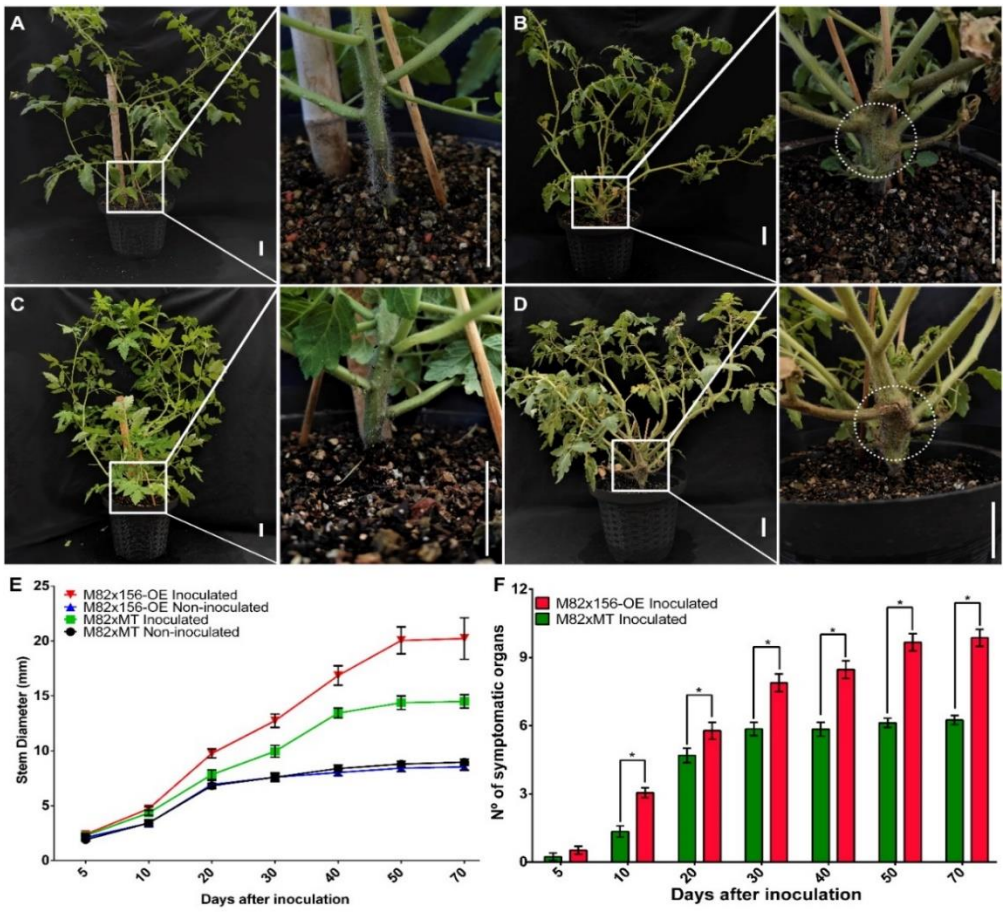

Figure 13 - Characterization of symptoms induced by *M. perniciosa* in F1 progeny of MT crossed with M82 plants (M82xMT) and 156-OE crossed with M82 plants (M82x156-OE). (A) M82xMT non-inoculated plant and zoomed inoculation site at 50 dai. (B) M82xMT inoculated plant and zoomed inoculation site at 50 dai. (C) M82x156-OE non-inoculated plant and zoomed inoculation site at 50 dai. (D) M82x156-OE inoculated plant and zoomed inoculation site at 50 dai. (E) Mean of the stem diameter showing that M82x156-OE inoculated plants has more severe stem swelling symptom compared with M82xMT from 20dai. (F) Average of number of symptomatic organs per plant, showing that from 10 dai, M82x156-OE inoculated plants displayed more symptomatic organs than M82xMT inoculated plants. Dashed circle highlights tissue death in M82x156-OE inoculated plants compared to M82xMT inoculated plants. Asterisks indicate results statistically different according with t test. (n = 20, $\alpha = 0.05$). Scale bar = 4 cm.

Symptoms of swelling and branching induced by *M. perniciosa* in M82 hybrids were comparable to those on inoculated MT and 156-OE transgenic plants (Figure 13a-d), showing that the increased symptom severity in 156-OE plants is not related to MT background, once M82x156-OE inoculated plants had stem swelling and number of symptomatic tissue behavior

in relation to M82xMT (Figure 13e, f) similar to 156-OE plants in relation to MT inoculated (Figure 11e, f). More importantly, the pathogen transition to necrotrophic stage was also observed by necrotic lesions in M82x156-OE inoculated hybrid similar to 156-OE inoculated plants (Figures 13d, 11). Altogether, our results suggest that some miR156-targeted SBP genes are play an important role in this interaction, not only in symptom development, but potentially in the fungi stage transition both in *T. cacao* and *S. lycopersicum*.

4.6 *M. perniciosa* infection prevent fruit set in symptomatic branches of 156-OE plants, and affects fruit quality

M. perniciosa is a pathogen known to alter fruit development and induce parthenocarpy in symptomatic fruits of *T. cacao* infected with C-biotype spores and tomato inoculated with S-biotype spores (PURDY; SCHMIDT, 1996; MELNICK et al., 2012; COSTA et al., 2021). In this work, noticed that only fruits originated from broom-like branches of MT inoculated and from non-inoculated 156-OE plants displays similar morphology (Figure 5e, f). Since 156-OE inoculated plants are highly susceptible to *M. perniciosa* (Figures 10-12), we investigated the effect of infection on fruit yield and quality in both inoculated genotypes in comparison with the non-inoculated controls. We noticed a decrease of yield in 156-OE inoculated plants compared with 156-OE non-inoculated and MT inoculated plants (Figure 14a). Thus, we decided to look the reproductive development from normal branches (asymptomatic) and symptomatic broom-like branches in MT and 156-OE and compared with control plants.

Surprisingly, we noticed that 156-OE broom-like branches present flower abortion, and do not produce fruits. Flowers from symptomatic branches of 156-OE inoculated plants showed signals of nutrient deficiency at their floral peduncles (Figure 14c), while such signals were absent in asymptomatic branch flowers (Figure 14d). Such difference is probably due to the nutrient sink formation in symptomatic swelled symptoms, as previously described by our group (PASCHOAL, 2018). Therefore, only asymptomatic branches from 156-OE presented fruit set (Figure 14). We also analyzed fruit weight, the number of seeds, and the °brix (soluble solids content) of fruits from inoculated and control MT and 156-OE plants (Figure 14f). Thus, we collected fruits separately from broom-like branches, and fruits from asymptomatic branches of MT.

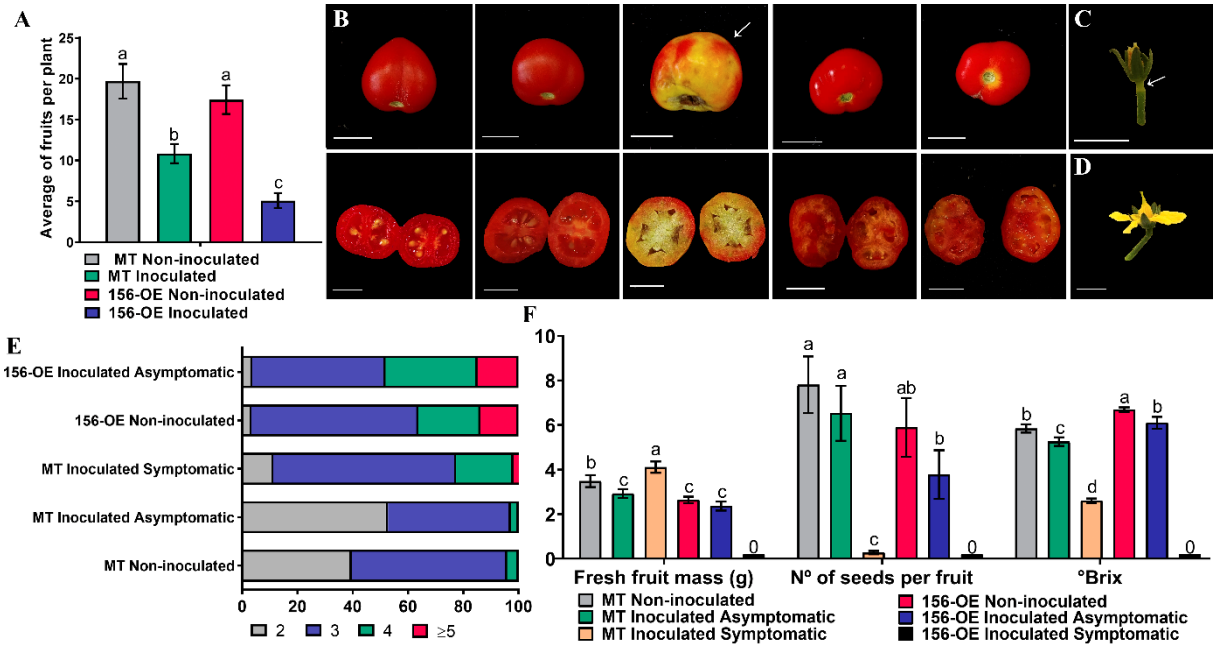

Figure 14 - Characterization of fruits from MT control plants and *M. perniciosa* S-biotype infected plants, compared to 156-OE control plants and infected plants (A) Average of fruits per plant. (B) From left to right, fruits of MT control, asymptomatic, symptomatic, 156-OE control fruit and asymptomatic, respectively. Below, from left to right, longitudinal cuts of MT control, asymptomatic, symptomatic and control 156-OE fruit, from inoculated MT and 156-OE plants and control fruits from non-inoculated MT and 156-OE plants. (C) Flower from symptomatic branches of 156-OE inoculated plants, showing signals of nutrient deficiency at 30 dai. (D) Flower from asymptomatic branches of 156-OE inoculated plants, not showing signals of nutrient deficiency at 30 dai. (E) Percentage of the number of locules found in fruits of MT non-inoculated plants, asymptomatic fruits of MT plants infected with *M. perniciosa* S-biotype, symptomatic fruits of MT plants infected with *M. perniciosa* S-biotype, fruits of 156-OE non-inoculated plants, and asymptomatic fruits of 156-OE plants infected with M. perniciosa S-biotype. (F) Average fruit weight, number of seeds and °brix. Different letters indicate statistically different results according to the Tukey test ($\alpha = 0.05$). n = 30 plants. Scale bar = 1cm. Arrows point to fruit uneven ripening (B), and to floral peduncle with signals of nutrient deficiency (C).

Since 156-OE inoculated plants do not produce fruits from broom-like branches (Figure 14), we were able to only collect fruits from asymptomatic branches. The increased locule number of MT inoculated plant fruits was previously reported by our group (COSTA et. al, 2021), however, in this work we noticed that only fruits from broom-like branches had such change (Figure 14e). Fruits from MT inoculated plants derived from asymptomatic branches presented normal morphology. Moreover, we observed that fruit from broom-like branches had an increased weight (Figure 14f), which could be explained by the increase of locule number. However, when we evaluated the °brix, we found that broom-like branches-derived fruits (symptomatic fruits) have a low accumulation of soluble solids compared to fruits from control plants and fruits from asymptomatic branches of inoculated plants (Figure 14f), despite having higher average of fresh fruit mass.

Our group recently established that the symptomatic swelled stem act as a sink by altering sugar composition and accumulation, besides the carbon reallocation to the

symptomatic stem (PASCHOAL, 2018) We speculate that soluble sugars are probably being directed and retained to symptomatic regions, not allowing them to accumulate in the fruit. Interestingly, symptomatic fruits have no seeds, which could potentially be related to the low °brix (D'AOUST; YELLE; NGUYEN-QUOC, 1999; reviewed by KANAYAMA, 2017). These sugar-related symptoms and fruit alterations found in symptomatic fruits from MT inoculated plants suggest that the formation of swelled symptoms could be one of the reasons of the decreased productivity found in inoculated plants, as suggested by our group (PASCHOAL, 2018). Interestingly, the formation of a sink in symptomatic tissues have also been suggested to occur in *T. cacao* infected tissue, in which similar fruit alterations are induced by the pathogen, including parthenocarpy (absence of viable seeds) (SCARPARI et al., 2005; MELNICK et al., 2012; TEIXEIRA et al., 2014).

Another symptom observed is uneven ripening in MT symptomatic fruit (Figures 4l, 14b), which resemble described characteristics of infected cacao fruits (RUDGARD, 1989). We were not able to evaluate the characteristics of symptomatic fruits from 156-OE plants, as they did not present fruit set from broom-like branches. Flowers from symptomatic branches of 156-OE infected plants display signs of nutrient deficiency ended in aborted flowers (Figure 14c), while flowers from asymptomatic branches of the same plants did not have change and produced fruits (Figure 14b, d). Our group has recently showed that the swelled stem of infected MT has an increase of nitrogen transport-related genes expression and increased accumulation of nitrogenated compounds, suggesting that the symptomatic stem is also a nitrogen sink (PASCHOAL, 2018). Therefore, the signs of deficiency observed in floral peduncles of symptomatic branches of 156-OE inoculated plants could be nitrogen deficiency (Figure 14c). This led to a great reduction in the average of fruits per plant in 156-OE inoculated plants compared to MT inoculated plants (Figure 14a). Thus, we analyzed fruits from non-symptomatic branches. Similarly to fruits of 156-OE control plants, fruits from asymptomatic branches of 156-OE inoculated plants showed less fruit fresh mass compared to MT (Figure 14f).

Regarding the °brix of 156-OE fruits, while control fruits showed higher soluble sugars than MT control plants, 156-OE asymptomatic fruits showed a slightly reduction of soluble sugars compared to control (Figure 13d). Such reduction also occurs to MT asymptomatic fruits compared to MT control (Figure 13d). This is possibly because it was not formed in a nutrient-sink symptomatic branch and so sugars were available to fruit set but were slightly affected by the infection still, probably due to redirecting of sugars to the symptomatic areas of the plant. However, the reduction of °brix in asymptomatic 156-OE fruits made these fruits have the same

°brix than MT control fruits. Moreover, 156-OE asymptomatic fruits displayed less average of seeds per fruit compared to MT control, like the previously described (SILVA et al., 2014a) (Figure 14f). 156-OE asymptomatic fruits also had no difference in seed number comparing to 156-OE control fruits, which also occurred in MT (asymptomatic fruits compared to MT control fruits) (Figure 14f). The higher °brix of 156-OE control fruits suggest that more sugars are being directed to those fruits than in MT control. Because the pathogen causes a sugar sink in swelled symptomatic regions (PACHOAL, 2018), this higher sugar content in 156-OE fruits could be stopping in the sink regions, which could assist the pathogen to grow, possibly causing a stronger sink that prevents fruit set in 156-OE symptomatic branches.

Our data suggests that although *M. perniciosa* reduce the productivity of inoculated MT plants, only fruits from symptomatic tissues display the changes characteristic to the disease, while asymptomatic fruits have a small reduction of °brix. Similarly, *T. cacao* fruits from infected plants display symptoms only in infected/symptomatic fruits, also producing healthy fruits in infected plants (MELNICK et al., 2012). In addition, we showed that the increased susceptibility caused by the repression of miR156-targeted *SPL/SBP-box* genes in 156-OE inoculated plants resulted in the abortion of flowers, preventing fruit set. Therefore, we asked ourselves which *SlSBP* that is repressed by the pathogen infection is important for the swelled symptom formation and why, to investigate in the future if the fruit set is only related to the severity of the swelled symptom.

4.7 Tomato *SlSBP15* gene overexpression reduces pathogen colonization and symptom severity

Our findings showed that the strong repression of miR156-targeted *SPL/SBP-box* genes make tomato plants more susceptible to the disease caused by *M. perniciosa* (Figures 10-14). We also detected in mRNA-seq data that the repression of *SPL/SBP-box* genes is part of the pathogen infection strategy (Figures 7, 8, 12). *TcSPL9* and *SlSBP15* are among repressed *SPL/SBP-box* genes and are orthologs of arabidopsis *AtSPL9* and rice *OsSPL14* (SALINAS et al., 2012). These genes were already described to participate in plant-pathogen interactions (WANG et al., 2018; LIU et al., 2019; YIN et al., 2019; ZHANG et al., 2020a). Moreover, *SlSBP*3, which is also repressed by the pathogen (Figure 7), was previously described to participate in ovary and fruit development (SILVA et al., 2014a), and *SlSBP3* repressed plants (156-OE) display similar changes in fruits to *M. perniciosa* induced fruit symptoms (Figure 5). Therefore, we inoculated shoot apex and the first two axillary meristems of MT, 156-OE, and

plants that overexpress versions of *SlSBP3* and *SlSBP15* resistant to the miR156 regulation (rSBP3-OE and rSBP15-OE, respectively) (Figure 15). Afterwards, we evaluated symptom occurrence, severity, progression, and the relative pathogen genomic DNA for each genotype (Figure 16).

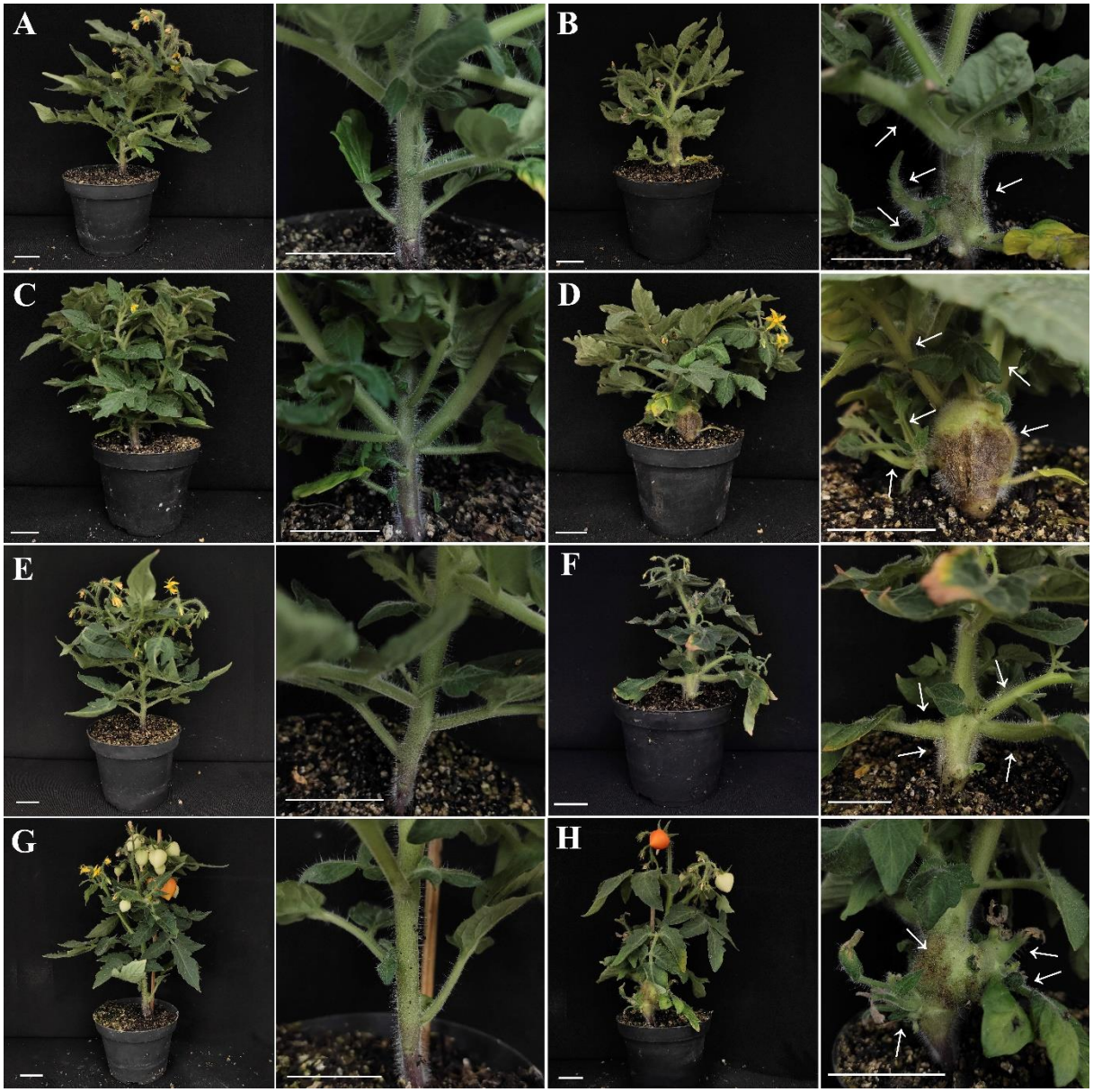

Figure 15 - Overexpression of *SlSBP*15 reduces stem swelling symptom (A) MT non-inoculated plant and zoomed inoculation site at 40 dai. (B) MT inoculated plant and zoomed inoculation site at 40 dai. (C) 156-OE plant non-inoculated plant and zoomed inoculation site at 40 dai. (D) 156-OE inoculated plant and zoomed inoculation site at 40 dai showing necrotrophic lesions. (E) rSBP15-OE non-inoculated plant and zoomed inoculation site at 40 dai. (F) rSBP15-OE inoculated plant and zoomed inoculation site at 40 dai showing reduced swelling. (G) rSBP3-OE non-inoculated plant and zoomed inoculation site at 40 dai. (H) rSBP3 inoculated plant and zoomed inoculation site at 40 dai. Scale bar = 2cm. Arrows point to symptomatic tissues.

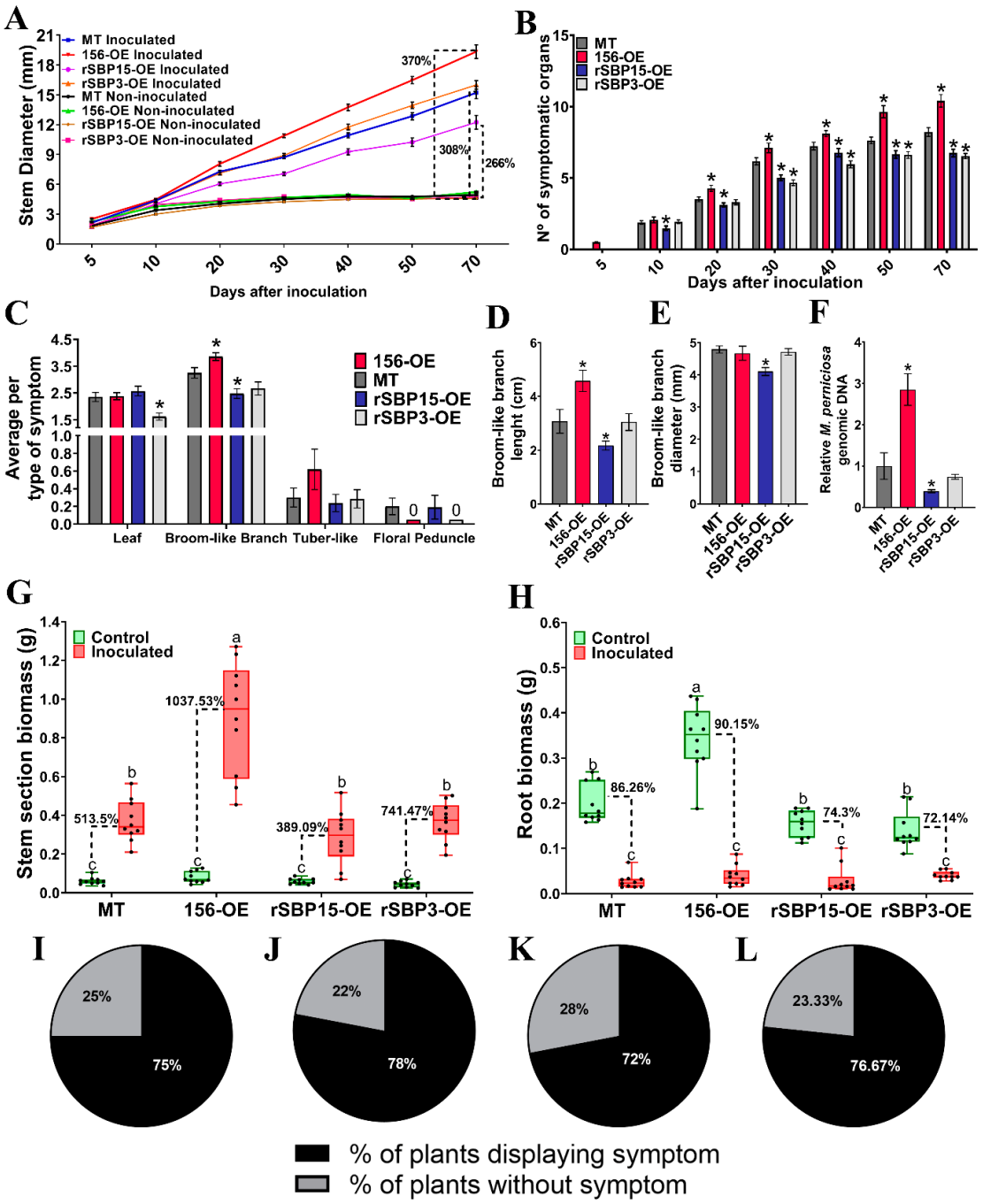

Figure 16 - rSBP15-OE inoculated plants display less symptomatic organs, reduced symptom severity, and pathogen colonization (A) Mean of the stem diameter showing that rSBP15-OE inoculated plants displayed reduced swelling severity from 20dai. (B) Mean of symptomatic organs per inoculated plant showing that, from 10 dai, rSBP15-OE plants displayed less symptomatic organs than MT. (C) Average frequency of each type of symptom found in inoculated plants at 70 dai. (D) Average length of inoculated plant Broom-like branches at 70 dai. (E) Average diameter of inoculated plant Broom-like branches at 70 dai. (F) *M. perniciosa* genomic DNA was quantified in inoculated plants by qPCR at 40 dai relative to plant genomic DNA. MT inoculated plants were used as the reference sample (set to 1.0). (G) Stem biomass (between the first and second leaves) of control and inoculated plants at 70 dai. (H) Root biomass of control and inoculated plants at 70 dai. (I-L) Percentage of inoculated plants that displayed symptoms at 20 dai for the MT (I), 156-OE (J), rSBP15-OE (K), and rSBP3-OE (L) genotypes. Error bars represent standard error (B-F). Asterisks indicate statistically different means according to the t-test at 5% of significance. Different letters show statistically different means according to the Tukey's test. Dashed lines with percentage values highlights the difference between control and inoculated plants of each genotype. n = 40 (A-E, I-L) or n = 5 (F) or n = 10 (G, H).

We did not detect any difference in incidence of symptoms (Figure 16i, l) and in stem swelling between MT and rSBP3-OE plants (Figures 15a, b, g, h, 16a). However, after 30 dai, rSBP3-OE inoculated plants had fewer symptoms than MT inoculated plants (Figure 16b). In addition, at 45 dai, only some small black and/or brown regions were detected in MT and rSBP3-OE genotypes (Figure 15b, h) and those did not grow or changed thought time, as previously described to occur in infected MT plants (DEGANELLO et al., 2014; COSTA et al., 2021). Meanwhile, 156-OE plants had similar results to our previous experiments (Figures 10, 11, 15c, d, 16a, b), including the formation of necrotrophic lesions in swelled tissues (Figure 15d).

Strikingly, rSBP15-OE plants showed increased resistance against the pathogen by having a reduced swelling of the stem from 20 dai (Figure 16a) and forming less symptoms than MT from 10 dai (Figures 15e, f, 16b). Moreover, at the last measurement time (70 dai), this genotype had only a 266% increase in the stem diameter, comparing to control plants, while MT and rSBP3-OE had about 308% increase and 156-OE had 370% (Figure 16a). Similar result of apparent resistance was achieved by the overexpression of arabidopsis *AtSPL9* and rice *OsSPL14* (*IPA1*) (WANG et al., 2018; LIU et al., 2019; YIN et al., 2019; ZHANG et al., 2020a). These *SlSBP*15 orthologs was described to diminish the main symptom size (in this case lesions in leaves) caused by *Pseudomonas syringae* in arabidopsis (YIN et al., 2019), and by *Xanthomonas oryzae* and *Magnaporthe oryzae* in rice (WANG et al., 2018; LIU et al., 2019; ZHANG et al., 2020a). Because this genotype seems to show resistance against this pathogen, we counted the occurrence of each symptom and measured the length and the diameter of symptomatic branches of all genotypes (Broom-like branches). In addition, we quantified the relative pathogen genomic DNA content compared to MT, rSBP3-OE, and 156-OE inoculated plants (Figure 16c-f).

Interestingly, rSBP15-OE plants formed fewer broom-like branches, and those that had formed were smaller in size and diameter compared to other genotypes (Figure 16c-e). Such data suggests that this genotype could have a reduced susceptibility to *M. perniciosa*. Consistently, the opposite results were found in 156-OE plants, which are more susceptible (Figure 16c-e). 156-OE plants had more broom-like branches than others that were longer but not thicker than broom-like branches of MT plants, indicating a potentially more vigorous symptom. Although not statistically significant, 156-OE plants formed slightly more tuber-like symptoms (Figure 2q, 16c). Interestingly, the formation of such structures has been demonstrated to be increased by repression of *SPL/SBP* genes, which affect cytokinin (CK)-related response in tomato and potato plants (EVIATAR-RIBAK et al., 2013;

BHOGALE et al., 2014). Consistently, Costa et al. (2021) showed that infection by *M. perniciosa* alters the cytokinin (CK) signaling pattern. Thus, the slightly more frequent formation of tuber-like structures in 156-OE could be due to the SPL/SBP-repression affecting CK-response. But to asses if this relation applies to the formation of these structures in response to *M. perniciosa* infection need to be further investigated.

Meanwhile, rSBP3-OE plants had less symptom formation, showing less symptomatic leaves than other genotypes (Figure 16c). In addition, this gene was repressed by *M. perniciosa* infection (Figure 7). However, we did not find any difference in swelling severity and pathogen colonization for this genotype (Figure 16). Thus, this gene could have a role in this plant-pathogen interaction, but still not clear rather this gene only affect the formation of symptomatic leaves or if other SPL/SBPs are needed to act with this gene and affect pathogenesis. Therefore, we chose to not continue investigating this genotype. When we quantified the pathogen DNA content in infected samples, we noticed that, as expected by the reduced symptoms, rSBP15-OE plants had lower pathogen colonization than MT (Figure 16f), reinforcing the idea that this genotype is more resistant to this disease. Meanwhile, as abovementioned, rSBP3-OE plants had no difference in colonization comparing to MT (Figure 16f), and156-OE plants had higher colonization, which has also occurred in our previous experiment (Figure 10).

Regarding changes in biomass (Figure 16g, h), Costa et al. (2021) suggested that *M. perniciosa* causes an increase of stem biomass and reduction of root biomass by modulating host CK levels. This change affects the cytokinin-auxin balance, resulting in a reduction in the growth of the root system, a novel symptom described in this interaction (COSTA et al., 2021). Curiously, we detected a significative change in 156-OE plants root biomass (Figure 16h). This genotype showed a higher reduction of the root system biomass, reducing 90.15% comparing to control (Figure 16h). Such data suggests an increased susceptibility in this genotype that could be result of a hormonal unbalance, as previously suggested by our group (COSTA et al., 2021). Moreover, 156-OE inoculated plants displayed a higher stem biomass (the infection site, between the first and second leaf), with more than 1000% increase compared to control (Figure 16g). Both changes in biomass could be result of cytokinin unbalance caused by the pathogen (COSTA et al., 2021). Thus, a higher difference in 156-OE plants could be result of an intensified CK unbalance, since repression of *SPL/SBP*s has been described to affect CK-response (EVIATAR-RIBAK et al., 2013; BHOGALE et al., 2014; ZHANG et al., 2015; DU et al., 2017). Although no significant difference was detected between all other genotypes in the same condition (control compared to control and inoculated compared to inoculated),

the inoculation has increased stem biomass and reduced root biomass for all genotypes (Figure 16g, h).

Altogether, our results show that *SlSBP15* may have an important role in this interaction. We saw that rSBP15-OE plants had reduced symptom severity and also had reduced *M. perniciosa* genomic DNA, which can be interpreted as reduced colonization (Figure 16). Interestingly, the resistance to *M. perniciosa* found in some *T. cacao* genotypes is also associated with reduced pathogen growth and number of green brooms, in addition to reduced stem swelling and broom sizes (BAKER; HOLLIDAY, 1957; WHEELER, 1996; SILVA et al., 2002; SURUJDEO-MAHARAJ et al., 2003; SENA; ALEMANNO; GRAMACHO, 2014). All of these characteristics can be detected in rSBP15-OE inoculated plants (Figure 16), suggesting that this gene could be a possible source of resistance. We do not discard the possible role of other *SPL/SBP-box* genes, like *SlSBP3*, in this interaction, but, because of the increased resistance observed, we chose to further investigate the role of the *SlSBP15* gene. Moreover, our group has recently demonstrated that cytokinins (CK) is crucial for the formation of the swelling symptoms (COSTA et al., 2021). This pathogen causes a cytokinin unbalance to form nutrient sinks that could be used by the pathogen as nourishment (PASCHOAL, 2018; COSTA et al., 2021). Thus, we asked ourselves if the attenuated symptoms in rSBP15-OE plants have a relation with CK-related gene expression, since its orthologs have been related to CK response (BHOGALE et al., 2014; ZHANG et al., 2015; DU et al., 2017).

4.8 *SlSBP15* overexpression attenuate symptoms induced by *M. perniciosa* by modulating cytokinin-response-related gene expression

As previously mentioned, our group have established that cytokinin (CK) is crucial for symptom formation (COSTA et al., 2021). We demonstrated that MT infected tissue have a deregulation of CK-related genes. The CK biosynthesis *ISOPENTENYL TRANSFERASE* (*IPT*) and CK degradation *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) genes are repressed and induced, respectively (COSTA et al., 2021). Moreover, *T. cacao* infected green-brooms have the similar CK deregulation (TEIXEIRA et al., 2014). Regarding CK signaling, the infection of MT by *M. perniciosa* caused deregulation of tomato response regulators (RRs) (COSTA et al., 2021).

In addition, several *SlSBP*15 orthologs have been described to participate in CK-related response, like *AtSPL9*, *StSPL9*, and *UB3* (BHOGALE et al., 2014; ZHANG et al., 2015; DU et al., 2017). The arabidopsis *AtSPL9* has been described to participate in CK signaling by

physically interfering in the binding ability of *ARABIDOPSIS RESPONSE REGULATOR 2* (*ARR2*, a type-B response regulator) protein, downregulating type-A response regulators expression, and therefore, CK signaling (ZHANG et al., 2015). Moreover, the potato *StSPL9* seems to participate in CK-related response by activating some cellular division genes, but such function still needs to be further investigated (BHOGALE et al., 2014). Additionally, the maize *UNBRANCHED3* (*UB3*) gene has also been demonstrated to negatively regulate type-A response regulators, interfering in CK response-related gene expression (DU et al., 2017). *UB3* also downregulated CK biosynthesis by regulating the *LONELY GUY 1* (*LOG1*) gene expression (DU et al., 2017). Therefore, we observed the gene expression in our three genotypes in both conditions at 12 hai and 40 dai to check these CK-related genes that are important in this pathosystem (Figure 17).

Regarding the CK biosynthesis-related genes *IPT3* and *TOMATO LONELY GUY 1* (*TLOG1*), we noticed in both times that, while no difference was observed among all control samples for the *TLOG1* gene, 156-OE control samples had higher expression of *IPT3* (Figure 17a, b). The induction of CK biosynthesis-related genes in our plants that have low miR156-targeted *SPL/SBP-box* genes (156-OE) was expected, once this pathway was induced for arabidopsis (ZHANG et al., 2015), potato (BHOGALE et al., 2014), maize, and rice (DU et al., 2017) with low *SPL/SBP-box* genes levels.

Curiously, at 12 hai, all inoculated plants showed an induced *IPT3* compared to control, but 156-OE plants had a lower induction (Figure 17a). However, at the same time, MT inoculated plants did not change the *TLOG1* expression, 156-OE inoculated plants displayed a reduced expression, and rSBP15-OE inoculated plants had a higher expression compared to their controls (Figure 17a). Oppositely, at 40 dai we observed that all inoculated samples had repressed *IPT3* and *LOG1* genes (Figure 17b), similar to the described to occur in MT at 30 dai (Costa et al., 2021). The increase at 12 hai in *IPT3* for all inoculated genotypes and in *TLOG1* for rSBP15-OE could be result of a CK production compensation due to the increased CK degradation by induction of *CKX* observed in all inoculated samples (Figure 17a).

As abovementioned, we observed that all inoculated samples had an increase in *CKX2* expression, compared to their controls (Figure 17a, b). The *CKX2* induction regardless the reduced expression of CK-biosynthesis genes in 40 dai infected samples could be due to processing of fungi-produced CK (COSTA et al., 2021). However, we noticed that rSBP15-OE inoculated plants had a higher induction of *CKX2* than MT and 156-OE inoculated plants at both times (Figure 17a, b). In addition, we observed that although 156-OE inoculated plants had no difference in *CKX2* induction compared to MT inoculated plants at 12 hai (Figure 17a),

at 40 dai we observe a lower *CKX2* induction in this genotype (Figure 17b). Since CK delays senescence and cell death (GUO; GAN, 2011), such difference could be a possible decrease in pathogen-derived CK levels. This decrease could be due to the start of the necrotrophic stage (which cell death is induced) in 156-OE infected stems (Figures 11, 12, 15).

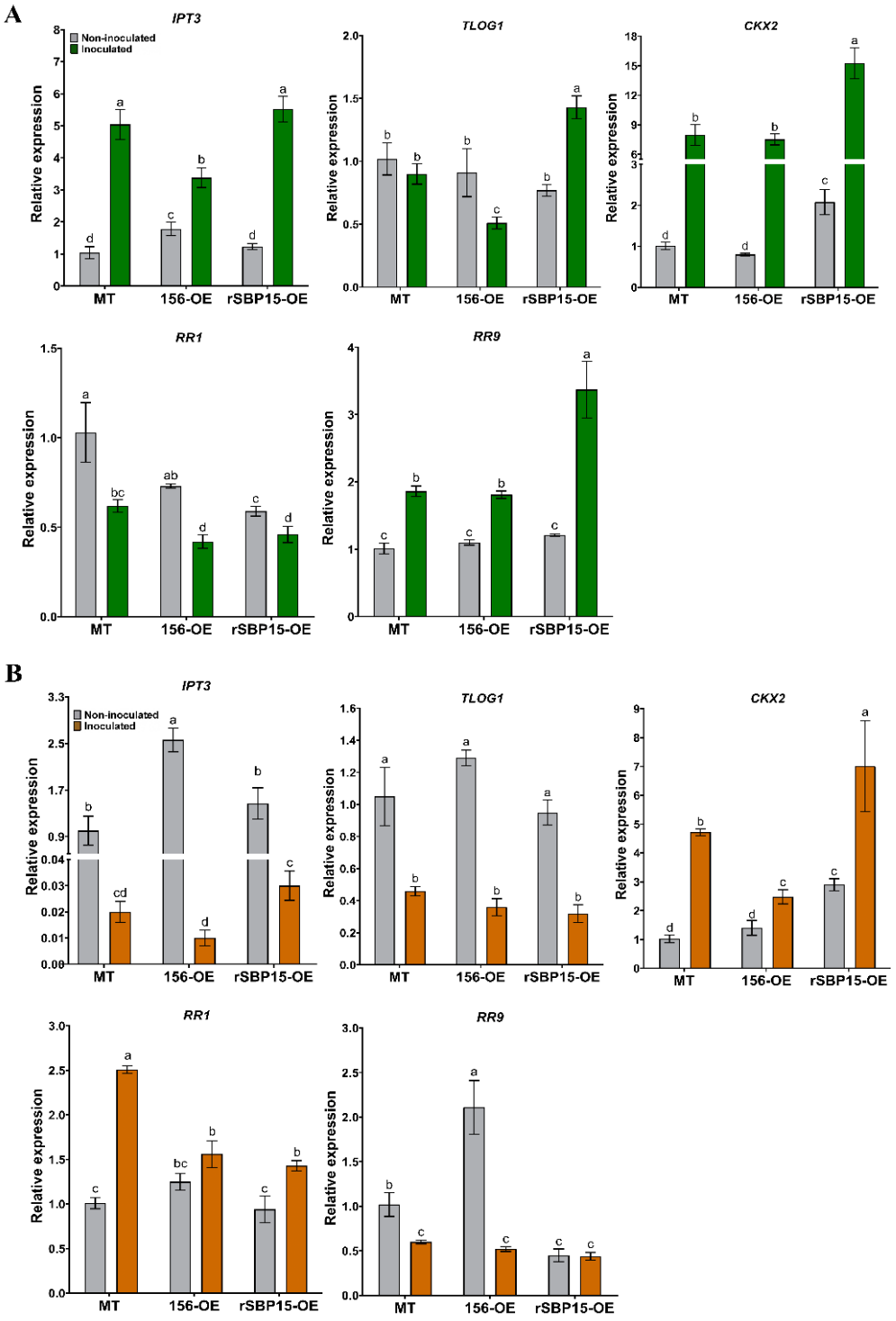

Figure 17 – *AtMIR156b* and *SlSBP15* overexpression altered cytokinin-related gene expression in tomato (A) Comparative expression analysis in tomato cv. MT shoots at 12 hai. (B) Comparative expression analysis in tomato cv. MT stems (between the first and second leaves) at 40 dai. The quantitative RT–PCR experiments used non-inoculated MT as the reference sample (set to 1.0). Error bars indicate standard error of three biological samples. Different letters show statistically different means according to the Tukey's test.

In non-inoculated samples, we saw that rSBP15-OE control plants have more *CKX2* transcripts than the other genotypes at both times (Figure 17a, b), which also was described in plants that overexpresses *SlSBP15* orthologs (BHOGALE et al., 2014; ZHANG et al., 2015; DU et al., 2017). Moreover, our group has demonstrated that overexpression of the Arabidopsis thaliana *AtCKX2* gene reduces the susceptibility of MT to *M. perniciosa* (COSTA et al., 2021). Thus, the increased *SlCKX2* expression in rSBP15-OE plants could explain the attenuated symptoms found in this genotype (Figures 15, 16, and 17).

Regarding the CK biosynthesis-related genes *IPT3* and *TOMATO LONELY GUY 1* (*TLOG1*), we noticed in both times that, while no difference was observed among all control samples for the *TLOG1* gene, 156-OE control samples had higher expression of *IPT3* (Figure 17a, b). The induction of CK biosynthesis-related genes in our plants that have low miR156-targeted *SPL/SBP-box* genes (156-OE) was expected, once this pathway was induced for arabidopsis (ZHANG et al., 2015), potato (BHOGALE et al., 2014), maize, and rice (DU et al., 2017) with low *SPL/SBP-box* genes levels.

Curiously, at 12 hai, all inoculated plants showed an induced *IPT3* compared to control, but 156-OE plants had a lower induction (Figure 17a). However, at the same time, MT inoculated plants did not change the *TLOG1* expression, 156-OE inoculated plants displayed a reduced expression, and rSBP15-OE inoculated plants had a higher expression compared to their controls (Figure 17a). Oppositely, at 40 dai we observed that all inoculated samples had repressed *IPT3* and *LOG1* genes (Figure 17b), similar to the described to occur in MT at 30 dai (COSTA et al., 2021). The increase at 12 hai in *IPT3* for all inoculated genotypes and in *TLOG1* for rSBP15-OE could be result of a CK production compensation due to the increased CK degradation by induction of *CKX* observed in all inoculated samples (Figure 17a).

As abovementioned, we observed that all inoculated samples had an increase in *CKX2* expression, compared to their controls (Figure 17a, b). The *CKX2* induction regardless the reduced expression of CK-biosynthesis genes in 40 dai infected samples could be due to processing of fungi-produced CK (COSTA et al., 2021). However, we noticed that rSBP15-OE inoculated plants had a higher induction of *CKX2* than MT and 156-OE inoculated plants at both times (Figure 17a, b). In addition, we observed that although 156-OE inoculated plants had no difference in *CKX2* induction compared to MT inoculated plants at 12 hai (Figure 17a), at 40 dai we observe a lower *CKX2* induction in this genotype (Figure 17b). Since CK delays senescence and cell death (GUO; GAN, 2011), such difference could be a possible decrease in pathogen-derived CK levels. This decrease could be due to the start of the necrotrophic stage (which cell death is induced) in 156-OE infected stems (Figures 11, 12, 15). In non-inoculated

samples, we saw that rSBP15-OE control plants have more *CKX2* transcripts than the other genotypes at both times (Figure 17a, b), which also was described in plants that overexpresses *SlSBP15* orthologs (BHOGALE et al., 2014; ZHANG et al., 2015; DU et al., 2017). Moreover, our group has demonstrated that overexpression of the Arabidopsis thaliana *AtCKX2* gene reduces the susceptibility of MT to *M. perniciosa* (COSTA et al., 2021). Thus, the increased *SlCKX2* expression in rSBP15-OE plants could explain the attenuated symptoms found in this genotype (Figures 15, 16, and 17).

Regarding the expression of CK response regulators (RRs), we evaluated the expression of the *RR1* and *RR9*, which are one representant of each type of RRs (HE et al., 2016). Both were described to be influenced by *M. perniciosa* infection (COSTA et al., 2021). While the type-A RR *SlRR1* gene was downregulated at 24 hai and upregulated at 30 dai, the type-B RR *SlRR9* showed opposite results (COSTA, 2017; COSTA et al., 2021). Consistently, our MT inoculated samples displayed the same expression pattern as 24 hai at 12 hai (Figure 17a), and as 30 dai at 40 dai (Figure 17b). Similarly, at 12 hai, 156-OE infected plants showed a repression and an induction of *RR1* and *RR9*, respectively (Figure 17a), but *RR1* repression was slightly higher than MT inoculated. However, at 40 dai, no difference in *RR1* expression was observed for this genotype. Moreover, at this time, 156-OE control plants showed an increased *RR9* expression, while inoculated plants showed the same repression as MT inoculated samples (Figure 17b). This increase in 156-OE control plants could be due to the higher expression of *IPT3*, which could be producing more CK in those plants, increasing *RR9* as part of CK-response. The increased CK synthesis and response in 156-OE plants could be facilitating the formation of more severe symptoms induced by *M. perniciosa* in 156-OE inoculated plants (Figures 10, 15, 16), since our group established the CK is important for symptom formation (COSTA et al., 2021).

Curiously, rSBP15-OE control plants showed a reduced expression of *RR1* at 12 hai and inoculated samples had even more repression of RR1 (Figure 17a). Such downregulation could be occurring by a similar mechanism presented by the interaction of arabidopsis *AtSPL9* (*SlSBP*15 ortholog) and *ARR2* (*SlRR9* ortholog) proteins, which negatively impact *ARR1* (*SlRR1* ortholog) and other CK-response-related genes expression (ZHANG et al., 2015). The expression of *RR9* in rSBP15-OE control plants had no difference compared to MT, but inoculated samples had the highest *RR9* expression (Figure 17a). The higher expression of *RR9* in rSBP15-OE inoculated plants could be the combination of the induction of *RR9* by the presence of pathogen-derived CK with an attempt to compensate the reduced *RR9* activity that could be caused by the interaction with *SlSBP15* protein, similar to the interaction between

AtSPL9 and *ARR2*. However, such possibility needs to be investigated. Moreover, at 40 dai, rSBP15-OE control plants showed no difference in *RR1* expression compared to MT (Figure 17b). But the expression of *RR9* was reduced, with similar expression of MT and 156-OE inoculated plants, and also rSBP15-OE inoculated plants (Figure 17b). Such reduction in *RR9* expression in control plants could be due to the increased *CKX2* gene expression observed in this genotype, which is lowering the CK availability in those plants, resulting in lowered *RR9* expression.

In summary, we observed that 156-OE and rSBP15-OE plants had opposite CK-related response. While 156-OE plants have higher levels of the CK-biosynthesis gene *IPT3*, rSBP15-OE plants have higher levels of the CK-degradation *CKX2* gene (Figure 17). This results in increased and reduced CK-related response in 156-OE and rSBP15-OE plants, respectively. Because CK was shown to be crucial to the *M. perniciosa*-host interaction (COSTA et al., 2021), this difference between both genotypes in CK-response gene expression matches the difference observed in symptom severity. However, as demonstrated for *SlSBP15* orthologs in rice and arabidopsis, the increased resistance and reduced pathogen colonization found in those plants might also be associated in immune response-related gene expression (WANG et al., 2018; LIU et al., 2019; YIN et al., 2019; ZHANG et al., 2020a). Therefore, we compared these genotypes in regards to defense-related genes.

4.9 *SlSBP15* attenuate *M. perniciosa* colonization by increasing immune response-related gene expression

As aforementioned, 156-OE and rSBP15-OE plants had more severe and attenuated symptoms, respectively, in response to *M. perniciosa* infection (Figure 16). Thus, we collected samples of infected and control plants at 12 hai and at 40 dai to check gene expression. We started by looking for defense-related genes that might be responsible for this difference in symptom severity (Figure 18). First, we confirmed that repression of *SlSBP15* is part of the infection from the first hours after inoculation in both MT and 156-OE, but no change was observed in rSBP15-OE (Figure 18a). Then we looked into the ERF family defense-related genes, like *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), *PATHOGENESIS-RELATED GENES TRANSCRIPTIONAL ACTIVATOR Pto-INTERACTING 5* (*PTi5*), which were described to positively regulate immune response against fungal pathogens (Figure 18) (ZHOU; TANG; MARTIN, 1997; HE et al., 2001; OUYANG et al., 2016; ZHANG et al., 2020b).

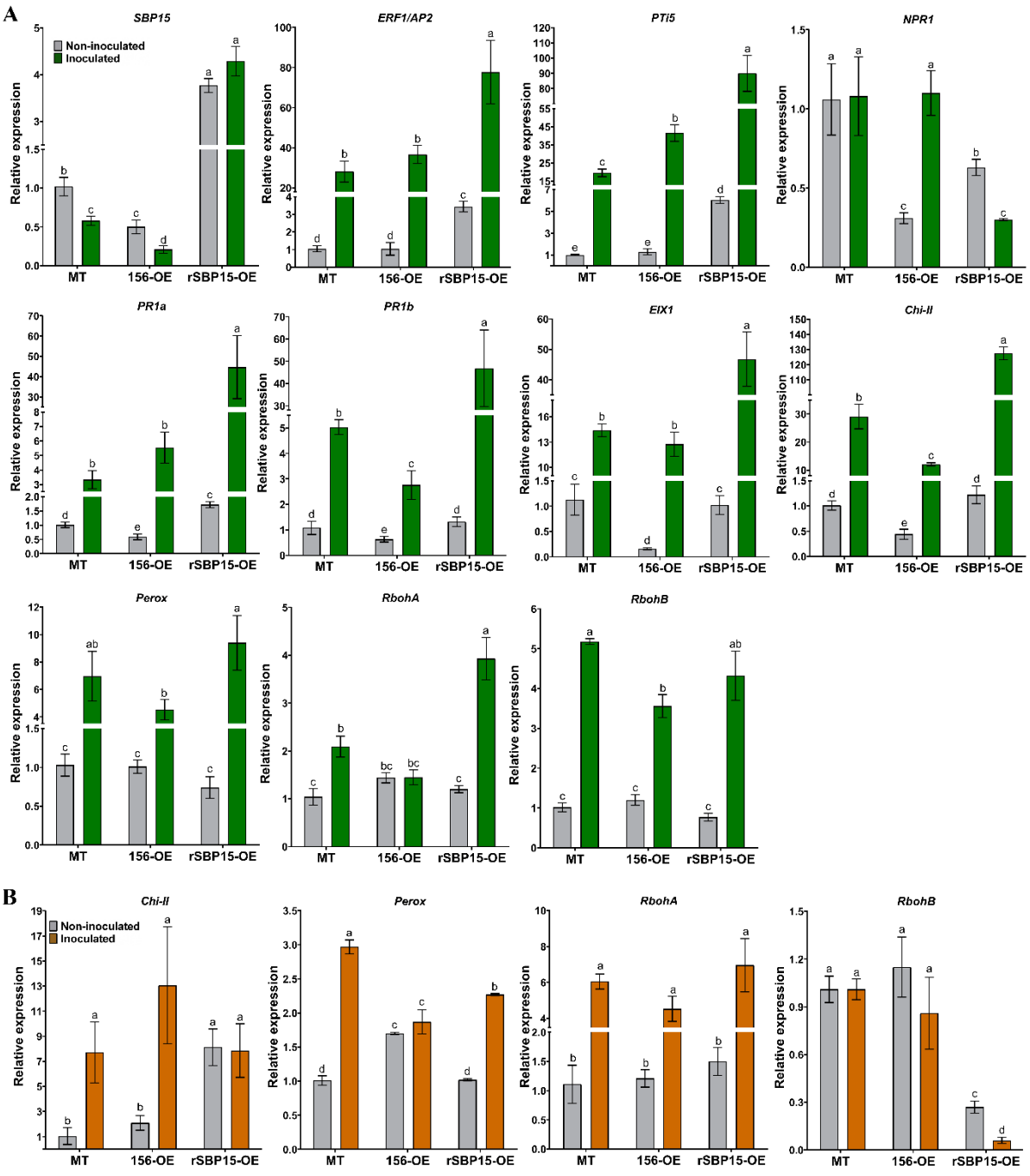

Figure 18 - *SlSBP15* overexpression increased defense-related and reduced ROS production-related gene expression (A) Comparative expression analysis in tomato cv. MT shoots at 12 hai. (B) Comparative expression analysis in tomato cv. MT stems (between the first and second leaves) at 40 dai. The quantitative RT–PCR experiments used non-inoculated MT as the reference sample (set to 1.0). Error bars indicate standard error of three biological samples. Different letters show statistically different means according to the Tukey's test.

We also looked into genes that are related to detection and production of fungal pathogen-associated molecular patterns (PAMP), such as ETHYLENE-INDUCING XYLANASE RECEPTOR 1 (*EIX1*) and *CHITINASE II* (*Chi-II*) (Figure 18) (BAR et al., 2010; CAO; TAN, 2019). Regarding the systemic acquired resistance (SAR) mediated by salicylic acid (SA), we looked into the expression of *NONEXPRESSOR OF PATHOGENESIS-*

RELATED GENES 1 (*NPR1*), which were related to *SlSBP15* orthologs and induces the expression of Pathogenesis-Related (PR) genes, triggering SA-related immune response (Figure 18) (BACKER; NAIDOO; VAN DEN BERG, 2019). We also evaluated the expression of the extensively related to positive regulation of immune response PR genes *PATHOGENESIS-RELATED GENE 1a* and *1b* (*PR1a* and *PR1b*) (Figure 18) (TORNERO et al., 1997; VAN LOON; VAN STRIEN, 1999). These genes already have been related to this plant-pathogen interaction and have been shown to be induced by *M. perniciosa* infection (DEGANELLO et al., 2014; TEIXEIRA et al., 2014; COSTA et al., 2021).

We noticed in our data that rSBP15-OE control plants have higher expression of *ERF1*, *PTi5*, and *PR1a* compared to control MT (Figure 18a). *ERF1* and *PTi5* are members of the APETALA2 (AP2) family of transcription factors. This superfamily is composed of ERF, AP2, and RELATED TO ABI3/VP (RAV) subfamilies (NAKANO et al., 2006). From these, the ERF subfamily was already related to plant development, ethylene-related responses, and stress responses (ELLIOTT et al., 1996; DUBOUZET et al., 2003; WARD et al., 2006; GUTIERREZ et al., 2007; QIN et al., 2007). From this subfamily, the *ERF1* gene was described to positively regulate defense response to pathogens in tomato, including *Botrytis cinerea*, reducing lesion size (OUYANG et al., 2016; ZHANG et al., 2020b). Likewise, the ERF family member *PTi5* gene participates in immune response by inducing the expression of PR genes (*e.g. PR1a* and *PR1b*) independently of *NPR1* gene expression, which triggers immune response (ZHOU; TANG; MARTIN, 1997; HE et al., 2001).

PR genes comprises a class of proteins that were described to be responsive to stress conditions (TORNERO et al., 1997; AKBUDAK; YILDIZ; FILIZ, 2020). More specifically, these proteins are induced by pathogen infection and assist in immune response with or without SA. Tomato *PR1a* and *PR1b* have extensively been demonstrated positively influence plant immune response (TORNERO et al., 1997; VAN LOON; VAN STRIEN, 1999). Therefore, the increased expression of these genes in rSBP15-OE plants could be responsible for the increased resistance of this genotype for the faster activation of defense-related routes, such as *PR1a*. As abovementioned, the induction of these genes has been demonstrated to assist in immune response and resistance to pathogenic attack (TORNERO et al., 1997; ZHOU; TANG; MARTIN, 1997; VAN LOON; VAN STRIEN, 1999; HE et al., 2001; OUYANG et al., 2016; ZHANG et al., 2020b). Moreover, *ERF1*, *PTi5*, *PR1a*, and *PR1b* were all induced in rSBP15-OE inoculated samples (Figure 18a). Such induced expression could be responsible to the attenuated symptom severity and also the less pathogen colonization found in those plants (Figure 16). The expression of *NPR1* (which are upstream of *PR1a* and *PR1b*) was lower in

rSBP15-OE control plants (Figure 18a). However, the induction of PRs could be by the increased expression of *PTi5*, that uncouples the PR gene expression from *NPR1* gene (ZHOU; TANG; MARTIN, 1997; HE et al., 2001). Consistently, 156-OE control plants display reduced expression of *NPR1*, *PR1a*, and *PR1b* (Figure 18a) defense-related genes, as demonstrated for other plants with low *SPL/SBP* levels (*e.g.* arabidopsis and rice) (LIU et al., 2019; YIN et al., 2019; ZHANG et al., 2020a). This could explain the increased susceptibility of this genotype shown in higher pathogen colonization and more severe symptoms found in this genotype (Figures 10-16).

Regarding the PAMP-related genes, our data showed that 156-OE plants display reduced expression of *EIX1* and *Chi-II*, which could also be responsible for the increased susceptibility of this genotype (Figure 18a). The ethylene-inducing xylanase (EIX) is a fungal protein that induced immune responses in tomato and tobacco. The *EIX1* gene encodes for a receptor plant protein that detects EIX in the apoplast (PAMP detection), triggering immune response (BAR et al., 2010). *M. perniciosa* is a fungus that resides in host apoplast during the biotrophic stage, therefore, the expression of *EIX1* could be associated with detection of the pathogen to trigger defense against it. Meanwhile, the *Chi-II* gene is responsible for the production of other PAMP. This gene encodes for a chitinase enzyme that catalyzes chitin. Because chitin is absent in plants, the chitinases enzymes participate in immune response by hydrolyzing the pathogen cell walls to release elicitors for defense reactions (CAO; TAN, 2019). Our group has demonstrated that an incompatible interaction between C-biotype *M. perniciosa* strain and MT plants led to accumulation of *Chi-II*, *PTi5*, and *EIX1* transcripts, and those plants did not present the characteristic swelling symptom (DEGANELLO et al., 2014). Therefore, lower expression of both these genes in 156-OE plants hinders the detection of the pathogen, which could be slowing immune response. In addition, rSBP15-OE inoculated plant showed the highest levels of both these genes (Figure 18a). Such induction probably is assisting this genotype to activate defense-related genes in response to *M. perniciosa*, leading to the reduced symptom severity observed (Figure 16), like to the reported to occur in the incompatible C-biotype *M. perniciosa* strain and MT interaction (DEGANELLO et al., 2014).

Because we observed that PRs are very induced in response to this pathogen, we looked into genes related to the accumulation of reactive oxygen species (ROS) in host. ROS accumulation in response to pathogens is usually related with PR-related immune responses (HERRERA-VÁSQUEZ; SALINAS; HOLUIGUE, 2015). Respiratory burst oxidase homologs (Rbohs) are key enzymes that catalyze the generation of ROS in plants for diverse cellular mechanisms and play multiple signaling roles in a wide range of organisms (LI et al., 2015).

In this context, tomato *RbohB* have been shown to be involved in wounding responses, development, tolerance to drought stress, and resistance to *Botrytis cinerea* (SAGI et al., 2004; LI et al., 2015). Meanwhile, although RbohA is also induced by B. cinerea infection, no significant difference in infection was detected by silencing this gene (LI et al., 2015). Therefore, this gene might have a role in other aspect of ROS accumulation, as shown for other arabidopsis Rbohs (LI et al., 2015).

Arabidopsis *AtSPL9* have been described to positively regulate PRs which could result in higher ROS accumulation in response to pathogen attack (YIN et al., 2019). Therefore, we hypothesized that its tomato homolog, *SlSBP15*, could have a similar function that would assist in immune response. To have an idea if any difference in the ROS production and processing was occurring in our experiment, we looked for *RbohA*, *RbohB*, and *Peroxidase* (*Perox*) gene expression at 12hai (Figure 18a) and at 40dai (Figure 18b). We chose RbohA because although it has no effect in disease resistance, it was highly induced in response to *B. cinerea* infection and also plays a role in ROS production (LI et al., 2015).

No difference was observed among control plants of 156-OE, rSBP15-OE, and MT genotypes in *RbohA*, *RbohB*, and *Peroxidase* (*Perox*) gene expression at 12 hai (Figure 18a). However, rSBP15-OE inoculated plants had higher expression of those genes at this time and increased ROS accumulation could assist in disease resistance against *M. perniciosa* biotrophic phase, similarly to described for *AtSPL9* (YIN et al., 2019). The lower induction of *RbohB* and the absent induction of *RbohA* in 156-OE inoculated plants could be result of the compromised immune response by lower expression of important defense-related genes in 156-OE plants (Figure 18), which was also demonstrated in arabidopsis (YIN et al., 2019). At 40 dai we notice that, while rSBP15-OE plants have lower expression of *RbohB*, MT and 156-OE plants had no difference in the expression, for both control and inoculated samples (Figure 18b). Repression of this gene have already been proved to confer resistance to *Botrytis cinerea*, a necrotrophic pathogen of tomato (LI et al., 2015). Thus, the repression of such gene in rSBP15-OE plants compared to MT could potentially be result of the reduced pathogen growth in this genotype and could be increasing the resistance against *M. perniciosa*. No difference was observed in *RbohA* expression between MT and rSBP15-OE control plants at 12 hai (Figure 18a). Similarly, at 40dai, MT, 156-OE, and rSBP15-OE inoculated plants had no difference among them (Figure 18b), and the same was among control samples of all genotypes (Figure 18b). This was expected, once this gene does not seem to participate in plant defense, potentially having roles in other aspects of ROS-related responses (LI et al., 2015).

The expression of the *Perox* gene was higher in inoculated plants (Figure 18a, b), as previously described to occur by our group (DEGANELLO et al., 2014). Such upregulation could be processing the ROS accumulated due to pathogen infection (ALMAGRO et al., 2009). Although no difference was detected in *Perox* expression among control samples of all genotypes at 12 hai (Figure 18a), we notice that 156-OE inoculated plants had lower induction of *Perox* by the infection. This lower induction could possibly be, as aforementioned, result of the compromised immune response in 156-OE plants (Figure 18). Since 156-OE plants have lower expression of defense related genes, they could be accumulating less ROS which would reduce *Perox* expression, which is usually downstream to some canonical immune responses (ALMAGRO et al., 2009).

However, at 40 dai 156-OE inoculated and control plants had a slight increase in *Perox* expression, however it still lower than MT inoculated plants and rSBP15-OE inoculated plants (Figure 16b). This low expression in 156-OE inoculated plants could be for the casement of ROS processing due to tissue death (Figures 11, 12, 15). Meanwhile, at 40 dai we also notice that rSBP15-OE inoculated plants had a lower induction of *Perox* than MT inoculated plants (Figure 18b). This could be due to the decrease of ROS accumulation in those plants, since they have less pathogen colonization and reduced symptoms (Figure 16). Altogether, since rSBP15-OE plants showed increased expression of defense-related genes (Figure 18) and attenuated symptoms (Figure 16), our results suggest that the repression of this gene by *M. perniciosa* infection (Figures 7, 8, 12) hinders plants immune response, increases CK-related genes, and decreases CK degradation, which could favor colonization and symptom formation (Figure 19).

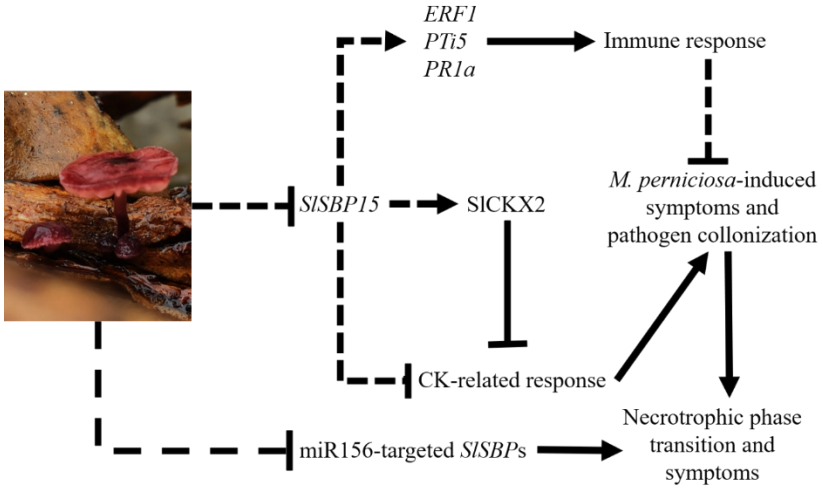

Figure 19 - *M. perniciosa* modulates *SlSBP*s to increase symptom severity and pathogen growth, leading to necrotrophic phase transition

5 CONCLUSIONS AND PERSPECTIVES

The use of MT as a tool to explore the *M. perniciosa*-host interaction has provided the possibility of more detailed and quicker studies of this complex interaction (DEGANELLO et al., 2014; PASCHOAL, 2018; COSTA et al., 2021). The transcript profiling of this interaction in MT and cacao (TEIXEIRA et al., 2014; COSTA et al., 2021) allowed us to see that *M. perniciosa* represses *SPL/SBP-box* genes throughout the interaction. Here, we show that the repression of *SPL/SBP-box* genes is an important part of *M. perniciosa* infection strategy. We showed that strong repression of miR156-targeted *SlSBP*s by miR156 (156-OE) in tomato results in increased susceptibility against *M. perniciosa*. Moreover, such repression led to higher pathogen colonization, and necrotrophic phase transition and completion for the first time reported in a tomato genotype. In addition, overexpression of a version of the mir156-targeted *SlSBP15*, resistant to miR156 regulation (rSBP15-OE), reduced both symptom severity and *M. perniciosa* colonization. Moreover, rSBP15-OE plants showed increased levels of defense-related genes and the *SlCKX2* cytokinin-degradation gene and showed downregulated *SlRR1* CK-response gene. Cytokinin is a plant hormone that is important to the formation of symptoms and for the susceptibility to *M. perniciosa* (COSTA et al., 2021).

Therefore, our findings suggest that *SlSBP15* might be another example of an *SPL/SBP-box* gene that could be used as a resistance source through genetic manipulation. However, further exploration is still needed to elucidate this mechanism in cacao plants to use it for *M. perniciosa* resistance. Moreover, the change to the necrotrophic stage induced by the repression of miR156-targeted *SlSBP* genes still needs to be further clarified. It will be relevant to establish which *SlSBP* gene is associated with *M. perniciosa* necrotrophic phase transition and how these and related genes affect the interaction. In this regard, high-resolution transcript profiling analysis (mRNA-seq) of this stage in 156-OE inoculated plants will shed light on genes associated with *M. perniciosa* phase transition in tomato plants. Considering that little is known regarding this transition and also to what extent *SPL/SBP-box* genes are associated with WBD development, understanding how *M. perniciosa* interferes with *SPL/SBP-box* pathways is also a pertinent future research topic. Overall, the results presented in this work establish a model for the study of the phase transition of this peculiar plant disease in tomato. In addition, we provided an insight into the relation of *SlSBP15* and CK-related response in an important tropical crop.

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ANNEX

Annex A – Collaboration Published during the Master's degree

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Research

Moniliophthora perniciosa, the causal agent of witches' broom disease of cacao, interferes with cytokinin metabolism during infection of Micro-Tom tomato and promotes symptom development

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Summary

• *Moniliophthora pemiciosa* causes witches' broom disease of cacao and inflicts symptoms suggestive of hormonal imbalance. We investigated whether infection of the tomato (*Solanum lycopersicum*) model system Micro-Tom (MT) by the Solanaceae (S)-biotype of *Moniliophthora pemiciosa*, which causes stem swelling and hypertrophic growth of axillary shoots, results from changes in host cytokinin metabolism.

• Inoculation of an MT-transgenic line that overexpresses the Arabidopsis *CYTOKININ OXIDASE*-2 gene (*35S::AtCKX2*) resulted in a reduction in disease incidence and stem diameter. RNA-sequencing analysis of infected MT and *35S::AtCKX2* revealed the activation of cytokinin-responsive marker genes when symptoms were conspicuous.

• The expression of an *Moniliophthora perniciosa tRNA-ISOPENTENYL-TRANSFERASE* suggests the production of isopentenyladenine (iP), detected in mycelia grown *in vitro*. Inoculated MT stems showed higher levels of dihydrozeatin and *trans*-zeatin but not iP. The application of benzyladenine induced symptoms similar to infection, whereas applying the cytokinin receptor inhibitors LGR-991 and PI55 decreased symptoms.

• *Moniliophthora perniciosa* produces iP that might contribute to cytokinin synthesis by the host, which results in vascular and cortex enlargement, axillary shoot outgrowth, reduction in root biomass and an increase in fruit locule number. This strategy may be associated with the manipulation of sink establishment to favour infection by the fungus.

Introduction

Pathogens have developed the ability to alter hormone signalling to promote virulence and/or induce susceptibility by altering host physiology (Spallek *et al.*, 2018; Han & Kahmann, 2019). Cytokinins are responsible for regulating several mechanisms concerning plant growth and development, including the maintenance and differentiation of root and stem meristems, cell division, vascular differentiation, delay of senescence, and source-sink relationships (Werner & Schmülling, 2009). More recently, the role of cytokinins in plant abiotic and biotic stress responses

Natural plant cytokinins include N^6-(Δ^2-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ). Cytokinins are derived from substitutions at the N^6-terminus of an adenine or from the degradation of transfer RNAs (tRNAs) (Sakakibara, 2006). In brief, isopentenyl transferases (IPTs) either catalyse the linking of an isopentenyl to adenosine diphosphate (ADP) or adenosine triphosphate (ATP) or metabolize tRNAs, producing iP and cZ, respectively. tZ is then derived from the hydroxylation of iP by cytochrome monooxygenase (Kiba *et al.*, 2013). DHZ is considered to be derived from the reduction of tZ (Martin *et al.*, 1989), but new evidence suggests