

**University of São Paulo
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**Deciphering the role of early molecular interactions between *Eucalyptus*
spp. x *Austropuccinia psidii* and its pathogenesis**

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Thesis presented to obtain the degree of Doctor in
Science. Area: Agricultural Microbiology

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RESUMO

Desvendando a patogênese e o papel das interações moleculares precoces entre *Eucalyptus* spp. x *Austropuccinia psidii*

Austropuccinia psidii é o agente causal da ferrugem das mirtáceas com crescimento biotrófico, ou seja, o patógeno depende dos tecidos do hospedeiro para crescer e se desenvolver. Os uredósporos de *A. psidii* infectam *Eucalyptus* por meio do contato inicial com a superfície do hospedeiro e também pela interação com a cutícula da folha que por sua vez fornece importantes sinais químicos e físicos capaz de desencadear o processo de infecção. Devido às características inerentes à cutícula de *Eucalyptus*, consideramos as hipóteses de que o mecanismo pré-formado, composto principalmente pelas ceras cuticulares, desempenha um papel crucial na resistência de *Eucalyptus* spp. contra *A. psidii*, e, também, é capaz de modular a expressão fúngica de genes associados a patogenicidade durante o estágio inicial de infecção de *A. psidii*. No capítulo 2, as ceras cuticulares de *Eucalyptus* spp. foram analisadas para determinar a composição/estrutura e sua correlação com suscetibilidade/resistência de *A. psidii*. Vinte e uma espécies de *Eucalyptus* foram classificadas em campo como resistentes ou suscetíveis. A análise de qPCR de seis *Eucalyptus* spp. revelou que o patógeno pode germinar na superfície de algumas espécies de eucaliptos sem se multiplicar no tecido hospedeiro. Foram identificados 26 compostos presentes na cutícula de *Eucalyptus* spp. e descobrimos o papel do ácido hexadecanóico na suscetibilidade de *E. grandis* e *E. phaeotricha* à ferrugem. Por meio da microscopia eletrônica de varredura encontramos uma correlação entre a morfologia epicuticular e a resistência contra *A. psidii*. No capítulo 3 para compreender a expressão gênica de *A. psidii* realizamos bioensaios (*in vitro*) contendo as ceras cuticulares de *E. grandis* (*E. g*), *E. urograndis* (*E. ug*) e *E. urophylla* (*E. u*). O tratamento com óleo mineral (MO) foi utilizado em todas as análises comparativas como controle negativo. A presença de ceras cuticulares de *E. g* induziu a expressão de genes que codificam proteínas relacionadas ao crescimento e colonização de *A. psidii*, como proteínas de ligação (*peptidylprolyl isomerase* e *ribosomal*) e proteínas de degradação da parede celular (*beta xylanase*). No entanto, outras proteínas patogênicas foram reprimidas na presença da cera cuticular de *E. g*, por exemplo, *triosephosphate isomerase, family 18 glycoside hydrolase, mitochondrial ATP carrier* e *glutamine-dependent NAD synthetase*. A análise de *E. ug* x MO resultou na ativação de proteínas associadas a transportadores e receptores de membrana, reparo de DNA e *glycine dehydrogenase*. Já a cera cuticular de *E. u* induziu a expressão de genes que codificam proteínas associadas a feromônios, cutinases e *prefoldin*. Pela primeira vez, está sendo apresentado a considerável variação interespecífica em espécies de *Eucalyptus* quanto à suscetibilidade a ferrugem, e, sua correlação com os compostos químicos de ceras cuticulares, os quais parecem ser um importante mecanismo de defesa pré-formado. Também foi revelado que as ceras cuticulares de *Eucalyptus* spp. são capazes de modular a expressão gênica de *A. psidii*, evidenciando o papel da interação molecular planta-patógeno precoce no desenvolvimento da ferrugem das mirtáceas.

Palavras-chave: Lipidômica, Transcriptoma, Fungo biotrófico, Mecanismo de defesa pré-formado

ABSTRACT

Deciphering the role of early molecular interactions between *Eucalyptus* spp. x *Austropuccinia psidii* and its pathogenesis

Austropuccinia psidii, the causal agent of myrtle rust, is a biotrophic pathogen, and therefore its growth and development depend on the host tissues. The uredospores of *A. psidii* infect *Eucalyptus* by engaging in close contact with the host surface and interacting with the leaf cuticle that provides important chemical and physical signals to trigger the infection process. Due to the inherent characteristics of the *Eucalyptus* cuticle, it was hypothesized that the preformed mechanism, comprised mostly by cuticular waxes, plays a crucial role in *Eucalyptus* resistance against *A. psidii* and its ability to modulate the expression of genes associated to the pathogenicity of *A. psidii* during the early stage of infection. In chapter 2, the cuticular waxes of *Eucalyptus* spp. were analyzed to determine their composition or structure and then correlated to susceptibility/resistance to *Austropuccinia psidii*. Twenty-one *Eucalyptus* spp. in the field were classified as resistant or susceptible. From these, the resistance/susceptibility level of six *Eucalyptus* spp. was evaluated in controlled conditions using qPCR, revealing that the pathogen can germinate on the eucalyptus surface of some species without multiplying in the host. CG-TOF-MS analysis detected 26 compounds in the *Eucalyptus* spp. cuticle and led to the discovery of the role of hexadecanoic acid in the susceptibility of *E. grandis* and *E. phaeotricha* to *A. psidii*. The scanning electron microscopy check revealed differences in *A. psidii* germination during host infection. It was found a correlation between epicuticular morphology and the resistance to *A. psidii*. In chapter 3, we investigated gene expression of *A. psidii* through bioassays *in vitro* containing cuticular waxes from *E. grandis* (*E. g*), *E. urograndis* (*E. ug*) and *E. urophylla* (*E. u*). Mineral oil (MO) treatment was used to all comparative analysis (negative control). The presence of cuticular waxes from *E. g* induced the expression of genes encoding proteins related to growth and colonization of *A. psidii* such as binding proteins (peptidylprolyl isomerase and ribosomal) and cell wall degrading proteins (beta-xylanase). However, other pathogenic proteins were repressed in presence of cuticular wax of *E. g*, for instance, triosephosphate isomerase, family 18 glycoside hydrolase, mitochondrial ATP carrier, and glutamine-dependent NAD synthetase. The *E. ug* x MO analysis resulted in DEGs associated with proteins related to membrane transporters and receptors, DNA repair and glycine dehydrogenase. As to the cuticular wax of *E. u*, it up-regulated the expression of genes encoding proteins associated with pheromone, cutinases, and prefoldin. Thus, for the first time, it was demonstrated a considerable interspecific variation in *Eucalyptus* species on the susceptibility to *A. psidii* and its correlation with cuticular waxes chemical compounds that seem to play a synergistic role as a preformed defense mechanism. We also demonstrated that *Eucalyptus* spp. cuticular waxes may modulate the *A. psidii* gene expression, suggesting the importance of early plant-pathogen molecular interaction to the development of myrtle rust.

Keywords: Lipdomic, Transcriptome, Biotrophic fungal, Preformed defense mechanism

1. INTRODUCTION

The planted forests are greatly important in ecosystems, they provide direct products, e.g. food, fuel and wood as well as environmental benefits such as including watershed protection, nutrient cycling, climate regulation, groundwater recharge, soil conservation, neutralization the negative effects of global warming and reduce the losses of natural forests. Moreover, planted forests have direct influence on economic, social and environmental development (Payn et al., 2015; Degroot et al., 2002; Laurance, 2007; Reddy et al., 2002).

It is estimated that between 1990 and 2010, the global area of planted forest increased by 7% up from 178 million hectares (ha) to 264 million ha (Jürgensen et al., 2014). A study conducted by FSC (Forest Stewardship Council) showed that in 2012 roundwood production from planted forest was 520 millions m³ and the forecast for 2050 will be 1082 million m³, demonstrating great increase of the segment.

Concerning the planted forest areas, China has the largest cultivated area with forest (77.1 million ha – 29.3%), Brazil is in the eighth position (7.4 million ha – 2.6%). However, Brazil occupies the first position in the annual productivity, ranking to roundwood (131.878 million m³). The largest producers (Brazil and United States) contributed with 43% of all produced timber, confirming the excellent management of planted forest in Brazil (Jürgensen et al., 2014; FAO, 2010).

Brazilian planted forests are composed by eucalyptus (76%), pinus (21%) and others (3%) (SNIF, 2016). Approximately 35% of the production is used as raw material to pulp and paper industries; 30% is composed by non-verticalized products; 13% to steel industry; 9% to TIMOs (Timber Investment Management Organizations); 6% to panel and flooring sectors; 4% of wood products and 3% others (IBGE, 2017). In 2014, the planted forestry sector was accountable for 1.1 % of total generated wealth in the country and 5.5 % of Gross Domestic Product. The international marketing of products from planted forests reached approximately US\$ 8 billion, around 3% Brazilian exportation, as well as generation of 5 billion direct and indirect jobs (Virgens et al., 2016; IBÁ, 2015).

The *Eucalyptus* genus is originated from Australia and belongs to Family of Myrtaceae, which has more than 900 described species (Brooker, 2000; Boland et al., 2006). Several eucalyptus species have been used such as planted forests in Brazil, for instance: *E. grandis*, *E. saligna*, *E. camaldulensis*, *E. tereticornis*, *E. urophylla*, *E. cloeziana*, *E. citriodora*, *E. maculata*, *E. pellita*, *E. brassiana*, *E. dunnii* and their several hybrids (Guimarães et al., 2010; Zauza et al., 2010). In Brazil, the expansion of planted forest is majorly due to the eucalyptus fast growth, high capacity of adaptation and production of wood of high quality. It is a consequence of continuous investments in research and development of genetic materials and improvement of silvicultural techniques (Ibá, 2015; Gonçalves et al., 2013).

The rise of planted forest sector is notable and shows the large growth potential for the next years. Nevertheless, the expansion of cultivated areas has favored the emergence of several diseases

that cause serious damage to the production of seedlings in clonal gardens and reforested areas, e.g. myrtle rust. Also, named eucalyptus rust, caused by *Austropuccinia psidii* (G. Winter) Beenken (Beenken, 2017).

A. psidii is a basidiomycete, previously known as *Puccinia psidii* Winter, the pathogen was newly redefined taxonomic position of Pucciniales family for Sphaerophragmiaceae family (Beenken, 2017). *A. psidii* is originated from South America. It was first described in Brazil by Winter in 1884 infecting guava (*Psidium guajava* L.) (Winter, 1884). Although the first recorded disease in *Eucalyptus* (*E. citriodora*) was in 1944, the first significant finding of economic damages in the crop occurred in 1973 in nurseries and plantations until eighteen months of age, in state of Espírito Santo (Joffily, 1944; Ferreira, 1983). Currently, *A. psidii* may be characterized as a cosmopolitan pathogen. It has been reported in different countries in America, including the Caribbean and Pacific Islands (Marlatt and Kimbrough, 1979; Coutinho et al., 1998; Uchida et al., 2006; Glen et al., 2007; Morin et al., 2012), Japan (Kawanishi et al., 2009), Australia (Carnegie et al., 2010), Africa (Roux et al., 2013) and China (Zhuang and Wei, 2011).

In 2000, a study was carried out and reported the areas with the highest risk of rust occurrence (*A. psidii*) in the Neotropical region. It was verified that Brazil has the largest region potentially affected by this phytopathogen (Booth et al., 2000). The researches conducted in seminal plantation of *E. grandis* in the state of Sao Paulo showed an incidence of rust in 35% of plants until 12 months of age, whose reduction was 35% in height and diameter of infected plants (Silveira and Higashi, 2003). These studies demonstrated the great epidemic potential of disease and the risk to productivity of eucalyptus planted forests as well to biodiversity. *A. psidii* can infect approximately 450 species, distributed in 73 genus of Myrtaceae, including *Eucalyptus*, *Corymbia*, *Eugenia* and *Melaleuca*, which demonstrates the serious global threat (Pegg et al., 2014; Carnegie and Cooper, 2011).

A. psidii attacks young, soft, actively-growing leaves, shoot tips and young stems. The presence of pathogen can be observed both in adult plants and in plantules in nursery stage (Ferreira, 1981). The first signs of rust infection are tiny spots or pustules loaded of yellow spores, characterizing the occurrence of disease (Ferreira, 1983). Symptoms can vary depending on the host species, susceptibility level within a host species, and age of the host leaf. In susceptible hosts, one-two weeks after infection, the attacked plant parts are atrophied and have areas with rust stains, in contrast in resistant hosts can occurs the hypersensitive response and the induction of cell death in plants (Ferreira, 1983; Glen et al., 2007).

The incidence and severity of disease depend on species, geographic region and time of year. *A. psidii* is very demanding as regards microclimate conditions and existence of juvenile organs (Abraf, 2013). Among *Eucalyptus* genus, the susceptible species are *E. grandis*, *E. cloeziana*, *E. phaeotricha*, *E. nitens* and *E. globulus*; and resistant: *E. pilularis*, *E. saligna*, *E. camaldulensis*, *E. urophylla*, *E. maculata*, *E. robusta*, *E. citriodora*, *E. tereticornis* and *E. torreliana* (Dianese et al., 1984; Ferreira, 1989, Santos et al. 2019).

The biotrophic pathogens such as *A. psidii* establishes intimate interactions with their hosts, whose success depends on the manipulation of host physiology (Eckardt, 2011). Concerning to *A. psidii*, little is known about genetic variability in pathogen populations and its correlation with the degree of host susceptibility (Quecine et al., 2014, Santos et al., 2019). Furthermore, there are no reports about the successful multiplication of uredospores in culture medium, hampering the molecular studies of this pathogen. This certainly contributes to the scarce number of studies related to genomic and transcriptomic of *A. psidii*, resulting in little knowledge about the genetics and pathogenicity mechanisms this fungus. Therefore, the understanding of *A. psidii* genetic approaches is critical for comprehension of host associations, especially in *Eucalyptus* plants, potential pathways of spread and climatic requirements, all of which facilitate the development of management strategies (Stewart et al., 2018).

The inter-intra specific genetic variability for resistance to rust in eucalyptus species allows the control of disease through clones, progenies and resistant species (Silva et al., 2014). However, the selection of resistant progenies from eucalyptus clones has been a challenge for forest industry and particular importance to eucalyptus breeding programs (Quecine et al., 2014). Little is known about the life cycle of *A. psidii*, which is essential to predict evolutionary potential and developing control strategies, as creation of durable host resistance or the removal of alternative hosts (Billiard et al., 2012; McDonald and Linde, 2002).

A. psidii has three known stages of life cycle: i) uredinial, mitotic and dicariotic stage used to differentiate other rust in mirtaceae; ii) telial stage – teliospores are diploid; and iii) basidial stage – basidiospores with one or two nuclei, its necessary for sexual reproduction (Maier et al., 2015; Morin et al., 2014). The optimum environmental conditions for development of spores in Brazil are 15-20°C (uredospores), 21-25°C (teliospores) and 21°C (basidiospores) (Aparecido, 2001). There are many controversies related to *A. psidii* life cycle, however the life cycle proposed by Glen et al. (2007) is more accepted by the scientific community. These authors consider this fungal as autoecious and macrocyclic rust, i.e. this pathogen complete life cycle in just one host and there is no evidence of spermacia/spermagonia and aecia/aeciospore stages. *A. psidii* is pleomorphic because can development/alter several forms, according to the life cycle stage, reproduction and environmental conditions. There is no information concerning the basidia stage on another host except in fruits of *Syzygium jambos* (McTaggart et al., 2018). Until now, only uredospores have been described as infective agents and sporulation structure in *Eucalyptus* species (Morin et al., 2014).

In the patossystem *Eucalyptus* spp. x *A. psidii* the host tissues penetration occurs differently of most other rust caused by fungi of order Pucciniales, with direct penetration through the cuticle and epidermis, following after appressorium formation (Xavier et al., 2001). Thus, the leaf surface features can strongly influence the development of infection structures and directly contributing to increase resistance or susceptible some species (Bushnell, 1972).

Constitutive or inducible plant defense mechanisms may be either structural or biochemical. Chemical composition and thickness of cuticular wax as well as the presence of trichomes can affect the pre-penetration, penetration and colonization process configuring important plant preformed mechanisms of resistance to phytopathogens (Wynn and Staples, 1981). There are also mechanisms involved in plant resistance after the interaction of pathogen with the host, such as cell wall appositions, tylosis, phenolic compounds accumulation, abscission layer, induction of reactive oxygen species, activation of defense genes, hypersensitivity reactions and others (Boava et al., 2010).

Among the possible plant preformed defense mechanisms, the composition of cuticular wax vary considerably within species, ontogeny and environmental growth conditions (Jenks and Ashworth, 1999). Commonly, the outermost plant barrier is the epidermis that is covered by a lipophilic layer composed mainly of cutin, hydroxylated acids, esterified aliphatic acids, ferulic acid, flavonoids and phenylpropanoids (Albersheim et al., 2011; Yeats and Rose, 2013). The level of plant resistance should be related with leaf cuticular thicknesses, conformations and chemical composition. Moreover, cuticular waxes are one of the first barriers to fungal infection (Stockwell and Hanchey, 1983; Smith et al., 2006).

The cuticle is a limiting region for PAMPs and DAMPs signaling and the host resistance. The increase of thickness as well as alterations in cuticular topography are plant responses associated with the presence of pathogens (Serrano et al., 2014; Marques et al., 2016). Many studies have demonstrated the cuticular waxes effects on pathogen germination such as Santos et al. 2019, Zabka et al., 2014, Hansjakob et al., 2011, Zabka et al., 2007, Reisige et al., 2006, Inuyang et al., 1999 and Kolattukudy et al., 1995.

The development of molecular biology allowed the emergence of omics approaches as powerful analyses tools. The mass spectrometry and the next-generation sequencing, for instance, RNASeq, allowed a better understanding about the first contact between plant x fungal pathogen mediating the morphogenesis of fungal structures and preformed defense mechanisms in the target plant tissue surface (Padliya et al., 2007; Naidoo et al., 2018). Since no single approach can fully clear up the complexity of living organisms, each tool does contributes synergistically to a better knowledge of pathosystems (González-Fernández et al., 2010).

The mass spectrometry is a powerful analytical technique to identify and quantify a diversity of compounds, as instance lipids, due to the combination of sensibility, specificity and selectivity in a short time of analysis as well as being an effective tool for studies with fungal pathogens (Baldwin, 1995; Hopfgartner and Varesio, 2012). Thus, the lipidomic researches play a vital role in identification the biochemical mechanism condition of lipid-related disease progressions through categorizing the changes found in lipid composition (Koriem, 2017). In spite of all those advantages, it is incipient the lipidomic studies of plant x microbe interaction.

The emergence of RNASeq enabled of novel gene discovery and has provided insight into the exploration and understanding of gene expression, allowing in turn to accelerate the description of

complex biological machineries used by fungi, specially by phytophagotogens (Janasz et al., 2018). The first study that used this technique was Velculescu et al. (1977), which analyzed the set of genes expressed from the model yeast during the fungal growth. The transcriptome analysis of 60.633 transcripts revealed 4665 genes, with expression levels varied, providing insight into global patterns of gene expression in yeast. Since then several studies with pathogen x plant interaction are been investigated such as Flor-Parra et al., (2006), López-Berges et al. (2010), Haddadi et al. (2015), Silva et al. (2017) among others.

Therefore, based on the scarcity of knowledge about the molecular mechanisms involved in the early interaction between *A. psidii* and *Eucalyptus* spp., this study aimed to analyze the lipid profile of the host and the transcriptomic of *A. psidii* modulated by cuticular wax from *Eucalyptus* spp. with different degree of susceptibility. Due to the inherent characteristics of the *Eucalyptus* cuticle, it was hypothesized that the preformed mechanism, comprised mostly by cuticular waxes, plays a crucial role in *Eucalyptus* resistance against *A. psidii* and it's able of modular the expression of genes associated to pathogenicity of *A. psidii* during the early stage of infection.

Thus, the specific objectives were to analyze the cuticular waxes composition and their structure of *Eucalyptus* spp. in relation with susceptibility/resistance to *A. psidii*. It was also evaluated the influence of cuticular wax from three *Eucalyptus* species (*E. grandis*, *E. urograndis* and *E. urophylla*) in *A. psidii* gene expression. In the chapter 2, it was demonstrated that *A. psidii* can germinate on *Eucalyptus* spp. surface of some species without multiplying in the host and revealed the role of hexadecanoic acid in the susceptibility of *E. grandis* and *E. phaeotricha* to *A. psidii*. It was provided the first report of considerable interspecific variation in *Eucalyptus* spp. on the susceptibility to *A. psidii* and its correlation with cuticular waxes chemical compounds, that seem to play a synergistic role as a preformed defense mechanism. Already, in the chapter 3, it was showed that the presence of cuticular wax from *E. g* induced the expression of genes encoding proteins related to growth and colonization of *A. psidii* such as binding proteins (peptidylprolyl isomerase and ribosomal) and cell wall degrading proteins (beta-xylanase). The *E. ug* x MO analysis resulted in DEGs associated to proteins related to membrane transporters and receptors, DNA repair and glycine dehydrogenase. The cuticular from *E.u* up-regulated the expression of genes encoding proteins associated to pheromone, cutinases and prefoldin. To our knowledge, this is the first report proving that *Eucalyptus* spp. cuticular waxes may act as preformed defense mechanisms stimulating the fungal germination as well as modulating the *A. psidii* gene expression, suggesting the importance of early plant-pathogen molecular interaction to development of myrtle rust.

2. CONCLUSIONS

Eucalyptus spp. has more than one mechanism (preformed and induced resistance) responsible for their resistance against *A. psidii*. The cuticular chemical composition is strongly related to the susceptibility of *E. grandis* and *E. phaeotricha*. However, our data suggest that there are many cuticular signals that act at different stages of fungal infection, uredospore germination, appressorium formation, invasion and survival on the eucalyptus leaves, corroborating the hypothesis that the *Eucalyptus* spp. resistance to *A. psidii* is related to synergistic preformed and induced resistance mechanisms that should be studied in more detail.

Despite considerable advances in understanding of pathosystems over the past few years through RNASeq analyses, the understanding concerning the biotrophic early interaction established between *A. psidii* - *Eucalyptus* spp. that cause the myrtle rust is still very limited. To our knowledge, this study is the first reporting at the molecular level of *A. psidii* transcriptome. This provides the basis of hypotheses regarding cuticular waxes as an important source of preformed defense, protecting the host surface as well modulating the gene expression of pathogen during the crucial early interaction.

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