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

Comparative epidemiology of grapevine and soybean rusts

Isabela Vescove Primiano

Thesis presented to obtain the degree of Doctor in
Science. Area: Plant Pathology

**Piracicaba
2019**

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*To my parents, Eduardo and Edenilze;
My brothers Eduardo and João Vítor;
My boyfriend Fábio*

I dedicate

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**“Do not wait for leaders; do it alone, person to person.
Be faithful in small things because it is in them
that your strength lies.”**

Mother Teresa

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RESUMO

Epidemiologia comparativa das ferrugens da videira e da soja

Phakopsora meliosmae-myrianthae, agente causal da ferrugem asiática da videira, e *Phakopsora pachyrhizi*, agente causal da ferrugem asiática da soja, ocasionam epidemias severas em seus hospedeiros. O comportamento dessas *Phakopsora* spp. parece não seguir o padrão de outras ferrugens, por exemplo apresentam elevada frequência de pústulas no limbo foliar concomitantemente à necrose foliar e desfolha precoce. Para elucidar a epidemiologia dessas ferrugens, este estudo teve como objetivos: (i) comparar o progresso da colonização de *P. meliosmae-myrianthae* e de *P. pachyrhizi*, em seus hospedeiros, pela determinação da biomassa fúngica via coloração histológica e via reação em cadeia da polimerase em tempo real (qPCR); (ii) comparar os efeitos de *Phakopsora* spp. na fotossíntese dos seus hospedeiros e os componentes monocíclicos: períodos de incubação, latente e infeccioso, número de uredínios e severidade da doença ao longo do tempo; e (iii) estimar as taxas relativas de desfolha ocasionadas pelas ferrugens da videira e da soja em função da severidade das doenças. Todos os patossistemas apresentaram crescimento da lesão. A colonização micelial não se estendeu além da borda da lesão. Não foi observado aumento no número de lesões ao longo do tempo, mas o número de novos uredínios de *P. pachyrhizi* e de *P. meliosmae-myrianthae* nas lesões aumentou em 9 e 19 vezes, respectivamente. Os períodos de incubação e latente foram coincidentes: 8 dias para ferrugem da videira e 13 dias para a ferrugem da soja. Os períodos infecciosos foram de, no mínimo, 21 dias para *P. meliosmae-myrianthae* e de 13 dias para *P. pachyrhizi* e foram compostos de vários picos de esporulação para ambas as ferrugens. Durante o monociclo, as duas ferrugens apresentaram aumento da severidade, com similar taxa de progresso, determinada pelo modelo monomolecular, de $0,06 \text{ dia}^{-1}$ e $0,05 \text{ dia}^{-1}$, para as ferrugens da videira e da soja, respectivamente. *Phakopsora meliosmae-myrianthae* e *P. pachyrhizi* reduziram, em média, 22% e 5% da taxa fotossintética líquida nas folhas infectadas antes do aparecimento dos sintomas, respectivamente. As taxas de desfolha da ferrugem da videira e da soja foram positivamente correlacionadas com a severidade média das doenças, de acordo com um modelo logarítmico. Nas folhas de videira e de soja sem sintomas, as taxas de desfolha foram de $0,05 \text{ dia}^{-1}$ e $0,06 \text{ dia}^{-1}$, respectivamente. Nas folhas de videira com severidade da doença entre 12,1 a 25%, a taxa de desfolha foi de $0,13 \text{ dia}^{-1}$ e nos folíolos de soja com severidade da doença entre 25 a 60%, foi de $0,12 \text{ dia}^{-1}$. Nossos resultados mostram que o comportamento epidemiológico de *P. meliosmae-myrianthae* é semelhante ao de *P. pachyrhizi*. O contínuo aumento do número de uredínios na lesão favorece um período infeccioso com vários picos de produção de urediniósporos, sendo um eficiente mecanismo de sobrevivência desses patógenos nos trópicos. Essas características podem estar diretamente relacionadas às frequentes epidemias ocasionadas por *Phakopsora* spp.

Palavras-chave: *Phakopsora euvitis*; Interação biotrófica; Expansão da lesão; *Vitis* spp.; *Glycine max*

ABSTRACT

Comparative epidemiology of grapevine and soybean rusts

Phakopsora meliosmae-myrianthae, a causal agent of Asian grapevine leaf rust, and *Phakopsora pachyrhizi*, a causal agent of Asian soybean rust, cause severe epidemics on their crop hosts. Both *Phakopsora* spp. seem to behave differently to other rusts, i.e. showing a high frequency of pustules on leaves concomitantly with host tissue necrosis and leading to premature defoliation. To shed light on the epidemiology of these rusts, this study aimed to: (i) compare the colonization progress of *P. meliosmae-myrianthae* and *P. pachyrhizi* on their hosts, by determination of fungal biomass via histological staining and quantitative polymerase chain reaction (qPCR); (ii) compare the effects of *Phakopsora* spp. on host photosynthesis and the monocyclic components: incubation, latent, and infectious periods, number of uredinia, and disease severity over time; and (iii) estimate the relative defoliation rate caused by Asian grapevine and soybean rusts and its relationship to a range of disease severity. All pathosystems showed lesion expansion. Mycelial colonization did not extend beyond the lesion border. No increase in the number of lesions was observed over time, but formation of new uredinia of *P. pachyrhizi* and *P. meliosmae-myrianthae* within an existing lesion, without the need for a new infection site, increased by 9- to 19-fold, respectively. Incubation and latent periods were coincident for 8 days in Asian grapevine leaf rust and 13 days in Asian soybean rust. Minimum infectious periods were 21 days for *P. meliosmae-myrianthae* and 13 days for *P. pachyrhizi*, and both pathogens presented several sporulation peaks. Both *Phakopsora* rusts showed an increase in disease severity during monocycle, with similar progress rates that were estimated with the monomolecular model as 0.06 and 0.05 day⁻¹ for grapevine and soybean rusts, respectively. *P. meliosmae-myrianthae* and *P. pachyrhizi* infection reduced relative photosynthetic rates by 22% and 5%, respectively, before the onset of symptoms. Defoliation rates of grapevine and soybean rusts were positively correlated with mean disease severity, according to a logarithmic model. On symptomless grapevine and soybean leaves, defoliation rates were 0.05 and 0.06 day⁻¹, respectively. On diseased grapevine leaves, defoliation rate was 0.13 day⁻¹ for leaves with disease severity between 12.1% and 25%. The rate of defoliation on soybean leaflets was 0.12 day⁻¹ when disease severity was between 25% and 60%. Our findings showed that the epidemiological behaviour of *P. meliosmae-myrianthae* is similar to that of *P. pachyrhizi*. The continuous increase in the number of uredinia within lesions ensures an infectious period with several urediniospore production peaks, which is an efficient survival mechanism for these pathogens in the tropics. This might be directly related to the frequent epidemics caused by *Phakopsora* spp.

Keywords: *Phakopsora euvitidis*; Biotrophic interaction; Lesion expansion; *Vitis* spp.; *Glycine max*

1. GENERAL INTRODUCTION

1.1. Life cycle of rusts

Rust pathogens belong to the class Pucciniomycetes, order Pucciniales, and phylum Basidiomycota. These organisms have a complex life cycle, with up to five distinct stages, which can occur on a single or on two unrelated hosts to complete their life cycle (Alexopoulos *et al.*, 1996; Massola, 2018). Rust pathogens are named autoecious when they complete their entire life cycle on a single host, and heteroecious when two hosts are needed to complete their life cycle. The life cycle stages of the causal agents of rusts are: spermogonial or pycnial stage (phase O), aecial stage (phase I), uredinial stage (phase II), telial stage (phase III), and basidial stage (phase IV). When all stages are present, the life cycle is named macrocyclic. Some rust fungi do not exhibit phase II and their life cycle is named demicyclic, while others do not present phases I and II and are considered microcyclic (Alexopoulos *et al.*, 1996). In heteroecious rust fungi, reproductive structures of the uredinial and telial stages, uredinia and telia respectively, are formed in the primary host. Reproductive structures of the spermogonial (pycnial) and aecial stages, spermogonia (pycnia) and aecia respectively, are formed in the secondary host (Alexopoulos *et al.*, 1996; Massola, 2018). For some rusts, the spermogonial and aecial stages are not known, as is the case for Asian soybean rust, caused by *Phakopsora pachyrhizi*, where the uredinial and telial stages are always present and have been so far the only ones reported in soybeans and other host plants (Agrios, 2005; Rupe & Sconyers, 2008).

Rusts are reported in several hosts of different botanical families (Agrios, 2005). However, there is much information on cereal rusts, for example on wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici*, which is used as a model of macrocyclic heteroecious rust fungi (Massola, 2018). Another macrocyclic heteroecious rust fungus is the causal agent of Asian grapevine leaf rust. Different pathogenic species of the genus *Phakopsora* are related to Asian grapevine leaf rust in Asia, Central America, and South America, for example, *P. ampelopsidis*, *P. vitis*, *P. montana*, and *P. meliosmae-myrianthae* (Chatasiri & Ono, 2008; Ono *et al.*, 2012; Okane & Ono, 2018). The classification depends on molecular analyses and on the host. *Phakopsora euvitis* was reclassified as *Phakopsora meliosmae-myrianthae*

(Ono *et al.*, 2012) with uredinial and telial stages on species of the genus *Vitis* and spermogonial and aecial stages on the tree *Meliosma myriantha* Sieb. & Zucc. that belongs to the family Sabiaceae. Molecular and phylogenetic analyses of the causal agents of Asian grapevine leaf rust have confirmed that Brazilian isolates belong to a different species to those which occur in North America. Brazilian isolates are grouped in the same clade as isolates from Thailand and were renamed *Phakopsora* sp. (Okane & Ono, 2018). In this work, we used *P. meliosmae-myrianthae* as the Latin name of the Brazilian isolate used in all trials.

Brazilian rust epidemics are caused by the spread of spores from the uredinial stage, the urediniospores, by the wind (Tessmann *et al.*, 2004; Massola, 2018). In Brazil, and in other tropical and subtropical areas where the secondary host does not occur, a diseased primary host ensures pathogen survival throughout the year. In grapevine, evergreen leaves that remain on the plants throughout the year are green bridges for pathogen survival between crops (Leu, 1988; Weinert *et al.*, 2003; Hennessy *et al.*, 2007). Similarly, *P. pachyrhizi* survives in voluntary soybean plants (soybean plants that grow spontaneously) or weeds throughout the year, given that no secondary host has been reported for this pathogen (Kelly *et al.*, 2015).

It is presumed that the urediniospores of *P. meliosmae-myrianthae* spread over long distances through air currents (Leu, 1988); however, no study has been conducted to investigate the aerobiology of this pathogen. The successful eradication of the disease in Australia (Daly & Tran-Nguyen, 2008) was the first example in the world of this method for rust control (Edwards, 2015), indicating that the dispersal of *P. meliosmae-myrianthae* is not as efficient as that of other rust pathogens. For *P. pachyrhizi*, wind is responsible for long-distance dispersal of urediniospores, as registered by the invasion of Florida in 2004 after hurricanes (Stokstad, 2004; Pan *et al.*, 2006). Rain also seems to be an important factor for short-distance dispersal of urediniospores (Barnes *et al.*, 2009), because *P. pachyrhizi* urediniospores tend to clump and stick together within lesions, and then rain splash is needed for their release (Melching *et al.*, 1979). Rain is also considered as an important dispersal agent of *Puccinia striiformis* urediniospores, the causal agent of stripe rust, probably due to the presence of a mucilaginous layer on the surface of the urediniospores that holds the spores of *P. striiformis* as a cluster (Rapilly, 1979; Geagea *et al.*, 1999; Sache, 2000).

1.2. Process of infection and colonization of rusts

The epidemiological behaviour of populations of pathogens on populations of plants can be better understood when complemented by knowledge of the pathogen's behaviour at a lower hierarchical level, for instance, the individual level. Information on infection and colonization processes at the cellular or tissue level can help to explain the population's behaviour (Bergamin Filho *et al.*, 2018).

Causal agents of rusts usually penetrate the host through the stomata (Figure 1), as in *Uromyces appendiculatus* and *U. fabae*, causal agents of bean rust. For these pathogens, stomata topography induces the formation of an appressorium, an initial structure of the infection process (Terhune *et al.*, 1991; Mendgen & Hahn, 2002). The exception has already been observed in the genus *Phakopsora*, in which *P. pachyrhizi* in soybean (Furtado *et al.*, 2009), *P. jatrophiicola* in *Jatropha gossypifolia* (Seier *et al.*, 2009), and *P. apoda* in *Pennisetum clandestinum* (Adendorff & Rijkenberg, 2000) present direct penetration by the host cuticle, but the hyphae grow in the mesophyll intercellular spaces, as in other rusts (Bonde *et al.*, 1976; Adendorff & Rijkenberg, 2000; Magnani *et al.*, 2007). In the case of *P. meliosmae-myrianthae*, infection occurs through stomata approximately 12 h post inoculation (Leu & Wu, 1983; Leu, 1988).

Figure 1 - Scheme of infection via stomata and intercellular colonization of rust: a urediniospore (U) secretes adhesive substances (AS), emits a germ tube (GT), and forms an appressorium (A) over the stomatal pore. Then, penetration hyphae (PH) are formed in the substomatal chamber and elongate into infection hyphae (IH). A haustorium mother cell (HMC) is formed in contact with the mesophyll host cell wall and gives rise to the haustorium (H). From the neckband (NB) and surrounding the haustorium, the extrahaustorial matrix (in blue), delimited by the extrahaustorial membrane, is produced (drawing adapted from Mendgen & Hahn, 2002).

Pathogens that are termed biotrophic, such as rust fungi, are obligate parasites and generally present little aggressive interaction when compared to non-obligate parasites (Mendgen & Hahn, 2002). Non-obligate parasites (hemibiotrophic and necrotrophic pathogens) are associated with more aggressive colonization and, generally, kill plant tissue before invasion (Perfect & Green, 2001; Amorim & Pascholati, 2018). All pathogens that belong to the order Pucciniales present intercellular colonization and formation of a specialized structure, the haustorium, to obtain nutrients from the host with no cell death (Staples, 2000; Voegelé & Mendgen, 2003). This order includes all causal agents of rusts, for example those from the family Phakopsoraceae, which includes the genus *Phakopsora*, or the family Pucciniaceae, which includes the genera *Uromyces*, *Puccinia*, and *Hemileia* (Alexopoulos *et al.*, 1996). Haustoria are more involved in primary metabolism to assimilate the nutrients from the plant cell, such as amino acids and sugars. Haustoria also produce and transport effectors (Garnica *et al.*, 2014; Amorim & Pascholati, 2018). No differences are observed between the categories of genes expressed in haustoria of *P. pachyrhizi* and *U. appendiculatus* (Link *et al.*, 2014), suggesting a similar pattern in the mechanisms of host resource acquisition among these pathogens.

Concurrently with the progress of intercellular colonization and haustoria formation of the rust pathogen into new host cells, the formation of lesions begins with reproductive structures on the abaxial leaf side. These lesions, or pustules, are typical symptoms of rusts, which may have a yellowish or orange colour, for example, those of Asian grapevine leaf rust, or a brownish colour, such as those of Asian soybean rust. In the case of Asian soybean rust, even in highly susceptible cultivars, a brownish colour is observed in the foliar tissue around new uredinia, characterized as necrotic tissue (Deverall *et al.*, 1977). Pustules can coalesce on the abaxial face, and areas on the adaxial side opposite the pustules also become necrotic as observed in grapevine and soybean rusts (Leu, 1988; Agrios, 2005).

1.3. Monocyclic components of rusts

Epidemics can be compared at different hierarchical levels (Kranz, 1988). Monocyclic components can be used to compare pathosystems at an individual level, and the variables can be: (i) incubation period, corresponding to the time interval

between pathogen inoculation and the appearance of symptoms; (ii) latent period, corresponding to the time interval between pathogen inoculation and the urediniospore production; (iii) infectious period, corresponding to the time interval of urediniospore production; (iv) number of lesions; (v) average size of lesion; and (vi) final biomass of each pathogen. Data from the literature can be useful for the analyses of comparative epidemiology; however, in the case of rusts caused by *Phakopsora*, the diversity of experimental conditions in several published reports and a lack of evaluation of certain monocyclic components do not allow this approach based only on literature information (Berger *et al.*, 1995; Alves *et al.*, 2007; Bonde *et al.*, 2007; Angelotti *et al.*, 2014). On the other hand, from the literature it is possible to obtain the weather conditions that are most favourable for disease development.

The interaction of leaf wetness, temperature, and cultivar susceptibility to rusts can influence monocyclic components. In susceptible soybean cultivars, the latent period of *P. pachyrhizi* ranged from 6 to 9 days, independently of the leaf wetness duration applied after inoculation (6 to 24 h) and incubation temperature (15 to 25 °C; Alves *et al.*, 2007). This temperature range was also ideal for *P. meliosmae-myrianthae* urediniospore germination and the appearance of symptoms of Asian grapevine leaf rust with wetness periods longer than 12 h (Angelotti *et al.*, 2014; Alves, 2015; Navarro *et al.*, 2015). The ideal temperature for the appearance of bean rust symptoms ranged from 16 to 21 °C (Bassanezi *et al.*, 1997); the ideal leaf wetness period was longer than 10 h (Coelho *et al.*, 2003). In grapevine 'Niagara Rosada' inoculated with *P. meliosmae-myrianthae*, the latent period ranged from 7 to 13 days; the temperature response curve of the latent period presented a typical asymmetric U-shape (Alves, 2015), as also observed for other rusts in other cultures (Zadoks & Schein, 1979; Kolnaar & van den Bosch, 2001; Hernandez-Nopsa & Pfender, 2014). When grapevine plants inoculated with *P. meliosmae-myrianthae* were incubated at minimum (15 °C), optimal (25 °C), and maximum (30 °C) temperature for disease development, the latent periods were 13 to 15, 6 to 7, and 6 to 9 days, respectively (Angelotti *et al.*, 2014; Alves, 2015). A tissue collapse expressed as foliar necrosis between lesions and defoliation was observed when plants were incubated at 30 °C (Alves, 2015).

Breeding programmes used to consider latent period as a quantitative trait to assist in typifying plant resistance to *Phakopsora* rust. This variable is in disuse, and evaluation of the number of uredinia within the lesions and the diameter of pustules is

most suitable for classifying cultivars (Bonde *et al.*, 2006; Angelotti *et al.*, 2008). The estimated average size of pustules or infection type is also used to classify bean plants as susceptible or resistant to *U. appendiculatus* (Acevedo *et al.*, 2013; Leitão *et al.*, 2013).

Lesion growth is an important component of rust epidemics in tropical conditions (Berger *et al.*, 1997). An increase in pustule area usually implies an increase in sporulation area. This behaviour appears to be associated with some rusts, such as Asian soybean rust (Bonde *et al.*, 2006; Salustiano *et al.*, 2007; Bergamin Filho, 2008). Lesion expansion, with satellite uredinia and a long infectious period, probably allows rusts to persist and remain a threat even under environmental conditions unfavourable for infection (Miles *et al.*, 2003). Therefore, this monocyclic component must be investigated in more detail (Sache & Vallavieille-Pope, 1993; Berger *et al.*, 1997). No quantitative studies have evaluated this epidemiological component for Asian grapevine leaf rust.

Lesion growth and pathogen colonization can be quantified by estimating diseased areas or by estimating fungal biomass in diseased hosts. Fungal biomass in plants can be indirectly estimated by quantifying specific components of the kingdom Fungi, such as the concentration of ergosterol (Gessner *et al.*, 1991) or chitin (Ayliffe *et al.*, 2013). Ergosterol is the most common sterol in fungal cell plasma membrane, and chitin is a cell wall component (Massola, 2018). Few studies have correlated monocyclic components (for example, disease severity levels) with ergosterol amount (Xue *et al.*, 2006). However, the advancement of molecular techniques allows quantification of fungal biomass based on specific genes of these organisms, such as the internal transcribed spacer (ITS) region (Gardes & Bruns, 1993; Manter & Vivanco, 2007; Tellenbach *et al.*, 2010; Weihmann *et al.*, 2016). Real-time polymerase chain reaction, also known as quantitative PCR (qPCR), is a useful technique that specifically detects, in each cycle, a fluorescent signal emitted by DNA probe fluorophores (TaqMan assay) or by a dye that interacts only with double-stranded DNA (SYBR green assay) during exponential phase amplification. Calibration curves of the relationship between the fluorescence values and the amount of fungal material allow the quantitative analysis of microorganism biomass (Tellenbach *et al.*, 2010; Haegi *et al.*, 2013; Feckler *et al.*, 2017).

1.4. Damage caused by rusts

Rust fungi affect several crops, such as cereals, legumes, and fruit, and affect different plant organs; however, their symptoms are more associated with leaves, and, consequently, they affect the photosynthetic process (Agrios, 2005). Low disease severity levels causing a high reduction in leaf photosynthesis can be explained by the concept of 'virtual lesion'. This concept is the ratio between the net photosynthetic rate of a diseased leaf (P_x) and the net photosynthetic rate of a healthy leaf (P_o) (Bastiaans, 1991). The relationship P_x/P_o as a function of disease severity (x) can be described by the equation $P_x/P_o = (1 - x)^\beta$. If the parameter β is greater than 1, there is a reduction of photosynthetic activity in the remaining green leaf tissue of diseased leaves, and the virtual lesion area is larger than the visual lesion area (Bastiaans, 1991). In hemibiotrophic and necrotrophic pathogens, such as *Colletotrichum lindemuthianum* and *Pyricularia oryzae*, the photosynthetic capacity of the remaining green leaf tissue is affected by the pathogen (Bastiaans, 1991; Bassanezi *et al.*, 2001). Measurements of leaf photosynthesis in soybean plants infected with *P. pachyrhizi* indicate that the impact of this biotrophic pathogen is greater than the visual lesion (Kumudini *et al.*, 2010). In contrast to soybean rust, bean plants infected with *U. appendiculatus* presented a β parameter similar to 1, indicating that this pathogen does not interfere in photosynthetic efficiency on asymptomatic areas of diseased leaves (Bassanezi *et al.*, 2001). Virtual lesions can be explained by the toxins produced and secreted by the pathogen, that diffuse to the surrounding area of the diseased tissues before symptoms appear (Bastiaans, 1991). However, this type of metabolite is not produced by rust pathogens (Amorim & Pascholati, 2018), and there is still no explanation for 'virtual lesion' in biotrophic pathogens (Lopes & Berger, 2001).

Plants infected by *Phakopsora* spp. present early leaf fall, as reported in grapevine and soybean (Leu, 1988; Yang *et al.*, 1990). In bean inoculated with *U. appendiculatus*, defoliation was related positively with the proportion of pustules accumulated in some experiments performed in a greenhouse (Mersha & Hau, 2008); however, no reduction in total leaf area was observed in field experiments (de Jesus *et al.*, 2001). The reduction in leaf area caused by early leaf fall may negatively reflect on the production quality and/or yield. Early defoliation can result in a lower soybean grain weight, uneven grape cluster maturation and reduced growth of

grapevine branches (Yang *et al.*, 1990; Mueller *et al.*, 2009; Sikora *et al.*, 2014; Vaillant-Gaveau *et al.*, 2014). In annual crops, the effects of leaf area reduction are observed in the same season, while in perennial crops, the effects may be cumulative over the years. For example, grapevine plants infected with *P. meliosmae-myrianthae* had their carbohydrate dynamics altered (Nogueira Júnior *et al.*, 2017), and this change in carbohydrate storage may affect plant development in the following season.

1.5. Objectives

The objective of this project was to unravel, at least in part, the mechanisms related to aggressiveness of rusts caused by pathogens of the genus *Phakopsora*. The specific objectives were to:

- (i) quantify colonization of *P. meliosmae-myrianthae* and *P. pachyrhizi* compared to *U. appendiculatus* in tissues of grapevine, soybean, and bean, respectively;
- (ii) compare the monocyclic components and photosynthetic efficiency of Asian grapevine leaf rust (*P. meliosmae-myrianthae*) and Asian soybean rust (*P. pachyrhizi*) under controlled conditions;
- (iv) estimate the defoliation rate of Asian grapevine leaf rust (*P. meliosmae-myrianthae*) and Asian soybean rust (*P. pachyrhizi*) in field trials.

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2. SATELLITE UREDINIA: AN IMPORTANT TRAIT RELATED TO THE DAMAGE CAUSED BY *PHAKOPSORA* SPP.

Abstract

Phakopsora pachyrhizi and *P. meliosmae-myrianthae* are the causal agents of Asian soybean rust and Asian grapevine leaf rust, respectively. Trying to understand the highly aggressive nature of both pathogens on their respective host plants, we took a close look at their plant colonization kinetics and compared them to the less aggressive bean rust pathogen, *Uromyces appendiculatus*. Colonization progress was monitored by determination of fungal biomass via histological staining and quantitative polymerase chain reaction (qPCR). After the first disease symptoms became visible, individual lesions of *P. pachyrhizi* on soybean leaves, *P. meliosmae-myrianthae* on grapevine leaves and *U. appendiculatus* on common bean leaves were evaluated every 3 to 4 days, and the number of uredinia was counted. Staining showed that mycelial colonization did not extend beyond the lesion border. The number of *P. pachyrhizi* and *P. meliosmae-myrianthae* uredinia within the lesions increased over time (on average 14-fold), whereas the number of *U. appendiculatus* uredinia remained the same. These findings were corroborated by qPCR which revealed a greater increase in fungal biomass for *Phakopsora* spp. than for *U. appendiculatus* until 12 days postinoculation. The high number of satellite uredinia within lesions ensures a long infectious period due to continuous host colonization without the need for secondary infections and might be directly related to the highly aggressive nature of these pathogens. In this study, we identified accelerated formation of satellite uredinia as an important feature of two *Phakopsora* spp. and evidenced its relation to the damage to important crop plants.

Keywords: Biotrophic pathogen; Rust fungi; *Vitis labrusca*; *Glycine max*

2.1. Introduction

Rusts are caused by obligate biotrophic fungi (Pucciniales, Basidiomycota) and can lead to significant damage to crop plants (Lorrain et al. 2018; Yamaoka 2014). Despite high crop losses, in general, rust fungi cause minor damage to host tissue, when compared to necrotrophic plant pathogens. In a biotrophic interaction, pathogens keep the host cells alive, unlike in necrotrophic interactions, where pathogens are dependent on dead plant tissue before invading it (Amorim and Pascholati 2018; Mendgen and Hahn 2002). Soybean rust, caused by *Phakopsora pachyrhizi*, is a globally and economically important rust disease (Godoy et al. 2016; Goellner et al. 2010; Yorinori et al. 2005). *Phakopsora pachyrhizi* does not exhibit

similar characteristics to other rust fungi: (i) it is not a specialized parasite, as it has an unusually broad host range within the Leguminosae family; (ii) it can directly penetrate plant epidermal cells by urediniospore-derived structures, while most rust fungi urediniospores penetrate the host through stomata; (iii) it produces satellite uredinia associated with lesion growth differently from others as one lesion has only one uredinium; (iv) it reduces photosynthetic efficiency, not only in the diseased area, as in most rust fungi, but also in green symptomless tissues surrounding lesions; and (v) it causes early leaf fall (Bonde et al. 2006; Edwards and Bonde 2011; Godoy et al. 2016; Jurick et al. 2008; Twizeyimana et al. 2014). *Phakopsora pachyrhizi* disease symptoms mainly start on the abaxial face of soybean leaves as small lesions, each one composed by a uredinium surrounded by a necrotic area, that expand and coalesce (Bonde et al. 2006; Godoy et al. 2016; Twizeyimana et al. 2014). The lesion growth of Asian soybean rust is due to the formation of satellite uredinia, which emerge within the lesion without the occurrence of new infections. The increment of satellite uredinia causes several peaks of sporulation through a long infectious period, and this increases the adaptability and the survival of the pathogen (Bergamin Filho and Amorim 1996; Bonde et al. 2006). This unique characteristic of Asian soybean rust is not observed in most rusts on crop plants. In common bean rust, caused by *Uromyces appendiculatus*, for example, lesion growth is due to expansion of the diseased area without the occurrence of new uredinia (Berger et al. 1995). The damage caused by *U. appendiculatus* on bean plants is not as great as the damage caused by *P. pachyrhizi* on soybean plants. The reduction of photosynthetic efficiency by *U. appendiculatus* on bean leaves is directly proportional to the diseased area and, even under high disease severity, there is no early leaf defoliation (Bassanezi et al. 2001).

Asian grapevine leaf rust, caused by *P. meliosmae-myrianthae* (syn. = *P. euvitis*), presents similar symptoms to Asian soybean rust, such as a high frequency of pustules per leaf, necrosis surrounding uredinia, and early leaf fall (Ono et al. 2012; Primiano et al. 2017; Scapin-Buffara et al. 2018). Like *P. pachyrhizi*, *P. meliosmae-myrianthae* significantly reduces the photosynthetic efficiency of grapevine leaves, even beyond the limits of the lesions (Kumudini et al. 2010; Nogueira Júnior et al. 2017). In addition, *P. meliosmae-myrianthae* alters the carbohydrate dynamics of grapevine plants and significantly reduces starch accumulation in the roots (Nogueira Júnior et al. 2017). Root biomass of grapevines

with high rust severity is reduced, which leads to a gradual decline in plant vigor in the subsequent seasons (Edwards 2015; Nogueira Júnior et al. 2018). Changes in photoassimilate distribution in plants (source) due to infection of biotrophic pathogens (sink) have often been reported (Voegelé and Mendgen 2003; Walters 1989). The vigorous mycelial growth represents a strong sink and, through a specialized infection structure, the haustorium, rust fungi can exploit host-derived resources (Garnica et al. 2014; Walters 1989). This source–sink relationship can be enhanced by urediniospore formation (Tremblay et al. 2012). Thus, satellite uredinia and massive urediniospore production should modify the metabolic flow in the host, resulting in severe damage to its hosts. Although the occurrence of satellite uredinia has already been reported in Asian grapevine leaf rust (Primiano et al. 2017), there are no studies that have precisely quantified the lesion growth of *P. meliosmae-myrianthae* on grapevine leaves. Comparative epidemiology is a powerful tool with great potential to advance the understanding of pathogen colonization strategies (Kranz 2003). In the present work, we have used the comparative epidemiology approach to quantify colonization of grapevine and soybean leaf tissues by *Phakopsora* spp., compared to colonization of common bean by *U. appendiculatus*.

2.2. Materials and methods

2.2.1. Host and inoculum production

Pathogen species used in this study were *P. meliosmae-myrianthae* (isolate AGLR064, syn. *P. euvitis*), the causal agent of Asian grapevine leaf rust; *P. pachyrhizi* (isolate Br05), the causal agent of Asian soybean rust, and *U. appendiculatus*, the causal agent of bean rust. Each isolate was maintained by weekly inoculations on their respective susceptible host plants. Grapevine cv. ‘Niagara Rosada’ (*Vitis labrusca*) plants grafted onto ‘IAC 766-Campinas’ were grown in a rust-free greenhouse at 21°C ($\pm 4^\circ\text{C}$) and 70% relative humidity ($\pm 12\%$). Soybean cv. ‘Abelina’ (*Glycine max*) and common bean cv. ‘Saxa’ (*Phaseolus vulgaris*) plants were grown in a plant growth room with long-day conditions (16 h of light/8 h of dark), at 22°C ($\pm 2^\circ\text{C}$) and 75% relative humidity. For inoculum maintenance, urediniospores were harvested and suspended in sterile water with 0.1% Tween. Leaves were uniformly inoculated by spraying 10^5 urediniospores·ml⁻¹

with a spray nozzle (glass thin-layer chromatography atomizer, order no. H451.1, Carl Roth GmbH + Co. KG, Germany) at 1 bar air pressure (Figure S1). Inoculated plants were kept in a moist chamber for 24 h postinoculation (hpi) in the dark. After the moist chamber period, grapevine plants were maintained in a plant growth room at 25°C and a photoperiod of 12 h/12 h, and soybean and bean plants were maintained in a plant growth room in the same conditions as previously described.

2.2.2. Lesion growth assay

For the lesion growth assay, grapevine ‘Niagara Rosada’ plants, soybean ‘Abelina’ plants and common bean ‘Saxa’ plants were inoculated with 2×10^4 urediniospores·ml⁻¹ of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus*, respectively. After inoculation, all plants were immediately transferred to a dark dew chamber (nearly 100% relative air humidity), incubated for 24 h, and then transferred to a growth room with controlled conditions. Grapevine plants were maintained at 25°C and a photoperiod of 12 h/12 h, and soybean and bean plants were kept at 22°C and a photoperiod of 16 h of light/8 h of dark. Irrigation was localized at the base of the plants. During the whole time of the experiments, there was no accumulation of free water on the leaves in order to avoid secondary infections.

Individual lesions of Asian grapevine leaf rust, Asian soybean rust and bean rust (Figure S2) were harvested every 3 or 4 days until a maximum of 33 days post first symptom (dps). For each harvest, 15 lesions, of which 5 lesions were from the same plant, were evaluated. Individual grapevine leaves and soybean and bean leaflets were considered as a replicate sample. Lesion areas and number of uredinia were evaluated by using a stereomicroscope, before and after 0.025% trypan blue staining (glycerol, lactic acid, water and ethanol in a ratio of 1:1:1:7). Grapevine rust samples were incubated in trypan blue solution for 24 h at 60°C, and soybean and bean rust samples for 1 h at 68°C. Lesions were cleared in chloral hydrate solution (2.5 g·ml⁻¹) for 7 to 10 days. Trypan blue staining in combination with chloral hydrate de-staining enables visualization of fungal growth inside leaf tissue (Maffi et al. 2011). Samples were stored in 25% glycerol, and all slides were set up with this solution. Images of grapevine lesions were taken on a Zeiss AxioLab.A1 microscope with Zen Blue imaging software, and images of soybean and bean lesions were

taken on a Leica MZ16 stereomicroscope with a Hitachi KP-FD140F-83 CCD camera using the DISKUS program (Technisches Büro Hilgers, Königswinter, Germany). Lesion areas were estimated using Zeiss Zen Blue or ImageJ2 (version 1.8.0) software (Rueden et al. 2017), and the number of uredinia was counted in a stereomicroscope. All experiments were performed twice.

In order to determine the relationship of non-stained lesion area with stained lesion area, and the relationship of the number of non-stained uredinia with stained uredinia, linear regressions of each pathosystem were performed, and slopes were compared to 1 by Student's t-test at a significance level of 0.05. Data from the mean stained lesion areas and from the mean number of stained uredinia were transformed in proportion, considering the highest value within the experiment, and linear regressions were also performed with this data over time. Parameters of the linear equations estimated for each pathosystem were compared by Student's t-test at 5% probability. All data analysis was performed using STATISTICA® software (version 7.0, StatSoft, Tulsa, USA).

2.2.3. Experimental design, inoculation and sample collection for qPCR assay

For the qPCR assay, grapevine rust, soybean rust and bean rust trials were carried out separately. Each trial was conducted in a completely randomized design. The abaxial face of the third and the fourth grapevine leaves 20 days after pruning, and the abaxial face of the first trefoil of soybean plants at growth stage V3 (approximately 21-day-old soybean plants) and bean plants at growth stage V4 (approximately 20-day-old bean plants) were spray-inoculated with 5×10^5 urediniospores·ml⁻¹ of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus*, respectively. All plants were incubated for 24 hpi in a dark moist chamber.

Leaf discs of 2 cm in diameter were harvested 2, 5, 7, 12 and 16 days postinoculation (dpi) from each pathosystem. Six discs were harvested per time point, of which 3 were used for symptom development observation, and the other 3, with a similar disease severity level, were used to quantify the fungal biomass by qPCR. In order to confirm symptom development, the three discs were stained with trypan blue solution as described for the lesion growth assay. For fungal biomass quantification by qPCR, leaf discs were snap-frozen in liquid nitrogen and stored at

–80°C for 20 days. After this period, all discs were removed from the ultra-low freezer and individually macerated (one sample = one biological replicate) under liquid nitrogen with a sterile mortar and pestle. All experiments were repeated once.

2.2.4. DNA extraction and primer design

For genomic DNA (gDNA) extraction, two modified cetyltrimethylammonium bromide (CTAB) methods were used. The protocol used for gDNA extraction of inoculated and non-inoculated leaves of *V. labrusca* was according to Lo Piccolo et al. (2012). The protocol used for gDNA extraction from urediniospores of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus* and from inoculated and non-inoculated leaves of *G. max* and *P. vulgaris* is described in the following part. Frozen powder of ground urediniospores and ground leaves was resuspended in 0.8 ml of pre-warmed (60°C) CTAB extraction buffer [2% CTAB, 10 mM Tris-HCl (pH = 8), 20 mM EDTA, 1.4 M NaCl, 0.2% β -mercaptoethanol, and 0.1 mg·ml⁻¹ proteinase K] and incubated at 60°C for 1 h. After incubation, with casual inversion, samples were mixed with 0.8 ml of chloroform/isoamyl alcohol (24:1) solution and centrifuged at 14,000 × *g* at 4°C for 15 min. The aqueous phase was transferred to a new tube and treated with 1 μ l of RNase at 37°C. After 30 min, 0.6 ml of isopropanol was gently added to each sample and it was stored overnight at –10°C. Samples were centrifuged for 15 min at 14,000 × *g* at 4°C to precipitate DNA, and the supernatant was removed. The pellet was washed once with cold 100% ethanol, air-dried at room temperature, and resuspended in 50 μ l of TE buffer (pH = 8). All samples were quantified using a NanoDrop 1000 spectrophotometer.

For quantitative polymerase chain reaction (qPCR) analysis of soybean rust and bean rust trials, an extra ethanol precipitation was necessary to remove PCR inhibitors. After dissolving the pellet in TE buffer, two volumes of 100% EtOH and one volume of 3 M Na-Ac (pH = 5) were added to each sample and incubated on ice for 1 h. After this period, samples were centrifuged for 30 min at 14,000 × *g* at 4°C, and the supernatant was removed. The new pellet was washed with 500 μ l of cold 70% ethanol, air-dried, and resuspended in TE buffer (pH = 8).

Primer pairs were designed to amplify a specific region of the plant *rbcL* gene (related to ribulose-1,5-biphosphate carboxylase oxygenase production), and a specific region of the pathogen, the ITS (internal transcribed spacer) region, based

on sequences deposited in GenBank for *V. labrusca*, *G. max*, *P. vulgaris*, *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus*. The primer pair designed for plant specificity had as the forward plant primer plant_fwd_1 (5' - CTTCTACTGGTACATG - 3'), and as the reverse plant primer plant_rev_1 (5' - GAAGTAAACATGTTAGTAACAGA - 3'). The primer pair used for rust specificity had as the forward rust primer rust_fwd_1 (5' - ATGGATCTCTAGGCTCTC - 3'), and as the reverse rust primer rust_rev_1 (5' - TTTCATGACACTCAAACAGG - 3'). Primer specificity was evaluated by qualitative and quantitative PCR. The primer pair plant_fwd_1 and plant_rev_1 specifically amplified part of the plant *rbcL* gene (175 bp fragment) of *V. labrusca*, *G. max* and *P. vulgaris*, and the primer pair rust_fwd_1 and rust_rev_1 amplified part of the fungal ITS region (148 bp fragment) of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus*. After sequencing, all results were compared to sequences of the *rbcL* gene region deposited in GenBank, and the gene organization presented 100% similarity (Figure S4).

2.2.5. Qualitative PCR assay

Gradient PCR with different annealing temperatures (52 to 60°C) and two MgCl₂ concentrations (0.6 and 0.8 µl of 50 mM MgCl₂) was performed to determine the optimal PCR conditions and confirm primer specificity for each primer pair (*data not shown*). Optimized PCR was conducted in 20-µl volumes containing 11.4 µl of water, 2 µl of 10× buffer, 0.6 µl of 50 mM MgCl₂, 2.5 µl of dNTP (2 µM), 1 µl of each primer (10 µM), 0.5 µl of SilverStar, 5 U·µl⁻¹ of DNA polymerase (Eurogentec Deutschland GmbH, Köln, Germany) and 1 µl of DNA or water (negative control) with cycling conditions of 94°C for 2 min, followed by 31 cycles of 95°C for 20 s, 60°C (grapevine rust) or 53°C (soybean and bean rust) for 15 s, 72°C for 10 s, and a final 5-min extension at 72°C. PCR products were visualized in 1.5% agarose gel with ethidium bromide staining under UV light. Bands were cut from agarose gel and purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendation before being sent out for sequencing. All nucleotide sequences were aligned and compared with sequences available in GenBank using BioEdit version 7.2.5.

2.2.6. qPCR analysis

Quantitative PCR was performed in a total volume of 10 μl containing 2 μl of nuclease-free water, 5 μl of 2x Luna[®] Universal qPCR mix (New England Biolabs GmbH, Frankfurt am Main, Germany), 0.5 μl of each primer (10 μM) and 2 μl of DNA or water (control). A thermal gradient (53 to 60°C) was applied to optimize the specificity of qPCR. The decision on the best qPCR conditions was based on analysis of primer–dimer formation observed in melting curves (*data not shown*). The optimal thermal cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C (grapevine rust), 56°C (soybean rust) or 53°C (bean rust) for 40 s, and 95°C for 10 s. Fluorescence at 520 nm was monitored for data collection during the extension phase. Melting curve analysis started at 60°C with increments of 0.5°C for 5 s up to 95°C. Gene quantification was performed in a CFX384 Real Time System (Bio Rad). For each sample (biological replicate), three technical replicates were performed.

To quantify the target gene by qPCR, standard curves were obtained for each host and each pathogen. Dilution series of pure *V. labrusca* gDNA (1.4 to 0.014 $\text{ng}\cdot\mu\text{l}^{-1}$), of pure *G. max* gDNA and of pure *P. vulgaris* gDNA, both from 100 to 0.001 $\text{ng}\cdot\mu\text{l}^{-1}$ (100, 10, 1, 0.1, 0.01 and 0.001 $\text{ng}\cdot\mu\text{l}^{-1}$), were used to obtain individual standard curves for each host with the primer pair plant_fwd_1/plant_rev_1. Dilution series of pure *P. meliosmae-myrianthae* gDNA (9.8 to 0.0098 $\text{ng}\cdot\mu\text{l}^{-1}$), of pure *P. pachyrhizi* gDNA and of pure *U. appendiculatus* gDNA, both from 100 to 0.001 $\text{ng}\cdot\mu\text{l}^{-1}$ (100, 10, 1, 0.1, 0.01 and 0.001 $\text{ng}\cdot\mu\text{l}^{-1}$), were used to develop individual standard curves for each pathogen with the primer pair rust_fwd_1/rust_rev_1. Calibration curves for each primer pair consisted of a linear regression based on quantification cycle value (Cq) *versus* logarithmic values of known quantities of gDNA (starting quantity – SQ) for absolute pathogen quantification (SQp) and absolute host quantification (SQh). The average of SQp and SQh for each technical replicate was calculated. The ratio between SQp and SQh for each biological replicate, and the relative fungal biomass per timepoint were determined.

2.3. Results

2.3.1. Lesion growth assay

The kinetics of colonization was monitored for all rust pathogens on their respective host plant by analysis of lesion area and counting of uredinia within each lesion. While the lesion area increased over time in all rust interactions, the increase in the number of uredinia which occurred in parallel to this lesion growth happened only in rusts caused by *Phakopsora* spp. (Figure 1). Trypan blue staining of samples enabled analysis of the emergence of new uredinia over time and its linkage to lesion enlargement. In the non-stained and non-cleared samples, particularly for the Asian grapevine leaf rust, it was not feasible to precisely quantify the number of uredinia for each individual lesion due to the massive urediniospore production arising from uredinia. Notably, telia of *U. appendiculatus* were observed after the eighth harvest (26 dpi) around the edges of bean rust lesions (Figure S3). For this pathosystem, the lesion area analysis was performed considering 10 timepoints but the number of uredinia was evaluated until telia formation.

Figure 1 - Asian grapevine leaf rust (*Phakopsora meliosmae-myrianthae* – A, D, G, J, M, P and S), soybean rust (*P. pachyrhizi* – B, E, H, K, N, Q and T) and bean rust (*Uromyces appendiculatus* – C, F, I, L, O, R and U) lesions before (left side) and after (right side) trypan blue staining at first (A, B and C), second (D, E and F), third (G, H and I), fourth (K, J and L), fifth (M, N and O), sixth (P, Q and R) and seventh (S, T and U) harvest with respective days post inoculation (DPI).. Images from the first experiment for each pathosystem. Scale bars: 500 μ m (A, B, D, E, G, H, J, K, M, N, P, Q, S and T) and 1000 μ m (C, F, I, L, O, R and U).

Slopes of linear regression of stained lesion area vs non-stained lesion area and number of stained uredinia vs number of non-stained uredinia for all pathosystems were different from 1 ($P < 0.05$, Figure 2). In spite of this, slopes of the lesion area were very close to 1 (between 1.05 and 1.08), and the intercepts close to zero (between 0.10 and 0.18) for all pathosystems (Figure 2A, C and E). On the other hand, slopes of the number of uredinia of *P. meliosmae-myrianthae* and *P. pachyrhizi* were higher than 1.65, and the intercept was above zero (Figure 2B and D). Correlation between the stained and non-stained number of uredinia showed that this variable is underestimated when evaluated without the staining process, whereas the mean values for non-stained lesion area were very close to the area colonized by

the mycelium inside the leaf. Considering this information, comparison of lesion growth over time between pathosystems was performed using only the data of stained lesion area and number of stained uredinia (Figure 3).

Figure 2 - Non-stained lesion area vs trypan blue-stained lesion area (A, C and E) and number of non-stained uredinia vs number of stained uredinia (B, D and F) of *Phakopsora meliosmaemyrianthae* in *Vitis labrusca* 'Niagara Rosada' (A and B), *P. pachyrhizi* in *Glycine max* 'Abelina' (C and D), and *Uromyces appendiculatus* in *Phaseolus vulgaris* 'Saxa' (E and F). Black circles correspond to trial 1, and white circles to trial 2. Dashed lines represent $y = x$. Solid lines represent linear regression ($y = ax + b$, where y is stained area or uredinia [proportion], x is time [days], ' a ' is the slope and ' b ' the intercept. R^2 is the determination coefficient) of pooled data.

The highest values of stained lesion area for the first and second experiments were, respectively, 4 and 2.69 mm² for Asian grapevine leaf rust, 3.81 and 4.93 mm² for Asian soybean rust, and 12.79 and 2.98 mm² for bean rust. Despite

the mean area difference between bean rust experiments, the linear regression rates for each experiment were similar (*data not shown*). Stained lesion areas for each pathosystem were pooled, and the slope of linear regressions for grapevine rust, soybean rust and bean rust were compared to each other (Figure 3). The growth rates for the lesion area of grapevine rust (0.027 – Figure 3A) and soybean rust (0.026 – Figure 3C) were higher than for bean rust (0.015 – Figure 3E).

Figure 3 - Lesion area (in proportion) after trypan blue staining (A, C and E) and uredinia (in proportion) after trypan blue staining (B, D and F) of *Phakopsora meliosmae-myrianthae* in *Vitis labrusca* 'Niagara Rosada' (A and B), of *P. pachyrhizi* in *Glycine max* 'Abelina' (C and D) and of *Uromyces appendiculatus* in *Phaseolus vulgaris* 'Saxa' (E and F). Black circles correspond to trial 1, and white circles to trial 2. Dotted lines correspond to linear regression of pooled data from both trials ($y = ax + b$, where y is stained area or uredinia [proportion], x is time [days], ' a ' is the slope and ' b ' the intercept. R^2 is the determination coefficient).

In both experiments of grapevine and soybean rust lesion growth, we observed a steady increase in the mean number of uredinia up to 33 dpi (Figure 3B

and D). The increase in the number of stained uredinia in the last harvest of grapevine rust and soybean rust was around 14 times higher than for the first harvest (1 dpi). This was not observed in the bean rust that showed an average increase in the number of uredinia of 1.6 times. Linear regressions of the number of stained uredinia of grapevine rust and soybean rust were significant ($P < 0.001$), and there was no difference between the experiments. Growth rate of the number of stained uredinia was similar between the rusts caused by *Phakopsora* species. In bean rust, there was no significant regression, indicating that there was no increase in the number of uredinia over time.

2.3.2. Quantification of fungal biomass by qPCR

Experimentally independent proof, that the increase in the number of uredinia was correlated with enhanced pathogen colonization, was gathered by qPCR and determination of the fungal DNA relative to the host DNA. To minimize PCR effects, e.g. those related to primer efficiency, we optimized PCR conditions in a way that the same primer combinations could be used for amplification of plant or fungal gene fragments in all three interactions (Figure S4).

Melting curves of each primer pair for each host and pathogen were analyzed to verify primer specificity (Figure S5). We observed a unique peak in the derivative melting curve of the plant primer used in *V. labrusca*, *G. max* and *P. vulgaris*, indicating the formation of a single product and the absence of primer–dimers. Melting curves of samples with the lowest concentrations of *P. pachyrhizi* DNA revealed the presence of nonspecific PCR products. This is a known feature of qPCR in cases where target DNA is limited, and excess primers might anneal to nonspecific sequences. Melting curve analysis of the rust primer pair used in *P. meliosmae-myrianthae* and *U. appendiculatus* did not present nonspecific products that could produce a bias in the quantification results, resulting in a defined peak on the derivative melting curve.

Serial dilutions were performed to verify primer sensitivity, where Cq values were obtained from a known starting quantity of gDNA (Figure S6). Cq values ranged from 10.6 to 21.8 cycles for the plant primer for *V. labrusca*, from 10.7 to 32.5 cycles for the plant primer for *G. max*, from 8.1 to 25.9 cycles for the plant primer for *P. vulgaris*, from 12.6 to 24 cycles for the rust primer for *P. meliosmae-myrianthae*, from

13.7 to 31.5 cycles for the rust primer for *P. pachyrhizi*, and from 8.9 to 25.1 cycles for the rust primer for *U. appendiculatus*. Standard curves of each primer pair for each host or pathogen were calculated by linear regressions between Cq and log-transformed SQ (Figure 4). High determination coefficients ($R^2 = 0.99$) between Cq values and gDNA amount were observed for all hosts and pathogens. Taken together, these values indicate that the primers and PCR conditions chosen were suited for this type of approach.

Figure 4 - Standard curves to verify primer sensitivity, with primer pair plant_fwd_1 and plant_rev_1 in gDNA of *Vitis labrusca*, *Glycine max* and *Phaseolus vulgaris* (A, C and E) and primer pair rust_fwd_1 and rust_rev_1 in gDNA of *Phakopsora meliosmae-myrianthae*, *P. pachyrhizi* and *Uromyces appendiculatus* (B, D and F). Quantification cycle value (Cq) indicates the number of cycles required to start to detect relative fluorescence units, and this value was used to obtain standard curves by the relation between Cq and the logarithm of starting quantity.

Using gDNA from infected plant tissue and subjecting it to qPCR analysis, we determined for both *Phakopsora* species similar kinetics of fungal biomass increase over time (Figure 5). From 7 to 12 dpi, soybean rust and grapevine rust presented a sharp increase in fungal biomass, differently from bean rust. Fungal biomass could

be assessed before the emergence of symptoms (Figure S7). Grapevine rust symptoms started at 7 dpi as small yellowish uredinia, and a fully susceptible reaction (so-called TAN lesion) also started in soybean plants at 7 dpi. In both *Phakopsora* species, pustules increased in size over time and released massive amounts of urediniospores. Symptoms of bean rust started at 5 dpi as small white specks which enlarged and formed reddish-brown pustules after 9 dpi in both experiments. Our results confirm that the increase observed for *Phakopsora* spp. uredinia within lesion areas is directly correlated with an enhancement of fungal biomass. Taken together, these findings further substantiate our previous finding that *Phakopsora* spp. do produce a steady or increasing level of secondary inoculum without the need for new infections.

Figure 5 - Fungal biomass (in proportion) over time measured by quantitative polymerase chain reaction (qPCR) 2 to 16 days postinoculation of *Phakopsora meliosmae-myrianthae* in grapevine 'Niagara Rosada' plants, *P. pachyrhizi* in soybean 'Abelina' plants, and *Uromyces appendiculatus* in bean 'Saxa' plants.

2.4. Discussion

In this study, we show for the first time that new satellite uredinia are associated with lesion growth of Asian grapevine leaf rust. Lesion growth ensures the progress of the disease even in the absence of weather conditions favorable for the

initiation of new infection events and the formation of novel uredinia. The emergence of satellite pustules is common among rusts caused by fungi of the genus *Phakopsora* (Bergamin Filho 2008; Seier et al. 2009) and may be associated with the intense damage that these pathogens cause in their hosts. Lesion growth and the continuous production of urediniospores are valuable components of the temporal dynamics of epidemics caused by *Phakopsora* spp. and should be strongly considered in works that focus on plant breeding for resistance to pathogens.

Grapevine and soybean rusts showed higher rates of increase of the mean lesion area and higher rates of increase of the mean number of uredinia per lesion than common bean rust. The low inoculum concentration and, consequently, the low density of lesions, used in this study allowed analysis of individual lesions. Frequently, a high initial inoculum leads to a high density of lesions that coalesce, which makes it then difficult to correctly identify the boundaries between them. Previous studies have reported continuous uredinia emergence of *P. pachyrhizi* on soybean, reaching a maximum of 14 uredinia per lesion, 7 weeks after inoculation (Melching et al. 1979). In our study, the maximum number of non-trypan blue-stained uredinia, around 5 weeks after inoculation, was 11 and 20 uredinia per lesion, for grapevine and soybean rust, respectively. After trypan blue staining, this value was 2.5 and 1.6 times higher than without staining, respectively, for grapevine and soybean rust. In the case of common bean rust, there was no difference in the number of stained and non-stained uredinia.

A high degree of correlation ($0.88 < R^2 < 0.97$) was observed between the lesion areas of the rusts before and after trypan blue staining. For all three rusts, the slope of the regression performed with lesion areas before and after staining was close to 1.0, indicating that the mycelial growth of the pathogens inside the leaf tissue corresponds to the visual symptom. Treatment with chloral hydrate solution resulted in translucent samples and promoted the observation of hyphae stained by trypan blue within the plant tissue (Loehrer et al. 2008). Trypan blue staining allowed a more precise quantification of the number of uredinia for two reasons: (i) it facilitated uredinia identification, even before their emergence; (ii) it removed the mass of urediniospores that blocked a clear observation of the symptoms. Besides usage of trypan blue to visualize fungal structures within host tissues (Bhadauria et al. 2010; Bonde et al. 2006; Maffi et al. 2011), this dye is also commonly used to indicate plant cell death. The latter type of reaction has also been observed after penetration of *P.*

pachyrhizi on non-host plants such as *Arabidopsis thaliana* or barley (Goellner et al. 2010; Hoefle et al. 2009; Loehrer et al. 2008) or soybean cultivars resistant to particular *P. pachyrhizi* isolates (Deverall et al. 1977; Keogh et al. 1980).

For the qPCR trials, primers were designed targeting a conserved region in the DNA sequence of all rust pathogens. This enabled pre-symptomatic monitoring of the colonization progress of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus* in the grapevine, soybean and common bean tissues, respectively, i.e. before the onset of symptoms. However, quantification of the pathogens by qPCR within the host tissue was laborious and complex. To increase qPCR reaction efficiency, it was necessary to adapt protocols for each pathogen–host interaction (Boyle et al. 2005). Hereby, different protocols for DNA extraction and additional cleaning steps to optimize the qPCR reaction of each pathosystem were performed. Another drawback of this technique is the variability in results between biological replicates, which did not provide a clear distinction in the amount of fungal biomass between pathosystems at the beginning of symptom development. qPCR-based calculation of *Colletotrichum graminicola* gDNA on maize has already shown a high degree of variation between replications (Weihmann et al. 2016). Although qPCR assays have paramount importance in the identification and pre-symptomatic detection of pathogens, their power for quantification of pathogen development over time is limited (Weihmann et al. 2016; Wunderle et al. 2012). In our work, the qPCR results generally substantiated the microscopy results, but in the last qPCR evaluation (16 dpi), despite lesion growth, there was a decrease in fungal biomass in rusts caused by *Phakopsora*. This may be related to the greater accumulation of qPCR inhibitory substances, such as phenolic compounds, in later stages of the diseases (Alonso-Villaverde et al. 2011; Lygin et al. 2009; Salzman et al. 1999). In spite of that, this technique is routinely and widely used to compare the resistance levels of plants to their pathogens, such as barley rust, caused by *Puccinia graminis* f. sp. *tritici* (Zurn et al. 2015), corn anthracnose, caused by *C. graminicola* (Weihmann et al. 2016), grapevine gray mold, caused by *Botrytis cinerea* (Diguta et al. 2010), and fusarium head blight of wheat, caused by *Fusarium graminearum* (Horevaj et al. 2011), but not to compare fungal biomass across pathosystems.

In this work, sporulation has not been quantified over time, but it is known that lesion growth allows continuous urediniospore production of pathogens (Bergamin Filho and Amorim 1996). Lesions of *P. pachyrhizi* in soybean, for

example, can produce urediniospores for up to 49 dpi (Melching et al. 1979). This secondary inoculum production, which remains for a long period of time, allows the re-establishment of an epidemic even after the prevalence of conditions unfavorable for reinfection (Bergamin Filho 2008; Salustiano et al. 2007; Yeh et al. 1982). Our hypothesis is that the continuous emergence of new uredinia implies a high demand for nutrient uptake (Tremblay et al. 2012) and, as a consequence, it causes great damage to the host. Leaves infected by biotrophic pathogens show both the accumulation and a dynamics shift of some nutrients, such as nitrogen and phosphorus (Murray and Ayres 1986). Barley leaves infected by *Erysiphe graminis* f. sp. *hordei* show nitrogen accumulation, as its translocation to the roots stops after sporulation. Nitrogen, which is normally recycled, becomes available for the pathogen (Walters 1989). Studies that investigate the sink–source relationship between pathogens of the genus *Phakopsora* and their hosts should be performed to verify the influence of the increase of number of uredinia on plant physiology.

In general, our approach of in-depth monitoring of satellite uredinia represents a significant breakthrough in our comprehension of the biology of *Phakopsora* pathogens and the great damage they cause to their hosts. Determination of the number of uredinia per lesion after trypan blue staining is a precise method to quantify resistance levels of grapevine to *P. meliosmaemyrianthae*. This methodology is already used to phenotypify the reaction of different soybean accessions when inoculated with *P. pachyrhizi* (Bonde et al. 2006). Susceptible soybean plants present, on average, 3 to 7 uredinia per lesion, and resistant plants present, on average, 0 to 2 small uredinia per lesion (Bonde et al. 2006). Our results should be considered in future studies involving automatic disease phenotyping or omics-based analyses of disease physiology in the genus *Phakopsora*.

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SUPPORTING INFORMATION

Figure S1. Plants for inoculum maintenance of soybean rust and bean rust in growth chambers with controlled conditions of temperature and photoperiod (A) and in an inoculation chamber (B).

Figure S2. Individual lesions of Asian grapevine leaf rust (*Phakopsora meliosmae-myrianthae*) in grapevine 'Niagara Rosada' (A and B), Asian soybean rust (*P. pachyrhizi*) in soybean 'Abelina' (C and D) and bean rust (*Uromyces appendiculatus*) in bean 'Saxa' (E and F) on abaxial (A, C and E) and on adaxial faces (B, D and F). Pustules formed only on the abaxial leaf face corresponded to chlorotic/necrotic lesions on the adaxial leaf face. Scale bars: 5 mm.

Figure S3. Bean rust (*Uromyces appendiculatus*) symptom before (left side) and after (right side) trypan blue staining at 8th (A), 9th (B) and 10th (C) harvest, corresponding to 26, 30 and 33 days post first symptom, respectively. Telia formation was observed on the edge of the lesions. Scanning electron microscope (D) and brightfield (E) images of teliospores (solid arrow; not echinulate and brownish spores) and urediniospores (dashed arrow, echinulate and yellowish spores) of *U. appendiculatus*. Scale bars: 1000 μ m for (A), (B), and (C); 40 μ m for (D) and (E).

Figure S4. Results of qualitative polymerase chain reaction (PCR) by agarose gel electrophoresis analysis (A). Specificity of the plant-specific primer pair plant_fwd_1 and plant_rev_1 (host primers) amplifying part of the plant *rbcL* gene only in host samples (Pv: *Phaseolus vulgaris*; Gm: *Glycine max*; and VI: *Vitis labrusca*) and of rust-specific primer pair rust_fwd_1 and rust_rev_1 (rust primers) amplifying part of the internal transcribed sequence – ITS – only in rust samples (Uap: *Uromyces appendiculatus*; Ppa: *Phakopsora pachyrhizi*; and Pmm: *P. meliosmae-myrianthae*). Bands from A were cut and sent for sequencing to confirm a partial nucleotide sequence of the *rbcL* gene (175 bp) of *V. labrusca*, *G. max* and *P. vulgaris* and partial nucleotide sequence of the ITS region (148 bp) of *P. meliosmae-myrianthae*, *P. pachyrhizi* and *U. appendiculatus* (B). Arrows indicate the position of the primer sequence (5' - 3').

Figure S5. Derived melting curves to verify primer specificity. Quantitative polymerase chain reaction (qPCR) with primer pair plant_fwd_1 and plant_rev_1 in gDNA of *Vitis labrusca*, *Glycine max* and *Phaseolus vulgaris* (A, C and E) and primer pair rust_fwd_1 and rust_rev_1 in gDNA of *Phakopsora meliosmae-myrianthae*, *P. pachyrhizi* and *Uromyces appendiculatus* (B, D and F).

Figure S6. Cycles related to relative fluorescence units (RFU) obtained by quantitative polymerase chain reaction (qPCR) with primer pair plant_fwd_1 and plant_rev_1 in gDNA of *Vitis labrusca*, *Glycine max* and *Phaseolus vulgaris* (A, C and E) and primer pair rust_fwd_1 and rust_rev_1 in gDNA of *Phakopsora meliosmae-myrianthae*, *P. pachyrhizi* and *Uromyces appendiculatus* (B, D and F).

Figure S7. Leaf discs with similar rust severity of samples used in qPCR from each pathosystem before (left side) and after (right side) trypan blue staining. Discs of grapevine leaf rust (A, D and G), soybean rust (B, E and H) and bean rust (C, F and I) harvested at different timepoints: 7 (A, B and C), 12 (D, E and F) and 16 (G, H and I) days postinoculation. Scale bars: 4 mm.

3. ADAPTATION OF *PHAKOPSORA* RUSTS TO THE TROPICS IS RELATED TO PATHOGEN SURVIVAL AND REPRODUCTION STRATEGIES

Abstract

Phakopsora pachyrhizi is the causal agent of Asian soybean rust and is an example of rust pathogen adapted to tropical regions where it causes severe epidemics. *P. meliosmae-myrianthae* is the causal agent of Asian grapevine leaf rust with symptoms similar to *P. pachyrhizi*, such as the presence of satellite uredinia and early leaf fall, that result in severe damage. To shed light on *P. meliosmae-myrianthae* adaptation to the tropics, we compared the monocyclic components (incubation period, latent period, infectious period, number of uredinia, and disease severity) of these *Phakopsora* species and the effects of these pathogens on host photosynthesis over time. Incubation and latent periods were coincident for 8 days in Asian grapevine leaf rust and 13 days in Asian soybean rust. Minimum infectious periods were 21 days for *P. meliosmae-myrianthae* and 13 days for *P. pachyrhizi*, and typical of tropical pathogens, presented several sporulation peaks. Both *Phakopsora* rusts showed an increase in disease severity with similar progress rates that were estimated with the monomolecular model as 0.06 day⁻¹ and 0.05 day⁻¹ for grapevine and soybean rusts, respectively. No increase in the number of lesions was observed from the first assessment, though the number of uredinia did increase over time. *P. meliosmae-myrianthae* and *P. pachyrhizi* infection reduced the net photosynthetic rates by 22 % and 5 %, respectively, before the onset of symptoms. How these pathogens withstand non-ideal environmental conditions to provoke secondary infections and how pathogen survival and reproduction are related to their tropical adaptations are discussed. We conclude that the epidemiological behaviour of *P. meliosmae-myrianthae* is similar to *P. pachyrhizi*.

Keywords: *Phakopsora euvitidis*; Pathogen adaptation; Infectious period; Lesion growth; *Vitis labrusca*

3.1. Introduction

Rusts caused by the genus *Phakopsora* are devastating diseases to crops and can cause yield reduction during the season, or even in the following seasons for perennial crops. Asian soybean rust, caused by *Phakopsora pachyrhizi*, and Asian grapevine leaf rust, caused by *P. meliosmae-myrianthae*, are examples of uncommon rust diseases. Both rusts have similar characteristics, including lesion growth associated with the emergence of new uredinia (Chapter 2), leaf tissue necrosis, significant CO₂ assimilation reductions in green tissues surrounding lesions,

and early defoliation (Yang *et al.*, 1990; Kumudini *et al.*, 2010; Godoy *et al.*, 2016; Nogueira Júnior *et al.*, 2017; Primiano *et al.*, 2017; Scapin-Buffara *et al.*, 2018). In grapevines infected with *P. meliosmae-myrianthae*, reduced carbohydrate reserves resulted in the reduction of grapevine vigour in years following epidemics (Nogueira Júnior *et al.*, 2017). Despite the economic importance of these diseases, many aspects of their epidemiology remain obscure (Lorrain *et al.*, 2018). Rusts are polycyclic diseases with many disease cycles during an epidemic (Madden *et al.*, 2007). To avoid the complexity of natural conditions, monocyclic processes are used to better understand rust population dynamics (Pivonia & Yang, 2006).

Polycyclic diseases are usually described in epidemiology by the "quintuplet epidemic" as consisting of the initial disease inoculum (y_0), latent period (p – time interval between inoculation and up to 50 % of lesions exhibiting sporulation onset), infectious period (i – time interval that the lesion produces spores), daily production of spores (N), and inoculum efficiency (E – proportion of urediniospores that cause new lesions) (Zadoks & Schein, 1980). In addition to these five components, lesion growth (k_{exp}) should also be considered a quantitative component in studies of pathogen aggressiveness to constitute the "epidemic sextuplet" (Berger *et al.*, 1997). An interesting aspect not yet addressed for rusts in the literature is that this sixth component (i.e., k_{exp}) is epidemiologically equivalent to the interaction of the infectious period with spore production (i.e., $i \cdot N$). In this way, the rust pathogens may present one peak of spore production when lesion growth is limited, or several peaks when lesion growth is expressive. Generally temperate rusts show one peak of spore production, while tropical rusts show several peaks through the infectious period (Berger *et al.*, 1995; Bergamin Filho, 2008). Temperate pathogens typically present low initial disease inoculum, a short latent period, short infectious period, high urediniospore production, and high inoculum efficiency. As a result, temperate rusts present with a high apparent rate of infection (r). Tropical pathogens, or those more adapted to the tropics, can present most of their monocyclic components different from the temperate pathogens (Zadoks & Schein, 1979; Kranz, 2003). In general, tropical pathogens have several sporulation peaks throughout their infectious period and present with lesion growth (Sache & Vallavieille-Pope, 1995). The apparent rate of infection of tropical rusts is normally lower than of temperate rusts (Bergamin Filho & Amorim, 1996).

The unique behaviour of tropical pathogens is partially explained by the climatic conditions of tropical or subtropical regions, which favour the presence of evergreen leaves, that is, a green bridge. In these regions, temperature is relatively uniform throughout the year and frost is infrequent. These conditions ensure a constant presence of host plants, and benefit especially pathogens, such as rust fungi, that depend on their host's survival. *Phakopsora pachyrhizi* is an example of a pathogen with a tropical epidemiological behaviour, as it presents lesion growth and several peaks of sporulation throughout the infectious period. These characteristics contribute to pathogen perpetuation, even in environmental conditions unfavourable to infection (Melching *et al.*, 1979; Bergamin Filho & Amorim, 1996).

Although there are several reports of the monocyclic components of soybean and grapevine rust in the literature, no comparative epidemiological analysis has verified whether *P. meliosmae-myrianthae* is classified as a pathogen adapted to the tropics. Data from monocyclic components are fragmented in the literature and in general are not comparable between pathosystems, either because the methodology of the experiments was variable or because data acquisition methods differed (Berger *et al.*, 1995; Alves *et al.*, 2007; Bonde *et al.*, 2007; Angelotti *et al.*, 2014). Further, photosynthetic rate reductions due to soybean and grapevine rusts was not evaluated during the rust monocycle (Kumudini *et al.*, 2010; Nogueira Júnior *et al.*, 2017). The aims of this work was to characterize the monocycle of Asian grapevine leaf rust in comparison to Asian soybean rust.

3.2. Materials and methods

3.2.1. Plant material and inoculation

Five soybean cv. M6410IPRO (*Glycine max*) seeds were sown in a 2-L pot containing a sterilized mixture of substrate Basaplant® and Fertsolo® (1:1, v/v). Substrate Basaplant® is a commercial substrate, made of processed peat, vermiculite, pine bark, and charcoal, with a pH of 5.8 (± 0.5) and EC (mS cm^{-1}) of 1.5 (± 0.3). Fertsolo® is a commercial mixture of earthworm humus, charcoal, coarse sand, sifted earth, processed pine bark, and bagasse. A total of 2.5 g of NPK (08:28:16) was added to each pot. At 15 days post seedling emergence, plants were thinned to one plant per pot based on appearance and homogeneity among pots.

Beginning at 20 days after seedling emergence, a 5-mL foliar fertilization with a nutrient solution containing 450 mg L⁻¹ of calcium nitrate, 300 mg L⁻¹ of potassium sulphate, 280 mg L⁻¹ of magnesium sulphate, 210 mg L⁻¹ of monopotassium phosphate, 30 mg L⁻¹ of iron, 25 mg L⁻¹ of micronutrients were weekly sprayed in each soybean plant. Grapevine cv. Niagara Rosada (*Vitis labrusca*) plants grafted on 'IAC 766-Campinas' were planted in 7-L pots containing a sterilized mixture of clay soil, manure, and sand (1:1:1). After pruning, a total of 20 g of NPK (08:28:16) was added to each pot. All grapevine and soybean plants were grown in a greenhouse at 23 °C ($\pm$ 3 °C) and 76 % relative humidity ($\pm$ 9 %). Grapevine 'Niagara Rosada' and soybean 'M6410IPRO' plants are highly susceptible to *P. meliosmae-myrianthae* and *P. pachyrhizi*, respectively.

Six pots of grapevine and soybean plants were spray-inoculated with 10⁴ urediniospores mL⁻¹ of *P. meliosmae-myrianthae* and *P. pachyrhizi*, respectively. Each isolate was maintained on the same cultivars used for experiments and urediniospores were freshly harvested from plants before inoculation. On average, germination of *P. meliosmae-myrianthae* urediniospores was 20 %, and of *P. pachyrhizi* urediniospores was 95 % on 1 % water-agar. A 5 mL suspension of each inoculum was pulverized with a spray nozzle at 1 bar for 10 sec in a 900 cm² area in which the third and the fourth grapevine leaves (20 days-old after pruning) and the first trefoil of soybean plants (approximately 35 days-old soybean plants) were arranged with the abaxial leaf surface facing upwards (Figure S1). Grapevine plants were kept in a dark moist chamber at 25 °C and soybean plants at 23 °C for 24 h. After this period, grapevine and soybean plants were maintained in plant growth chambers (Convion E-7, Winnipeg, Canada) with a 12-h photoperiod, and temperature and humidity as previously. As a control, three plants each of grapevine and soybean were mock-inoculated with sterilized water containing 0.1 % Tween.

3.2.2. Monocyclic components

A 6 cm² leaf area was delimited for evaluation of the monocyclic components. Within this area the incubation period (the time interval from inoculation until the onset of 50 % of the lesions), the latent period (the time interval from inoculation until the onset of 50 % of sporulating lesions), the number of lesions, the number of uredinia per lesion, and the disease severity (percentage of diseased leaf

area) were assessed. Every 3 or 4 days, this 6 cm² area of each leaf was photographed until a maximum of 34 days post inoculation (dpi). The number of lesions, the number of uredinia, and disease severity were estimated with ImageJ2 (version 1.8.0) software (Rueden *et al.*, 2017). The infectious period (the time interval from the beginning to the cessation of urediniospore production) and urediniospore germination were also evaluated, but in other leaves. Every 5 or 7 days, the inoculum produced in the delimited 6 cm² leaf area of each plant was collected with the aid of a brush and deposited in 1 mL of sterilized water with 0.1% Tween. We evaluated urediniospore production and urediniospore germination in this suspension. From each replicate, three 40 µL droplets of this urediniospore suspension were placed on 1 % water-agar in Petri dishes. Dishes were kept in the dark at 25 °C and at 23 °C for *P. meliosmae-myrianthae* and for *P. pachyrhizi* urediniospores germination, respectively. After 24 h, lactoglycerol was added to each droplet, interrupting the germination process. Percentage germination was assessed by counting 100 urediniospores per droplet, and the average of 3 droplets was calculated and used as the replicate value. Urediniospores production was evaluated using a Neubauer's chamber and the average of 3 evaluations was calculated and used as the replicate value.

3.2.3. Leaf gas exchange

This study was carried out to assess the influence of *Phakopsora* species on physiological aspects of each host. Net photosynthetic rate (P), stomatal conductance (g_s), intercellular CO₂ concentration (C_i), and transpiration rate (E) were evaluated at 6, 9, 15, 23, and 30 days post inoculation (dpi). Measurements were performed with an infrared gas analyser LI-6400XT equipped with the fluorometer head (6400-40, LI-COR Inc., Lincoln, NE, USA) using 1000 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR), 400 µmol mol⁻¹ ambient CO₂ concentration in the chamber, 500 µmol s⁻¹ airflow rate, and ambient air temperature (25 °C for grapevine plants and 23 °C for soybean plants). The evaluation of these variables was always performed on the same 2 cm² sampling area of a healthy (control) leaf and of an inoculated leaf. Following each measurement, the abaxial face of this area was isolated and photographed. Each image was analyzed using ImageJ2 (version

1.8.0) software (Rueden et al. 2017) and the severity of symptomatic leaves, including necrotic areas, was quantified.

3.2.4. Experimental design and data analysis

For all evaluations, a leaf per pot of grapevine and soybean plants was considered as a biological replicate. Six replicates were used in each experiment and the experimental design was completely randomized. All experiments were performed twice.

The number of uredinia and disease severity were first normalized to the highest value of each experiment (fixed as 1) and proportion data were analysed over time. Monomolecular model [$y(t) = y_{max} - (y_{max} - y_0) \exp(-r t)$] was fitted to data by nonlinear regression in STATISTICA® software (version 7.0, StatSoft, Tulsa, USA), where $y(t)$ corresponds to the (proportional) number of uredinia or disease severity; y_0 , to the initial inoculum; r , to the progress rate of the number of uredinia or of the disease severity; y_{max} , to the asymptote; and t , to time in days post inoculation. Regression parameters obtained from each of the two experimental replications were compared with Student's t tests and a new regression was performed with data pooled from both experiments when the parameters did not significantly differ. The estimated rate of each pathosystem was compared with a Student's t test with alpha set to 0.05 (Madden *et al.*, 2007).

All leaf gas exchange variables were transformed to proportions relative to the average value of 3 healthy leaves (P_x/P_o , g_{sx}/g_{so} , C_x/C_o , and E_x/E_o) where variables followed by an 'x' were measured on diseased plants and those followed by 'o' on healthy plants. Photosynthetic rate and disease severity were analysed over time with data pooled from both experiments. Linear regressions were performed using STATISTICA® software (version 7.0, StatSoft, Tulsa, USA). Slopes of each pathosystem were compared using a Student's t test with alpha set to 0.05.

3.3. Results

3.3.1. Monocyclic components

Synchronous incubation and latent periods were observed for each rust species, which means that all visible lesions had urediniospores. Latent periods were 7 and 8 days for experiments 1 and 2, respectively, for the Asian grapevine leaf rust, and 13 and 14 days for Asian soybean rust. The rate of progress for the number of *P. meliosmae-myrianthae* uredinia over time (0.03 day^{-1} , Table 1 and Figure 1a) was similar to that estimated for *P. pachyrhizi* in the first experiment (0.05 day^{-1} , Table 1 and Figure 1c), but differed from the second experiment (0.15 day^{-1} , Table 1 and Figure 1c). The progress rate of Asian grapevine leaf rust severity (0.06 day^{-1} , Table 1 and Figure 1b) was also similar to that of the Asian soybean rust severity (0.05 day^{-1} , Table 1 and Figure 1d).

Figure 1 - Uredinia progress curves as proportions (a and c) and disease severities (b and d) over time for *Phakopsora meliosmae-myrianthae* on grapevine 'Niagara Rosada' leaves (a and b) and *Phakopsora pachyrhizi* on soybean 'M6410IPRO' leaves (c and d). Both the first (black circles) and second (white circles) experiments are presented. Dashed and dotted lines correspond to monomolecular model fitted to the data. In c, dashed line corresponds to the first experiment and dotted line, to the second experiment.

Table 1 - Parameters and respective standard errors (in parentheses) estimated by non-linear regression with the monomolecular model, fitted to the proportion of uredinia or disease severity over time of Asian grapevine leaf rust caused by *Phakopsora meliosmae-myrianthae*, and of Asian soybean rust caused by *P. pachyrhizi*.

Parameters ^a	Uredinia (proportion)			Disease severity	
	AGLR ¹	ASR ²	ASR ³	AGLR ¹	ASR ¹
y_{max}	2.02	1.5	1.06	1.36	1.71
(error)	(-0.89)	(-0.38)	(-0.06)	(-0.13)	(-0.6)
y_0	-0.2	-1.09	-5.82	-0.77	-1.2
(error)	(-0.13)	(-0.48)	(-2.31)	(-0.13)	(-0.5)
r	0.03	0.05	0.15	0.06	0.05
(error)	(-0.02)	(-0.02)	(-0.02)	(-0.01)	(-0.02)
R^2	0.8	0.91	0.89	0.91	0.8

^a Coefficients of determination (R^2) and parameters were estimated with the monomolecular model $y(t) = y_{max} - (y_{max} - y_0) \exp(-r t)$ where, $y(t)$ corresponds to the proportion of uredinia or disease severity, y_{max} to the asymptote, y_0 to the initial inoculum, r to the proportional progress rate of the number of uredinia or of the disease severity, and t to time (in days post inoculation).

¹ analysis performed with pooled data from 2 experiments. AGLR = Asian grapevine leaf rust. ASR = Asian soybean rust.

² analysis performed with data from experiment 1

³ analysis performed with data from experiment 2

The number of sporulating lesions that first appeared in each experimental replication was constant throughout the evaluations (*data not shown*). A maximum average of 7.2 and 14.4 lesions of *P. meliosmae-myrianthae* and 37 and 36.5 lesions of *P. pachyrhizi* were counted per 6 cm² area in the first and second experiments, respectively (Figure S2). The number of urediniospores was quantified up to 31 dpi for Asian grapevine leaf rust and up to 27 dpi for Asian soybean rust, resulting in a 21-day infectious period for *P. meliosmae-myrianthae* and 13-day infectious period for *P. pachyrhizi*. Frequent brushing caused leaf injury preventing continued spore harvesting for both rusts. In spite of this, the urediniospore yield was on average 1,394 and 2,316 cm⁻² for *P. meliosmae-myrianthae* for the first and second experiment, respectively. *Phakopsora pachyrhizi* urediniospores production varied on average from 1,145 to 1,450 urediniospores cm⁻² on soybean leaves, for experiments 1 and 2, respectively (Figure 2). Urediniospore germination varied over time between 3.5 % and 35.5 % for *P. meliosmae-myrianthae* and between 9.6 % and 76 % for *P. pachyrhizi*.

Figure 2 - Urediniospore production over time for *Phakopsora meliosmae-myrianthae* in grapevine 'Niagara Rosada' leaves (circles) and *Phakopsora pachyrhizi* in soybean 'M6410IPRO' leaves (triangles). The first (black circles and black triangles) and second (white circles and white triangles) experiments are both presented. Bars represent the standard error ($n = 6$).

3.3.2. Leaf gas exchange

Photosynthetic rate (P_o), stomatal conductance (g_{so}), intercellular CO_2 concentration (C_{io}), and transpiration rate (E_o) of healthy grapevine and soybean leaves were obtained at each time point (6, 9, 15, 23, and 30 dpi; Table 2) and compared with values of inoculated plants to obtain relative values over time (Figure 3 and Figure S3).

Figure 3 - Asian grapevine leaf rust (a, *Phakopsora meliosmae-myrianthae*) and Asian soybean rust (b, *Phakopsora pachyrhizi*) progress and relative net photosynthetic rate (Px/Po) over time on grapevine 'Niagara Rosada' plants (c) and soybean 'M6410IPRO' plants (d) in the first (dark circles) and in the second (white circles) experiments. Bars represent the mean standard error ($n = 6$). Dashed lines represent values of diseased plants equal to healthy plants. Dotted lines represent the linear regression fit of pooled data from both experiments ($y = ax + b$, where y is disease severity or relative net photosynthesis, x is time [days post inoculation], ' a ' is the slope, and ' b ' the intercept; R^2 is the coefficient of determination).

The net photosynthetic rate decreased 22 % in inoculated grapevines before the onset of symptoms. Similarly, Px in inoculated soybeans was reduced 6 % relative to healthy plants. In the area evaluated with the infrared gas analyser, the estimated average of Asian grapevine leaf rust severity was 0.72 % at 9 dpi. Disease severity increased linearly at a rate of 0.16 day^{-1} , reaching 4.08 % at the last evaluation (Figure 3a). The estimated Asian soybean rust severity was 0.03 % at 10 dpi and the progress rate was significantly lower (0.03 day^{-1}) than that of Asian grapevine leaf rust (Figure 3b). As a consequence, estimated disease severity at the last evaluation (30 dpi) was 0.63 % (Figure 3b). Px/Po decreased linearly over time for both rusts at similar rates (Figure 3c and d).

Table 2 – Net photosynthetic rate (P_o), stomatal conductance (g_{s_o}), intercellular CO_2 concentration (C_o), and transpiration rate (E_o) of healthy grapevine 'Niagara Rosada' plants and soybean 'M6410IPRO' plants.

	Net photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) ^a	Estomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) ^a	Intercellular CO_2 concentration ($\mu\text{mol mol}^{-1}$) ^a	Transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) ^a
Grapevine	9.65 - (12.37) - 15.6	0.08 - (0.15) - 0.25	126.77 - (232.8) - 281.67	1.98 - (3.45) - 5.32
Soybean	11.3 - (14.57) - 18.7	0.21 - (0.28) - 0.30	267.6 - (292.4) - 306.3	4.14 - (5.58) - 7.21

^a minimum and maximum values over the evaluations, mean in parentheses.

The relative intercellular CO_2 concentration (C_x/C_o) remained constant over time for both rusts and it was not influenced by disease severity (Figure S3). Relative stomatal conductance (g_{s_x}/g_{s_o}) and transpiration (E_x/E_o) presented similar patterns (Figure S3) since these variables are positively related (Bassanezi *et al.*, 2002). For both rusts, stomatal conductance and transpiration rate slightly decreased during disease progress, mainly after the onset of pustules. Variation between experiments was observed, as observed in other studies.

3.4. Discussion

Asian grapevine leaf rust has similar monocyclic components as Asian soybean rust and other tropical rusts, particularly (i) increasing numbers of uredinia and disease severity without new infections, and (ii) continuous production of viable urediniospores, with several spore production peaks, throughout the infectious period. This pattern of sporulation leads to an increase in the chances of pathogen survival since the pathogen produces spores for a long time course. The continuous moderate production of viable urediniospores allows the pathogen to surpass periods of drought or high temperatures that are unsuitable for new infections and represents an evolutionary advantage for tropical rusts. Without severe winters and with the presence of evergreen leaves throughout the year, tropical rusts remain endemic, even with lower urediniospore yields than temperate rusts (Sache & Vallavieille-Pope, 1995). As a consequence, epidemics of tropical rusts are less explosive than epidemics of temperate rusts (Bergamin Filho & Amorim, 1996).

To achieve this comparative perspective about *Phakopsora* spp. epidemiology and to quantify monocyclic components across-pathosystems accurately, the methodology used in our experiments was standardized (Kranz,

2003). As a first step, inoculations with low concentrations of urediniospores were used. For assessing monocyclic components of Asian soybean rust, inoculations with a low-density of urediniospores are recommended to avoid lesion coalescence and to precisely identify each individual lesion (Zanatta *et al.*, 2012). High lesions density is generally associated with a decrease in uredinium size and in urediniospore production, as observed in the barley – *Puccinia hordei* and wheat – *Puccinia triticina* pathosystems, respectively (Teng & Close, 1978; Robert *et al.*, 2002, 2004; Pariaud *et al.*, 2009). In addition, the evaluation of the monocyclic components was always carried out in the same area of the leaf using non-destructive sampling throughout the experimental period in order to precisely quantify the growth of individual lesions.

Previous studies determined the effect of several environmental variables on the infection process of *P. pachyrhizi* on soybean leaves and of *P. meliosmae-myrianthae* on grapevine leaves (Marchetti, 1976; Adendorff & Rijkenberg, 2000; Naruzawa *et al.*, 2006; Magnani *et al.*, 2007; Furtado *et al.*, 2009; Gomes *et al.*, 2011; Navarro *et al.*, 2015; Alves, 2015). Optimal germination in *P. pachyrhizi* occurs at 22.5 °C (range of 10 to 27.5 °C) with a 24 h wetness period even though a small quantity of urediniospores germinate after 6 h (Marchetti, 1976; Melching, 1989). Optimal germination in *P. meliosmae-myrianthae* occurs at 20–25 °C (range of 10 to 30 °C) with 24 h wetness in the dark (Naruzawa *et al.*, 2006; Alves, 2015). After successful infection, plant tissue is colonized by the pathogen and lesions can be observed. In our work, incubation and latent periods were similar for each *Phakopsora* rust. Asian grapevine leaf rust symptoms and spore production started 8 days post inoculation at 25 °C, on average. The Asian grapevine leaf rust latent period under equivalent inoculation conditions observed in other reports was similar to our data (Angelotti *et al.*, 2014; Alves, 2015). Incubation and latent periods of Asian soybean rust are in general 6 to 10 days post inoculation at 22 °C (Pivonia & Yang, 2006; Alves *et al.*, 2007; Danelli & Reis, 2016). In our work, sporulating uredinia of Asian soybean rust were observed 13 days post inoculation at 23 °C. Since experiments were conducted under optimum environmental conditions for infection, the difference in the latent period of Asian soybean rust observed in this study in relation to the literature may be due to the interaction of the pathogen with the specific cultivar M6410IPRO.

Phakopsora rusts severity increased over time despite the number of lesions remaining constant throughout the experiment. This increase in the disease severity

was due to increased lesion area caused by an increase in the number of uredinia. Lesion growth is typical of tropical rusts, such as myrtle rust caused by *Austropuccinia psidii*, and coffee rust caused by *Hemileia vastatrix* (Coutinho *et al.*, 1998; Salustiano *et al.*, 2007). Both rusts cause severe damages to their hosts with significant impacts to agriculture. Coffee rust is a remarkable rust with a lesion expansion rate that can reach 0.14 to 0.16 mm² day⁻¹ (Salustiano *et al.*, 2007; Avelino *et al.*, 2015). Myrtle rust also presents with lesion expansion and several sporulation peaks throughout its infectious period (Castro *et al.*, 1984; Coutinho *et al.*, 1998).

Long infectious periods up to 36 days are reported for Asian soybean rust (Melching *et al.*, 1979; Yeh *et al.*, 1982). The infectious period of both *Phakopsora* rusts, estimated in our work, were also long but estimations had to be interrupted by leaf senescence and the difficulty of harvesting spores on damaged plant tissues. Leaf damage caused by the urediniospores removal technique is an inconvenience that limits the assessment period of the disease. Most temperate rusts have short infectious periods, for example, wheat rust caused by *Puccinia recondita* f. sp. *tritici* and *P. striiformis* presented infectious periods up to 12 days (Sache & Vallavieille-Pope, 1993). Several peaks of urediniospores production were quantified for both Asian grapevine leaf rust and Asian soybean rust throughout their infectious periods. This behaviour is not observed in temperate rusts, such as brown barley rust caused by *Puccinia hordei*, which presented only one peak of urediniospores production through its infectious period (Teng & Close, 1978). In general, temperate rusts produce more urediniospores than tropical rusts regardless of lesion density (Sache & Vallavieille-Pope, 1995). *Puccinia recondita* f. sp. *tritici* in wheat plants had a sporulation peak of 22,500 to 36,500 urediniospores per cm² at 16 days post inoculation (Sache, 1997). The maximum daily yield of urediniospores observed in this study was 4,218 and 1,851 urediniospores per cm² for *P. meliosmae-myrianthae* and *P. pachyrhizi*, respectively. Besides the several spore production peaks throughout the infectious period, our study also found that the *Phakopsora* urediniospores remained viable over the infectious period. There is no correlation between viability and infectivity of *P. pachyrhizi* urediniospores since the same level of infectivity was observed for urediniospores with 35 % and 72 % germination rates on detached soybean leaves (Park *et al.*, 2008). The authors suggest that germ tube growth is a more important factor than spore viability since urediniospores with fast-

elongating germ tubes will have better chances of a successful infection than those with slow-elongating germ tubes. In our study, the urediniospores germination rate varied from 3.5 % to 35.5 % and from 9.6 % to 76 % for, respectively, *P. meliosmae-myrianthae* and *P. pachyrhizi*. No visual differences in the length of the germ tubes were observed. This suggests that urediniospores produced during the infectious period under favourable conditions for infection can infect healthy tissues and produce new uredinia.

The photosynthetic rates were strongly reduced in grapevine and soybean leaves inoculated with *P. meliosmae-myrianthae* and *P. pachyrhizi*, respectively. Even before the onset of symptoms, the net photosynthetic rate of 'Niagara Rosada' leaves inoculated with *P. meliosmae-myrianthae* was reduced by 22 %. At the end of the experiments when the mean grapevine rust severity reached 4.2 %, the net photosynthetic rate was reduced 52 %. Our data confirm previous observations showing grapevine photosynthetic rate reductions even in areas distant from those invaded by *P. meliosmae-myrianthae* (Nogueira Júnior *et al.*, 2017). Morphological changes, such as hypertrophy of mesophyll cells, decreased intercellular air spaces, and chloroplast degeneration with starch accumulation were related to the severe photosynthesis reductions in grapevine leaves showing rust symptoms (Nogueira Júnior *et al.*, 2017). The effect of *P. pachyrhizi* on the net photosynthetic rate of soybean plants was less pronounced, but still greater than that observed in other rust-infected plants (Shtienberg, 1992; Bassanezi *et al.*, 2001). Soybean net photosynthetic rate was reduced 5 % before the onset of symptoms. At the end of the experiments when the mean soybean rust severity was 0.73 %, the net photosynthetic rate reduction reached 19 %. Previous studies estimated that net photosynthetic rate reductions of 20 % in soybean plants occurred only when rust severity reached 10 % (Kumudini *et al.*, 2010). However, high variability in photosynthetic response was observed in actual data, from 0 to 50 % reduction when the disease severity ranged up to 10 % (Kumudini *et al.*, 2010). Net photosynthetic rate reductions before the onset of symptoms or beyond the area of lesions is more frequently observed in necrotrophic or hemibiotrophic pathogens than in biotrophic pathogens. A steep decrease in the net photosynthetic rate of common bean infected with *Colletotrichum lindemuthianum* was reported even at low disease severity (Bassanezi *et al.*, 2001). Many necrotrophic pathogens produce toxins whose effects extend beyond colonized areas and allow the pathogen to access resources by

destroying host tissue (Berger *et al.*, 1995; Newton *et al.*, 2010). This phenomenon is not reported in biotrophic pathogens, which in general do not cause collateral damage and feed on living host cells (Newton *et al.*, 2010).

The continuous spore production and the lack of periods with environmental conditions unfavourable to *P. pachyrhizi* survival were responsible for soybean rust epidemics in Brazil at the beginning of 21st century. As a consequence, a public policy was adopted in Brazil to reduce the survival of *P. pachyrhizi* inoculum and thus delay the Asian soybean rust outbreak in the subsequent season (Li *et al.*, 2010; Godoy *et al.*, 2015, 2016). This strategy is called the soybean-free period and consists of a mandatory period, for a minimum of 60 days, of total soybean plant absence in fields, either cultivate or volunteer soybean plants (Godoy *et al.*, 2015, 2016; Langenbach *et al.*, 2016).

In several regions of Brazil grapevine is produced in a two growing-season per year system with an overlap or short interval between the seasons (Scapin-Buffara *et al.*, 2018). Build-up of *P. meliosmae-myrianthae* inoculum under these conditions is likely connected to this year-round green-bridge and the wide disease dispersal in the Brazilian territory (Primiano *et al.*, 2017). As was already implemented for soybean, a polyetic epidemiological approach could verify the importance of the previous growing season as source of inoculum and identify new management strategies to reduce the disease incidence in the viticulture industry.

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SUPPORTING INFORMATION

Figure S1. Selected area (900 cm²) for spray inoculation in grapevine 'Niagara Rosada' leaves (a) and in soybean 'M6410IPRO' leaflets (b).

Figure S2. Lesion growth of *Phakopsora* rusts. The number of lesions was constant, but each lesion grew in size and number of uredinia over time. Asian grapevine leaf rust (*Phakopsora meliosmae-myrianthae* – dark arrows) and Asian soybean rust (*Phakopsora pachyrhizi* – white arrows) lesions on grapevine 'Niagara Rosada' plants (a and c) and soybean 'M6410IPRO' plants (b and d), respectively. dpi = days post inoculation.

Figure S3. Asian grapevine leaf rust (*Phakopsora meliosmae-myrianthae*) and Asian soybean rust (*Phakopsora pachyrhizi*) progress and changes in photosynthetic variables over time on grapevine 'Niagara Rosada' plants (a, c, and e) and soybean 'M6410IPRO' plants (b, d, and f) in the first (dark circles) and in the second (white circles) experiments. The relative photosynthetic variables were relative stomatal conductance (g_{sx}/g_{so} – e and f), relative intercellular CO_2 concentration (C_{ix}/C_{io} – g and h), and relative transpiration (Ex/E_o – i and j). Bars represent the mean standard error ($n = 6$). Dashed lines represent values of diseased plants equal to healthy plants.

4. DEFOLIATION RATE SHIFTS CAUSED BY PHAKOPSORA RUSTS

Abstract

Symptoms of Asian grapevine leaf rust and Asian soybean rust, caused by *Phakopsora meliosmae-myrianthae* and *P. pachyrhizi*, respectively, are associated with early host leaf fall. Early defoliation implies that photoassimilate reallocation to storage organs is reduced, and consequently, that yield losses are incurred. The defoliation rate is an important parameter to incorporate into models simulating yield losses as a damage mechanism, but there are no current estimates related to *Phakopsora* rusts. Thus, this work aimed to estimate the relative defoliation rates caused by Asian grapevine and soybean rusts and their relationships to a range of disease severities. Field trials consisted of grapevine and soybean plants inoculated, respectively, with *P. meliosmae-myrianthae* and *P. pachyrhizi* urediniospore suspensions at varying concentrations. Portions of the vineyard and of the soybean field were fungicide-sprayed to evaluate natural leaf senescence. Every three or four days, the disease severity in each leaf or leaflet was evaluated on 1326 grapevine leaves and on 643 soybean leaflets. Defoliation rates ($RRDEF_t$) were calculated with the model $RRDEF_t = ((\ln(L_{t1}) - \ln(L_{t0})) / (t_1 - t_0))$, where L is the total number of leaves at time t . Defoliation rates in grapevine and soybean were positively correlated with the mean disease severity according to a logarithmic model. On symptomless grapevine and soybean leaves, defoliation rates were 0.05 day^{-1} and 0.06 day^{-1} , respectively, while on diseased grapevine leaves (disease severity between 12.1 and 25 %) it was 0.13 day^{-1} and on soybean leaflets (disease severity between 25 and 60 %) it was 0.12 day^{-1} .

Keywords: *Vitis labrusca*; Biotrophic pathogens; Simulation model; *Phakopsora euvitis*

4.1. Introduction

Asian soybean rust is caused by *Phakopsora pachyrhizi* and is a severe disease that can result in yield losses as high as 80 %. Once the disease is established, control is difficult (Sikora *et al.*, 2014; Godoy *et al.*, 2016). Asian grapevine leaf rust is also caused by a fungus from the genus *Phakopsora* and is extremely severe on its host. The causal agent of Asian grapevine leaf rust, *P. meliosmae-myrianthae*, infects susceptible plants of the genus *Vitis*, for example *V. labrusca*. Conditions favouring infection with *P. meliosmae-myrianthae* are similar to those favourable to *P. pachyrhizi* infection on soybean leaves, as both have optimal temperatures of 23–25 °C and favour leaf wetness periods greater than 6 h (Marchetti, 1976; Bonde *et al.*, 2007; Alves *et al.*, 2007; Angelotti *et al.*, 2014b). Both

rusts were detected in Brazil in 2001 and spread to the main Brazilian grapevine and soybean growing regions (Tessmann *et al.*, 2004; Yorinori *et al.*, 2005; Godoy *et al.*, 2016; Primiano *et al.*, 2017).

Symptoms of grapevine and soybean rusts appear as small chlorotic lesions on the adaxial leaf surface that correspond to sporulating pustules on the abaxial surface (Marchetti *et al.*, 1975; Leu, 1988). As the symptoms develop, pustules coalesce, host tissue becomes necrotic, and early leaf fall occurs (Leu, 1988; Agrios, 2005). Net photosynthetic rates are reduced in both hosts, even at low disease severity levels (Kumudini *et al.*, 2008; Nogueira Júnior *et al.*, 2017; Chapter 3). Grapevines artificially inoculated with *P. meliosmae-myrianthae* presented a linear reduction in leaf area with increasing disease severity (Nogueira Júnior *et al.*, 2017). The combined decrease in photosynthesis and leaf area of grapevine plants reduces the translocation of photoassimilates to storage organs. This was quantified as a negative linear correlation between decreasing root biomass and increasing disease severity (Nogueira Júnior *et al.*, 2017). Artificial defoliation of grapevine plants causes a reduction in yield and quality of the berries in years subsequent to the leaf removal. The yield reduction is due to the low quantity of carbohydrates stored in the roots during the dormant period, and consequently, low supplies are available for plant development in the following season (Vaillant-Gaveau *et al.*, 2014). Early defoliation is negatively correlated with yield in the same season for soybean, due to the low amount of carbohydrates translocated and stored in the grains (Mueller *et al.*, 2009).

Damages caused by plant diseases can be estimated with mechanistic simulation models, such as Ricepest and Wheatpest (Wilocquet *et al.*, 2000, 2002). However, prior to the development of simulation models that account for damage mechanisms, it is necessary to simulate healthy host growth. An example of a model that simulates healthy growth of annual plants is GENECROP (Savary & Willocquet, 2014). Based on the GENECROP model, the model GENECROP-P was developed to simulate the growth dynamics of grapevine within and over years (Nogueira Júnior *et al.*, 2018). This model simulates the growth of this perennial plant over several years by considering the carbohydrate flow between the organs of the plant (fruit, branch, root, trunk, and leaf) and, after leaf senescence, the reallocation of carbohydrates to the storage organs (root and trunk). Following the design of the base host growth simulation model, damage mechanisms caused by diseases, senescence acceleration for example, can be incorporated into the model.

One of the damage mechanisms caused by *P. meliosmae-myrianthae* and *P. pachyrhizi* on their hosts is senescence acceleration, which is associated with early leaf fall and negatively related to grapevine and soybean yields. In order to simulate yield losses caused by *Phakopsora* rusts, it is necessary to estimate a parameter representing the rate of leaf fall that is related to disease severity and incorporate this parameter into the simulation model (Willoquet *et al.*, 2004; Allorement *et al.*, 2005). There is a common sense that early grapevine leaf fall is correlated with Asian grapevine leaf rust severity, but no quantitative estimations were reported (Leu, 1988; Angelotti *et al.*, 2014; Scapin-Buffara *et al.*, 2018). Estimations of defoliation in soybean plants or experimental plots due to Asian soybean rust are not uncommon (Yang *et al.*, 1990; Mueller *et al.*, 2009; Hirano *et al.*, 2010), but the relative rate of defoliation as a function of disease severity has never been quantified. The objective of this paper was to quantify the relationship between grapevine and soybean rust severities and the relative defoliation rates of grapevine and soybean.

4.2. Materials and methods

4.2.1. Field trials

Field trials (Figure S1) were carried out in Piracicaba municipality, São Paulo, Brazil (22°42'35" S; 47°37'35.9" W; 546 m a.s.l) to assess defoliation rates caused by grapevine and soybean rusts. Weather data were obtained from an automatic weather station operated by the Department of Biosystems Engineering (The ESALQ Weather Station) and located in Piracicaba, São Paulo.

4.2.1.1. Vineyard trials

Trials to assess defoliation rate related to Asian grapevine leaf rust were conducted on a vineyard planted in 2015. Grapevines cv. Niagara Rosada (*Vitis labrusca*) grafted on grapevine cv. IAC-766 Campinas [(*V. riparia* – *V. rupestris* x *V. cordifolia* 106-8 Mgt) x *V. caribaea*] were grown in a vertical shoot positioning system at 2 m x 1 m spacing and provided with a daily 30 min drip irrigation. The first trial was carried out over the 2016–2017 season, and the second trial in the 2017–2018 season. Grapevines from both trials were pruned at the beginning of August

(08/03/2016 and 08/10/2017). A 5 % solution of the plant growth regulator Hydrogen Cyanamide (Dormex®) was brushed on the buds after pruning to stimulate dormancy and standardize bud break. Fertilizer was applied throughout the season in 3 stages. The first application was performed one month before pruning with 100 g tanned manure, 150 g urea, 600 g single superphosphate, and 160 g potassium chloride were applied per plant. The second fertilizer application was performed at bud break by distributing 120 g urea and 160 g potassium chloride per plant. The third application was performed at phenological stage 75 when berries were pea size (Lorenz *et al.*, 1995) by distributing 120 g urea and 160 g potassium chloride per plant.

The vineyard field had 5 rows and 25 plants per row. Data collection was performed on 45 plants selected from 3 rows (15 plants per row) and 2 shoots per plant (total of 679 leaves in the 2016–2017 season and 647 leaves in the 2017–2018 season). Shoots with more than 7 leaves, all healthy, were used as selection criteria. Five treatments were distributed in each row and applied to 3 neighbouring plants (total of 9 plants or 18 shoots per treatment). The treatments were (i) a fungicide spray, (ii) inoculation with a *P. meliosmae-myrianthae* suspension at 10^3 urediniospores mL⁻¹, (iii) inoculation with a *P. meliosmae-myrianthae* suspension at 10^4 urediniospores mL⁻¹, (iv) inoculation with a *P. meliosmae-myrianthae* suspension at 10^5 urediniospores mL⁻¹, and (v) a water spray. Treatments ii to v received 220 mL plant⁻¹ of spore suspension or water. The purpose of the inoculations was to achieve plants with different rust severities. In treatments where fungicide was sprayed, the objective was to quantify the natural senescence of grapevine leaves. Inoculations were performed two weeks before harvest; on 12/06/2016 and 11/28/2017 for the experiments conducted in the 2016–2017 and 2017–2018 seasons, respectively. Plants in treatments (ii), (iii), (iv), and (v) were sprayed with fungicide from the beginning of leaf emission up to 3 weeks prior to inoculation. Plants in treatment (i) were fungicide-sprayed throughout the grapevine season. Fungicide sprayings were performed according to diseases occurrence and rainfall frequency. In the 2016–2017 season, fungicides spraying was carried out once a week, while in the 2017–2018 season, spraying was performed only every 15 or 21 days due to lower rainfall frequency. Quinone outside inhibitor (QoI) fungicides and demethylation inhibitor (DMI) fungicides were sprayed in rotation as preventive treatments to control rust, anthracnose caused by *Elsinoë ampelina*, and leaf spot

caused by *Isariopsis clavispora*. In the 2017–2018 season, a methyl benzimidazole carbamate (MBC) fungicide was also sprayed 3 times after fruiting with a 15-day interval between applications to control leaf spot. Oomycete inhibitor fungicides were sprayed throughout the season to control grape downy mildew, caused by *Plasmopara viticola*.

4.2.1.2. Soybean field trial

Soybean cv. M6410IPRO seeds were planted 45 cm apart in 5, 45 m long, rows to achieve a plant density around 300,000 plants ha⁻¹ (15 plants m⁻¹). Soybean seed treatment was performed before sowing using a co-formulated mixture of pyraclostrobin with thiophanate-methyl and fipronil (2 mL kg⁻¹ of Standak Top®) and a peat inoculant of *Bradyrhizobium elkanii* strains SEMIA 587 and SEMIA 5019 (4 g kg⁻¹ of Adhere®). Fertilizer was applied to the sowing furrow with a 209 kg ha⁻¹ dose of NPK (08:28:16) according to recommendations based on soil analysis. After sowing, a pre-emergent herbicide treatment of glyphosate in combination with diclosulam and clethodim was applied for weed control. Weekly centre pivot irrigation was performed.

The soybean field was subdivided into 5, 9 m², plots and 6 plants per plot were chosen (30 plants in total) for evaluation. The treatments performed on soybean plants were the same as those performed on grapevine plants (see topic above), except using *P. pachyrhizi* in place of *P. meliosmae-myrianthae*. *P. pachyrhizi* inoculations were performed when the soybean plants were in the full flowering stage (R2). Fungicides were sprayed on the plot that aimed to quantify natural leaf senescence [treatment (i)]. Three fungicide sprays were initiated at the V8 vegetative growth stage (in which the seventh trifoliolate leaf is fully developed) and applied with a 15-day interval. The first spray was made with MBC fungicide, the second with QoI associated with succinate dehydrogenase inhibitors (SDHI), and the third with QoI associated with DMI. Assessments were carried out on the 30 selected plants. The severity of Asian soybean rust was evaluated in each leaflet of each trifoliolate leaves on the main stem.

4.2.2. Defoliation rate estimation

The severity of the grapevine and soybean rusts were estimated based on visual assessments of symptomatic grapevine leaf or individual soybean leaflet area using diagrammatic scales every 3 or 4 days until leaf fall (Godoy *et al.*, 2006; Angelotti *et al.*, 2008). Defoliation rates associated with the severity of grapevine and soybean rusts were calculated in two ways: with, and without, consideration of leaf position within the plant. In the first case, the leaves were initially grouped into three plant strata – top, middle, and bottom thirds – and then grouped by disease severity levels. In the second case, all leaves were grouped by disease severity levels and then defoliation rates were calculated. The maximum disease severity observed in each leaf and leaflet was used for grouping into the different disease severity levels. Grapevine leaves were grouped into 6 disease severity levels (0 to 0.09 %, 0.1 to 1 %, 1.1 to 5 %, 5.1 to 12 %, 12.1 to 25 %, and > 25.1 %) and soybean leaflets were grouped into 7 disease severity levels (0 to 0.09 %, 0.1 to 5 %, 5.1 to 12 %, 12.1 to 25 %, 25.1 to 45 %, 45.1 to 60 %, and > 60.1 %). The relative defoliation rate was defined as the proportion of defoliated leaf per day. It was calculated for each disease severity level using the model $RRDEF_t = ((\ln(L_{t1}) - \ln(L_{t0})) / (t_1 - t_0))$, where $RRDEF_t$ corresponds to the relative defoliation rate (day^{-1}) for each evaluation day, L to the total number of leaves or leaflets on the evaluation day, and t to time in days (Willocquet *et al.*, 2004).

After excluding rates equal to zero, the mean $RRDEF_t$ values for all assessment periods corresponded to the $RRDEF$ of each disease severity level. Data for $RRDEF$, considering or not each third of the plant, were plotted in relation to the average disease severity of each class. The model $y = a * \ln(x + 1) + b$ was fit to the data using nonlinear regression in STATISTICA® software (version 7.0, StatSoft, Tulsa, USA), where y corresponded to the relative defoliation rate ($RRDEF$) and x to the severity (as percentage) of grapevine or soybean rusts (Willocquet *et al.*, 2004). The model parameters obtained for each third, or for all leaves, were compared to each other using a Student's t test with alpha set to 0.05. If the parameters did not significantly differ, the data were pooled and a new regression was performed.

4.3. Results

4.3.1. Weather conditions and symptoms development

The two grapevine seasons targeted by the study were characterized as having similar air temperatures in the period between inoculation and first symptom appearance (Figure 1). Mean air temperatures were 23.4 °C (± 1.7 °C) and 24.1 °C (± 1.4 °C), maximum air temperatures were 30.4 °C (± 2.3 °C) and 31.1 °C (± 1.8 °C), and minimum air temperatures were 19 °C (± 2 °C) and 18.7 °C (± 2 °C) in the 2016–2017 (Figure 1a) and 2017–2018 (Figure 1b) seasons, respectively. There were 8 and 4 days with more than 1 mm of rain between inoculation and first symptom appearance in the 2016–2017 and 2017–2018 seasons, respectively. The amount of rain differed between seasons, with 81.3 mm (ranged from 2.8 to 24.1 mm.day⁻¹) in 2016–2017 and 19.6 mm (ranged from 1.7 to 9.9 mm.day⁻¹) in 2017–2018. For the 2016–2017 season, the first symptoms of Asian grapevine leaf rust were apparent at 13 days post inoculation (dpi) and mean disease severities were 0.3 % and 3 % in the plants inoculated with 10⁴ and 10⁵ urediniospores mL⁻¹, respectively. Plants inoculated with 10³ urediniospores mL⁻¹ presented symptoms at 16 dpi with 1 % mean disease severity. Symptoms in water-sprayed grapevine plants were observed at 33 dpi. For the 2017–2018 season, the first symptoms of Asian grapevine leaf rust did not appear until 17 dpi in plants inoculated at the highest inoculum concentration. Symptoms of natural infection in water-sprayed grapevine plants were observed only at 48 dpi. Within the period between symptom appearance and complete leaf fall, the maximum, minimum, and mean air temperatures were similar to the previous period in both seasons. The amount of the rain after appearance of the first symptoms in 2016–2017 was higher (635.2 mm) than in the 2017–2018 season (457.6 mm), as were the number of days with more than 1 mm of rain in this period, 45 and 36 days, in the 2016–2017 and 2017–2018 seasons, respectively.

Figure 1 - Weather conditions during the grapevine experiments. Maximum, mean, and minimum temperatures and rainfall (mm) registered post inoculation of grapevine cv. Niagara Rosada plants with *Phakopsora meliosmae-myrianthae* during the 2016–2017 (a) and 2017–2018 (b) seasons.

The mean air temperature in the period between *P. pachyrhizi* inoculation and the appearance of the first Asian soybean rust symptom was 24.7 °C (± 1.3 °C). The maximum and minimum air temperatures within this period were 31.7 °C (± 1.6 °C) and 19.8 °C (± 0.9 °C), respectively (Figure 2). There was 157 mm of rainfall (varied from 4.8 to 33.5 mm.day⁻¹) and 9 days with more than 1 mm of rain. There was 39.88 mm of rainfall on the inoculation day, though plants were inoculated

in the late afternoon when plants had already dried. Although these weather conditions favour *P. pachyrhizi* germination and leaf infection, symptoms were only observed after 27 dpi in all plots. The amount of rain post symptom appearance until complete soybean defoliation was 0.3 mm.

Figure 2 - Weather conditions during the soybean experiment. Maximum, mean, and minimum temperatures and rainfall (mm) registered post inoculation of soybean cv. M6410IPRO plants with *Phakopsora pachyrhizi*.

4.3.2. Defoliation rate estimation

A positive correlation was observed between the severity of Asian grapevine leaf rust and relative defoliation rate whether plant strata were considered or not (Figure 3). Logarithmic regression parameters of bottom, middle, and top thirds of grapevine leaves differed between the seasons. Thus, it was not possible to pool data from both grapevine seasons and to perform a new regression by plant strata (Figure 3a and Table 2). However, when considering all grapevine leaves, logarithmic regression parameters were similar for both 2016–2017 and 2017–2018 seasons and a single regression with pooled data from both seasons was performed (Figure 3b and Table 1).

Figure 3 - Relationship between Asian grapevine leaf rust severity (%) resulting from *Phakopsora meliosmae-myrianthae* infection and relative defoliation rate (day⁻¹) considering (a) three strata of grapevine cv. Niagara Rosada plants [bottom (diamonds), middle (triangles), and top (circles)] or considering all leaves (b, squares). Black symbols and dotted lines refer to the 2016–2017 season and white symbols and solid lines to the 2017–2018 season. Lines in (a) correspond to non-linear regressions for each strata and lines in (b) correspond to a non-linear regression with data pooled from all leaves and both trials ($y = a * \ln(x + 1) + b$, where y is the relative defoliation rate and x is the disease severity).

Table 1 - Coefficients of determination, parameters, and respective standard errors (in parentheses) estimated with non-linear regressions of the logarithmic equation adjusted to the relative defoliation rate of grapevine cv. Niagara Rosada to the severity of Asian grapevine leaf rust caused by *Phakopsora meliosmae-myrianthae*

	Thirds	R^2	Estimated parameters ^a	
			a	b
Season 2016-2017	Top	0.70	0.01 b (0.005)	0.05 a (0.012)
	Middle	0.88	0.02 ab (0.004)	0.05 a (0.009)
	Bottom	0.73	0.03 ab (0.009)	0.04 a (0.020)
	All leaves	0.89	0.02 ab (0.003)	0.04 a (0.008)
Season 2017-2018	Top	0.94	0.03 a (0.005)	0.07 a (0.009)
	Middle	0.45	ns	0.07 a (0.012)
	Bottom	0.43	ns	0.06 a (0.009)
	All leaves	0.93	0.03 a (0.005)	0.05 a (0.010)

^a Coefficients of determination (R^2) and parameters were estimated from the model $y = a * \ln(x + 1) + b$, where y corresponds to the relative defoliation rate and x to the percentage severity of Asian grapevine leaf rust. Values followed by the same letter in the same column are not significantly different ($\alpha < 0.05$).

ns = values not significantly different from zero.

The relative defoliation rates of Asian soybean rust, considering the top third, middle third (Figure 4a), or all leaves (Figure 4b) were also positively correlated with disease severity. The model did not fit to data from the bottom third.

Figure 4 - Relationship between Asian soybean rust severity (%) resulting from *Phakopsora pachyrhizi* infection and relative defoliation rate (day^{-1}) considering (a) three strata of soybean cv. M6410IPRO plants [bottom (diamonds), middle (triangles), and top (circles)] or considering all leaflets (b, squares). Lines in (a) and (b) correspond to non-linear regressions ($y = a * \ln(x + 1) + b$, where y is the relative defoliation rate and x is the disease severity) for the top third (dotted line), middle third (dashed line), or all leaflets (solid line).

Table 2 - Parameters and respective standard errors (in parentheses) estimated with logarithmic regressions fitting the relative defoliation rate of soybean cv. M6410IPRO to the severity of Asian soybean rust caused by *Phakopsora pachyrhizi*

Thirds	R^2	Estimated parameters ^a	
		a	b
Top	0.73	0.062 a (0.026)	0.004 a (0.077)
Middle	0.70	0.036 a (0.016)	0.119 a (0.047)
Bottom	ns		
All leaflets	0.79	0.022 a (0.007)	0.053 a (0.022)

^a Coefficients of determination (R^2) and parameters were estimated from the model $y = a * \ln(x + 1) + b$, where y corresponds to the relative defoliation rate and x to the percentage severity of Asian soybean rust. Values followed by the same letter in the same column are not significantly different ($\alpha < 0.05$).

ns = values not significantly different from zero.

4.4. Discussion

Our study focused on establishing a relationship between relative defoliation rate and *Phakopsora* rust severity. A positive correlation between disease severity and relative defoliation rate was observed for both rusts with similar coefficients: 0.026 and 0.022 for grapevine and soybean rusts, respectively. Yang *et al.* (1990)

reported a significant linear regression between soybean rust severity and defoliation, both as percentages, but there was high variability in the data and the authors did not recommend using defoliation to predict yield losses caused by Asian soybean rust. In the case of Asian grapevine leaf rust, investigations directly or indirectly correlating disease severity with leaf fall are even rarer (Nogueira Júnior *et al.*, 2017). An increase in Asian grapevine leaf rust severity in potted and artificially inoculated plants was linearly related to the decrease in leaf area and root dry matter (Nogueira Júnior *et al.*, 2017). Reduced accumulation of carbohydrates stored in the roots for the subsequent grapevine season was also reported in studies that used defoliation as a cultural practice. In this case, grapevine defoliation resulted in reductions to reproductive variables, as for example inflorescence numbers, in the subsequent season (Noyce *et al.*, 2016; Frioni *et al.*, 2018). The complexity of determining the relationship between relative defoliation rate and disease severity mainly involves the sample unit analysed and the internal and external plant factors inducing leaf senescence under field conditions.

Assessment of rust severity progression over time is usually based on the average values of leaf disease severity using the shoot or the plant as the sampling unit (Madden *et al.*, 2007). High heterogeneity in rust severity values was observed within grapevine shoots (Figures S2 to S6) and within soybean plants (Figures S7 to S11) on any given assessment date. Frequently, a wide range of disease severity was observed within grapevine shoots or soybean plants, for instance from 0 to 60 % disease severity (see date 9 for soybean plants in Figure S8). Because of the high heterogeneity in disease severity values within a sampling unit (*data not shown*), no positive correlation was established between disease severity and defoliation rate when shoots or plants were used as the sample unit. Classifying leaves or leaflets into disease severity levels and disregarding the shoot or plant unit is highly recommended (Willocquet *et al.*, 2004). Thus, the analysis is more accurate and allows detection of the positive correlation between the relative defoliation rate and disease severity, as observed in the present work.

Plant senescence is a natural and organized process that corresponds to the final stage of specific organs, such as grapevine leaves, or of the whole plant as occurs in annual plants, such as soybean (Woo *et al.*, 2013). This process implies a programmed degradation and degeneration of plant cell structures through which macromolecules, such as proteins, carbohydrates, lipids, and nucleic acids are

hydrolysed and undergo reallocation to storage organs (Lim *et al.*, 2007). Grapevine plants store these hydrolysed molecules in trunks and roots, and soybean plants in seeds. Thus, grapevine leaves fall and the plant begins a period of dormancy, while for soybean plants this leads to the death of the entire plant (Woo *et al.*, 2013). Senescence is triggered by various endogenous or exogenous environmental signals (Buchanan-Wollaston, 1997). Several internal synchronized signals activate leaf senescence, for example increased concentrations of different plant hormones in the leaf, such as ethylene, jasmonic acid, abscisic acid, and salicylic acid (Woo *et al.*, 2013). In addition to the hormonal balance, the nutritional status of the plant can also trigger senescence, for example the accumulation of sugars and lipid-soluble products above tolerable levels in the leaf reduces photosynthetic activity and induces leaf senescence (Lim *et al.*, 2007; Wang *et al.*, 2015). Pathogen attack is an exogenous environmental signal that can induce senescence, and as a consequence early leaf fall (Buchanan-Wollaston, 1997). This premature initiation of senescence will interfere with nutrient reallocation to storage organs as observed previously in grapevine and soybean rusts (Mueller *et al.*, 2009; Nogueira Júnior *et al.*, 2017). Other exogenous environmental factors that induce leaf senescence were also observed over the seasons in this study, for example, defoliating insects. These did not interfere in defoliation rate analysis since individual leaves were used as sample unit. Thus, it is possible to identify and remove from the analysis all leaves that presented other external interference besides rust severity. Weather conditions and nutrient limitations are also considered exogenous environmental factors that influence leaf senescence. In this work, both grapevine and soybean plants were irrigated and fertilized in order to minimize the influence of these abiotic factors.

The defoliation rates of healthy grapevine and soybean leaves were similar to those with less than 5 % disease severity. Plants can compensate for a certain proportion of lost capacity, at least at low levels of diseased tissue, because the resources not used by diseased tissue are reallocated to healthy tissue (Seem, 1988). *Phakopsora* rusts caused substantial defoliation in grapevine and soybean at disease severities higher than 5 %. For a disease severity of 10 %, the estimated defoliation rate was 0.11 day⁻¹ with Asian grapevine leaf rust and 0.10 day⁻¹ with Asian soybean rust. These defoliation rates are in the same range as those caused by angular leaf spot of common bean, caused by *Phaseariopsis griseola*, a necrotrophic pathogen (Wilocquet *et al.*, 2004). These findings will allow easy

incorporation of a parameter associated with senescence acceleration into simulation models to predict yield losses. These improved yield predictions aid in the development of public policies, research priorities, and improved management practices for *Phakopsora* rusts (Wilocquet *et al.*, 2000).

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SUPPORTING INFORMATION

Figure S1. Experimental fields of grapevine cv. Niagara Rosada (a) and soybean cv. M6410IPRO (b) plants used to assess defoliation rates related to grapevine rust, caused by *Phakopsora meliosmae-myrianthae*, and soybean rust, caused by *P. pachyrhizi*.

Figure S2. Schematic representation of 3 representative plants of fungicide spray treatment from grapevine experimental field assessed throughout the 2016/17 season. No disease was reported. dps = days post first symptom.

Figure S3. Schematic representation of three grapevine cv. Niagara Rosada plants inoculated with 10^3 *Phakopsora meliosmae-myrianthae* urediniospores mL⁻¹ in which the severity levels of Asian grapevine leaf rust were assessed throughout the 2016–2017 season. dps = days post first symptom.

Figure S4. Schematic representation of three grapevine cv. Niagara Rosada plants inoculated with 10^4 *Phakopsora meliosmae-myrianthae* urediniospores mL⁻¹ in which the severity levels of Asian grapevine leaf rust were assessed throughout the 2016–2017 season. dps = days post first symptom.

Figure S5. Schematic representation of three grapevine cv. Niagara Rosada plants inoculated with 10^5 *Phakopsora meliosmae-myrianthae* urediniospores mL⁻¹ in which the severity levels of Asian grapevine leaf rust were assessed throughout the 2016–2017 season. dps = days post first symptom.

Figure S6. Schematic representation of three representative grapevine cv. Niagara Rosada plants from the water spray treatment in which the severity levels of Asian grapevine leaf rust resulting from *Phakopsora meliosmae-myrianthae* infection were assessed throughout the 2016–2017 season. dps = days post first symptom.

Figure S7. Schematic representation of one representative plant from the fungicide spray treatment in which the severity levels of Asian soybean rust resulting from infection with *Phakopsora pachyrhizi* were assessed over time. dps = days post first symptom.

Figure S8. Schematic representation of one soybean cv. M6410IPRO plant inoculated with 10^3 *Phakopsora pachyrhizi* urediniospores mL^{-1} in which the severity levels of Asian soybean rust were assessed over time. dps = days post first symptom.

Figure S9. Schematic representation of one soybean cv. M6410IPRO plant inoculated with 10^4 *Phakopsora pachyrhizi* urediniospores mL⁻¹ in which the severity levels of Asian soybean rust were assessed over time. dps = days post first symptom.

Figure S10. Schematic representation of one soybean cv. M6410IPRO plant inoculated with 10^5 *Phakopsora pachyrhizi* urediniospores mL⁻¹ in which the severity levels of Asian soybean rust were assessed over time. dps = days post first symptom.

Figure S11. Schematic representation of one soybean cv. M6410IPRO plant from the water spray treatment in which severity levels of Asian soybean rust resulting from *Phakopsora pachyrhizi* infection were assessed over time. dps = days post first symptom.