UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
CAMPUS DE ILHA SOLTEIRA

LOANE DANTAS KRUG

AEROBIOLOGY OF THE ASIAN SOYBEAN RUST PATHOGEN FOR MONITORING INOCULUM DISPERSAL AND RT qPCR DETECTION OF THE SdhC I86F SUBSTITUTION ASSOCIATED WITH RESISTANCE TO SDHI FUNGICIDES IN POPULATIONS OF *Phakopsora pachyrhizi* FROM BRAZIL

Ilha Solteira - SP
2024

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Tese apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Engenharia de Ilha Solteira, para obtenção do título de Doutor(a) em Agronomia

Área de Concentração: Sistema de Produção

Orientador(a): Prof. Dr. Paulo Cezar Ceresini

Coorientador(a): Prof. Dr. Felipe Rafael Garcés-Fiallos

Ilha Solteira - SP
2024

FICHA CATALOGRÁFICA
Desenvolvida pela Diretoria Técnica de Biblioteca e Documentação

K94a

Krug, Loane Dantas.

Aerobiology of the asian soybean rust pathogen for monitoring inoculum dispersal and RT qPCR detection of the SdhC i86F substitution associated with resistance to sdhi fungicides in populations of *Phakopsora pachyrhizi* from Brazil / Loane Dantas Krug. -- Ilha Solteira: [s.n.], 2024

63 f. : il.

Tese (doutorado) - Universidade Estadual Paulista. Faculdade de Engenharia de Ilha Solteira. Especialidade: Sistemas de Produção, 2024

Orientador: Paulo Cezar Ceresini
Coorientador: Felipe Rafael Garcés-Fiallos
Inclui bibliografia

1. Monitoramento de patógenos. 2. Dispersão de inóculo. 3. Detecção molecular. 4. Quantificação de DNA fúngico. 5. Condições ambientais. 6. Estratégias inteligentes de manejo de doenças.

Elaborada por Raiane da Silva Santos - CRB-8/9999

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Ilha Solteira

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Aerobiology of the Asian soybean rust pathogen for monitoring inoculum dispersal and RT qPCR detection of the SdhC I86F substitution associated with resistance to SDHI fungicides in populations of Phakopsora pachyrhizi from Brazil

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Aprovada como parte das exigências para obtenção do Título de Doutora em Agronomia, área: Sistemas de Produção pela Comissão Examinadora:

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Ilha Solteira, 21 de agosto de 2024

Dedico este trabalho à minha família

AGRADECIMENTOS

A Deus que me sustentou emocionalmente.

A minha família, que sempre me incentivou e me deu todo o suporte para poder concluir essa jornada, principalmente aos meus pais Julio Cesar Krug e Marcia B. Dantas Krug e minhas irmãs Juliana D. Krug Sales e Amabile Dantas Krug.

Gostaria de agradecer ao Dr. Paulo Cézar Ceresini por sua orientação e ensinamentos durante este longo período de formação acadêmica.

A toda equipe do laboratório de Fitopatologia Molecular, pelas contribuições nos experimentos e no desenvolvimento da tese.

Em especial ao grupo da ferrugem, sem eles esse trabalho não seria possível: Lucas M. de D. P. Gonçalves, Amanda R. Acerbi, Rafael R. de L. Montan, Davi J. Ferreira e Felipe Rafael Garcés-Fiallos.

Aos meus amigos que me ajudaram em todas as etapas desse trabalho: Samara Campos Nunes Vicentini, Abimael Gomes da Silva e Katherin Castro-Ríos.

A todos os produtores e institutos de pesquisa pelas amostras disponibilizadas.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Esta pesquisa foi financiada pela FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil), Números de Auxílio: 2017/50456-1, 2018/21197-0, 2019/12509-1, 2020/07611-9 e 2021/03402-9; CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Número de Auxílio: Pq-1C 311895/2022-0; CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) e CNPq (Chamada 3/2022 - Programa PIBITI/CAPES 001); e Newton Fund/BBSRC (Biotechnology and Biological Sciences Research Council, Reino Unido), Número de Auxílio: BB/S018867/2 concedido pelo BBSRC no Programa de Resistência a Antimicrobianos e Resistência a Inseticidas em Pecuária e Agricultura BBSRC-FAPESP.

RESUMO GERAL

A ferrugem asiática da soja, causada pelo fungo *Phakopsora pachyrhizi* (*Pp*), é a principal doença que afeta a produção de soja no Brasil, gerando perdas significativas e custos de manejo estimados em aproximadamente 2 bilhões de dólares por safra. Desde sua introdução no Brasil na safra 2001/2002, a doença se espalhou rapidamente, atingindo praticamente todas as regiões produtoras e desafiando constantemente os agricultores. A resistência generalizada do patógeno a fungicidas, particularmente aos grupos QoI, DMI e SDHI, tem comprometido o controle químico, exigindo novas estratégias de manejo. Este estudo focou no monitoramento das populações aéreas de *P. pachyrhizi* em Londrina, Paraná, entre 2019 e 2021, utilizando um ciclone de alto volume para coleta de ar e PCR quantitativo em tempo real (qPCR) para detecção de DNA do patógeno. A análise das amostras revelou a presença de *P. pachyrhizi* em 24,73% das amostras, com picos significativos em março de 2020 e 2021, coincidentes com o período crítico de enchimento de grãos. As variáveis climáticas, como precipitação, umidade relativa e velocidade do vento, mostraram correlações significativas com a presença de DNA do patógeno, com a precipitação acumulada apresentando uma correlação negativa, e a umidade relativa e a velocidade do vento mostrando correlações positivas em determinados períodos. Além disso, a resistência a fungicidas tem sido uma preocupação crescente. A resistência a fungicidas QoI e DMI diminuiu a eficácia do controle químico, enquanto novos fungicidas SDHI também enfrentaram resistência. A detecção da mutação SdhC-I86F, associada à resistência a SDHI, foi prevalente em populações amostradas entre 2020/21 e 2022/23 em diferentes estados brasileiros, confirmando a persistência desta substituição. As análises também mostraram que as populações fenotipadas tinham sensibilidade reduzida ao fungicida fluxapiroxade. Os resultados destacam a eficácia da metodologia empregada para monitoramento e detecção de *P. pachyrhizi* e fornecem informações valiosas para práticas de manejo mais eficientes, que podem ajudar a reduzir a dependência de fungicidas e promover uma agricultura mais sustentável.

Palavras-chave: monitoramento de patógenos; dispersão de inóculo; detecção molecular; quantificação de DNA fúngico; Condições ambientais; estratégias inteligentes de manejo de doenças; *Phakopsora pachyrhizi*; Substituição SdhC-I86F; Fluxapiroxade; Inibidores da succinato desidrogenase.

ABSTRACT

Asian soybean rust, caused by the fungus *Phakopsora pachyrhizi* (Pp), is the main disease affecting soybean production in Brazil, leading to significant losses and management costs estimated at approximately US$ 2 billion per crop. Since its introduction to Brazil in the 2001/2002 growing season, the disease has spread rapidly, affecting nearly all soybean-producing regions and consistently challenging farmers. The widespread resistance of the pathogen to fungicides, particularly to QoI, DMI, and SDHI groups, has compromised chemical control, necessitating new management strategies. This study focused on monitoring airborne populations of *P. pachyrhizi* in Londrina, Paraná, between 2019 and 2021, using a high-volume cyclone for air collection and quantitative real-time PCR (qPCR) for pathogen DNA detection. Sample analysis revealed the presence of *P. pachyrhizi* in 24.73% of samples, with significant peaks in March 2020 and 2021, coinciding with the critical grain-filling period. Climate variables, such as rainfall, relative humidity, and wind speed, showed significant correlations with the presence of pathogen DNA, with accumulated rainfall presenting a negative correlation, and relative humidity and wind speed showing positive correlations at certain periods. Furthermore, fungicide resistance has become an increasing concern. Resistance to QoI and DMI fungicides has reduced the efficacy of chemical control, while new SDHI fungicides have also faced resistance. The detection of the SdhC-I86F mutation, associated with SDHI resistance, was prevalent in populations sampled between 2020/21 and 2022/23 across various Brazilian states, confirming the persistence of this substitution. The analyses also showed that phenotyped populations had reduced sensitivity to the fungicide fluxapyroxad. The results highlight the effectiveness of the employed methodology for monitoring and detecting *P. pachyrhizi* and provide valuable information for more efficient management practices, which may help reduce dependence on fungicides and promote more sustainable agriculture.

Keywords: pathogen monitoring; inoculum dispersal; molecular detection; fungal DNA quantification; environmental conditions; smart disease management strategies; *Phakopsora pachyrhizi;* SdhC-I86F substitution; fluxapyroxad; succinate dehydrogenase inhibitors.

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1 AEROBIOLOGY OF THE ASIAN SOYBEAN RUST PATHOGEN IN PARANÁ, BRAZIL: A REVISITATION OF ARCHIVED FUNGAL DNA SAMPLES

ABSTRACT

Asian soybean rust, caused by the fungus *Phakopsora pachyrhizi*, is the main disease affecting soybean production in Brazil, resulting in yield losses and management costs estimated at around 2 billion dollars per growing season. Since its introduction in the country in the 2001/2002 crop season, the disease has spread rapidly, affecting practically all producing regions and becoming a constant concern for farmers. The widespread resistance of the pathogen to fungicides, especially the QoI, DMI and SDHI groups, has compromised the effectiveness of chemical control, requiring the implementation of alternative management strategies. This study aimed to monitor the airborne populations of *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust, in the municipality of Londrina, Paraná state, between 2019 and 2021. Using a high-volume cyclone for air sampling and the quantitative PCR (qPCR) technique for pathogen DNA detection, samples collected during different periods, including the soybean growing season and the off-season, were analyzed. DNA quantification revealed that *P. pachyrhizi* was detected in 24.73% of the samples, with significant detection peaks occurring in March 2020 and 2021, critical periods for soybean grain filling. Analysis of climatic variables indicated important correlations between pathogen DNA presence and factors such as rainfall, relative humidity and wind speed. Accumulated rainfall showed a significant negative correlation with DNA presence, while relative humidity and wind speed showed positive correlations in certain periods. The results highlight the effectiveness of the collection and detection methodology used, which proved to be sensitive and reliable for the aerobiological monitoring of *P. pachyrhizi*. The information obtained contributes to the understanding of the aerobiology of *P. pachyrhizi* and can guide more informed and efficient management practices, aiming at reducing fungicide use and promoting more sustainable agriculture.

Keywords: pathogen monitoring; inoculum dispersal; molecular detection; fungal DNA quantification; environmental conditions; smart disease management strategies

RESUMO

A ferrugem asiática da soja, causada pelo fungo *Phakopsora pachyrhizi*, é a principal doença que afeta a produção de soja no Brasil, resultando em perdas de produtividade e custos de manejo estimados em cerca de 2 bilhões de dólares por safra. Desde sua introdução no país na safra 2001/2002, a doença se espalhou rapidamente, afetando praticamente todas as regiões produtoras e se tornando uma preocupação constante para os agricultores. A resistência generalizada do patógeno a fungicidas, especialmente os grupos QoI, DMI e SDHI, tem comprometido a eficácia do controle químico, exigindo a implementação de estratégias alternativas de manejo. Este estudo teve como objetivo monitorar as populações aéreas de *Phakopsora pachyrhizi*, agente causador da ferrugem asiática da soja, em Londrina, Paraná, entre 2019 e 2021. Utilizando um ciclone de alto volume para a coleta de ar e a técnica de PCR quantitativo em tempo real (qPCR) para a detecção de DNA do patógeno, foram analisadas amostras coletadas em diferentes períodos, incluindo a safra de soja e o vazio sanitário. A quantificação do DNA revelou que *P. pachyrhizi* foi detectado em 24,73% das amostras, com picos significativos de detecção ocorrendo em março de 2020 e 2021, períodos críticos de enchimento de grãos da soja. A análise das variáveis climáticas indicou correlações importantes entre a presença de DNA do patógeno e fatores como precipitação, umidade relativa e velocidade do vento. A precipitação acumulada apresentou uma correlação negativa significativa com a presença de DNA, enquanto a umidade relativa e a velocidade do vento mostraram correlações positivas em determinados períodos. Os resultados destacam a eficácia da metodologia de coleta e detecção utilizada, que se mostrou sensível e confiável para o monitoramento aerobiológico de *P. pachyrhizi*. As informações obtidas contribuem para a compreensão da aerobiologia de *P. pachyrhizi* e podem orientar práticas de manejo mais informadas e eficientes, visando a redução do uso de fungicidas e a promoção de uma agricultura mais sustentável.

Palavras-chave: monitoramento de patógenos; dispersão de inóculo; detecção molecular; quantificação de DNA fúngico; Condições ambientais; estratégias inteligentes de manejo de doenças.

1.1 INTRODUCTION

Since its arrival in Brazil in 2001, Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* (*Pp*), has become the main disease affecting this crop in the country. Under favorable climatic conditions and in the absence of fungicide treatments, ASR can result in production losses of up to 90% in susceptible varieties (Juliatti; Zambolim, 2021). Crop losses have been frequent over the years, ranging from a minimum of 363,500 tons in 2011/2012 to a historic maximum of 4.6 million tons in 2003/2004. These figures severely impact the country's economy, which is heavily reliant on commodity exports (Godoy *et al*. 2016; Juliatti; Zambolim, 2021).

With a national distribution from south to north, ASR outbreaks are common in Brazilian agroecosystems. The fungus can survive year-round on volunteer soybean plants (Fanaro *et al*. 2011; Yorinori, 2021). Since 2007, federal and state regulations have established the "sanitary void," a period of 60 to 90 days between harvests during which soybean cultivation is prohibited in certain regions. Non-compliance can result in fines ranging from 100 to 5,000 state tax units. For instance, in São Paulo, the unit is R$ 35.36, while in Mato Grosso, it is R$ 237.69. This interval aims to reduce the occurrence of soybean plants from grains that were not harvested in the previous season, thereby decreasing the survival of the inoculum between seasons and its onset (Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA) 2021; MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023). Despite these measures, 573 ASR outbreaks were reported in the last growing season (Consórcio Antiferrugem, 2023).

The management strategies employed for ASR include chemical control, use of resistant varieties, and management of the inoculum source, with the implementation of the sanitary void (Godoy *et al*. 2007). The use of fungicides has still been the most effective practice in managing the disease to maintain crop productivity levels (Klosowski, 2015). Registered products for the control of Asian soybean rust in Brazil mostly contain active ingredients from the groups of demethylation inhibitors (DMIs), represented by triazoles, external quinone inhibitors (QoIs), known as strobilurins, and succinate dehydrogenase inhibitors (SDHIs), also called second-generation carboxamides, which are formulated either alone or in mixtures (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023). However, the loss of efficacy of these fungicide groups has been reported by various research groups, associated with reduced sensitivity of *Pp* to these formulations, caused by the intensive use of products from the same chemical group and with the same mode of action (Schmitz *et al*., 2014; Godoy *et al*., 2015; Klosowski *et al*., 2016; Simões *et al*. 2018; Mello *et al*. 2021; Müller *et al*., 2021; Stilgenbauer *et al*., 2023).

To maintain the effectiveness of new and existing fungicides in managing ASR, the ideal scenario would be to completely prevent the emergence and spread of resistance while maintaining disease control. However, due to the high adaptive costs associated with this characteristic and the possibility of negative cross-resistance between different fungicides, achieving this reality becomes impractical (Brent; Hollomon, 2007; Mikaberidze *et al*., 2014; Hawkins; Fraaije 2018). Consequently, developing a set of measures aimed at delaying the emergence of resistance is increasingly relevant in this context, with a focus on validated experiments based on evolutionary principles that have shown promise for a range of pathosystems (Van den Bosch *et al*., 2014).

In this regard, the use of technologies that assist in detecting the presence of early symptoms and signs of the disease is of great interest and has a significant economic impact on production (Mumford; Norton, 1984). Rapid detection of the initial evidence of the pathogen’s presence in the field requires monitoring of crops and determining disease incidence in the laboratory to assess the economic threshold (ET) (Reis *et al*., 2022). The use of advanced devices to monitor pathogen populations allows for the quantification of inoculum levels and early detection of fungicide resistance alleles, along with disease forecasts that could be employed as strategic tools. These resources would be used to guide the implementation of resistance management measures based on optimizing fungicide inputs, such as product choice, timing, and number of applications (Vicentini *et al*., 2023). For example, an automatic air sampling device combined with DNA-based molecular detection can be used to quantify levels of airborne pathogen inoculum (Van der Heyden *et al*., 2021).

Disease forecasting models based on aerobiology differ from climate-based models, such as Sisalert (Plant Disease Epidemic Risk Forecasting System) (Fernandes *et al*., 2017). Epidemic models using aerobiological methods have been developed to assess the risk and seasonal forecasting of ASR, grounded in climate forecasts to calculate the long-distance movement of the pathogen (Isard *et al*., 2005;

Pan *et al*., 2006). The model elaborated by Pan and colleagues considers parameters such as inoculum source strength, spore production, survival, and deposition of the inoculum, which were estimated mechanically and empirically based on system knowledge and physical principles (Pan *et al*., 2006).

Research in fungal aerobiology has advanced globally over the past two decades, aiming to provide real-time quantitative measurements of airborne plant pathogen inoculum to enhance disease management (Van der Heyden *et al*., 2021). Since the introduction of the first automated device for detecting airborne spores of *Sclerotinia sclerotiorum* in 1952 (Hirst, 1952), significant innovations have been made in air sampling devices, increasing efficiency in spore capture for fungal aerobiology studies (West; Kimber, 2015). Recently, the combination of spore capture with quantitative polymerase chain reaction (qPCR) has proven effective in detecting and quantifying airborne spores of wheat pathogens such as *Puccinia striiformis* (Dedeurwaerder *et al*., 2011), *Mycosphaerella graminicola* (also known as *Zymoseptoria tritici*) (Fraaije *et al*. 2005; Duvivier *et al*., 2016), *Blumeria graminis* (Cao *et al*., 2016), *Pyricularia oryzae* Triticum lineage (Vicentini *et al*. 2023), and *Phakopsora pachyrhizi* in soybean (Reis *et al*., 2022). Additionally, DNA-based SNP detection allows for measuring the frequency of fungicide-resistant alleles in pathogen aerosol populations (Fraaije *et al*., 2005).

Pp spores are considered dry and are transported by the wind over long distances, making them collectible through air sampling. Early detection of inoculum through aerobiology, relative to the initial presence of the pathogen in the area, could serve as an alert system for ASR occurrence, assisting in decision-making regarding fungicide application, reducing yield losses while also decreasing environmental impact and fungicide resistance selection pressure (Reis *et al*., 2022).

To monitor airborne *P. pachyrhizi* spore populations in aerosol samples obtained in Londrina, a major soybean-producing region in Paraná state, we used a high-volume cyclone, an automated air sampling device, combined with a quantitative PCR (qPCR) assay. The study aimed to monitor the temporal dispersion of *Pp* airborne inoculum from 2019 to 2021, based on the revisit of archived fungal DNA samples (Vicentini *et al*., 2023) for target DNA detection of the fungus. It also aimed to assess the impact of climatic conditions on the dynamics of pathogen inoculum dispersion.

1.2 MATERIALS AND METHODS

This study was based on the revisit and analysis of archived DNA samples, used by Vicentini (2023) to describe the aerobiology of the ascomycete phytopathogenic fungus *Pyricularia oryzae* Triticum lineage, the agent of wheat blast. Airborne fungal spores were collected daily in 2 ml tubes using a high-volume automated cyclone provided by Agri Samplers Ltd (Cressex Enterprise Centre, Cressex Business Park, Lincoln Rd, High Wycombe, Buckinghamshire, HP12 3RL, UK) (Figure 1). The air flow was adjusted according to the manufacturer's standard recommendation, at 270 L/min. The high-volume cyclone sampling equipment captured aerosols in the Paraná region from 2019 to 2021, operating for 12 hours daily, from 6:00 AM to 6:00 PM. Sampling took place at the IDR Paraná Experimental Station, located in Londrina (23° 21' 34.2"S; 51° 09' 52.9"W), at a height of 5 meters above the ground and at least 2 km away from soybean-growing areas.

Figure 1 - (A) High-volume cyclone air sampler from Agri Samplers Ltd. (UK), installed in Londrina, PR, operating at the IDR Paraná Experimental Station (Institute of Rural Development of Paraná); (B) Front view of the sampler unit; (C) Control panel through which the sampler parameters are set; and (D) Details of the open spore sampler unit.

Fonte: Próprio autor.

1.3 FUNGAL DNA EXTRACTION FOR AEROBIOLOGY AND PURIFICATION

For DNA extraction, 0.5 g of sterilized glass beads (diameter 400–455 μm; Sigma, St. Louis, Missouri, USA) were added to each 2 mL tube, along with 440 μL of extraction buffer composed of 400 mM Tris-HCl, 50 mM EDTA pH 8, 500 mM NaCl, 2% polyvinylpyrrolidone, 5 mM 1,10-phenanthroline monohydrate, and, immediately before use, 0.1% β-mercaptoethanol. The tubes were then transferred to a FastPrep homogenizer (Savant FastPrep BIO101 Homogenizer, Thermo Fisher, Waltham, MA, USA) for three cycles at 6.0 m/s for 40 s, with cooling on ice for 2 min between cycles. Next, 400 µL of 2% SDS was added to the tubes, which were inverted to homogenize the solution and incubated at 65 °C in a water bath for 30 min, with shaking every 10 min. After this, 800 µL of phenol-chloroform (1:1) was added to each tube, which were briefly vortexed and centrifuged at 13,000 rpm for 10 min at 4 °C. In parallel, in another set of 1.5 mL tubes, 30 µL of 7.5 M ammonium acetate, 480 µL of isopropanol, and 1 µL of GlycoBlue were added. The supernatant from the centrifuged solution was then transferred to the new set of tubes and gently mixed, followed by storage at -20 °C overnight. The tubes were then centrifuged again at 13,000 rpm for 30 min at 4 °C, and the DNA pellet, visible by GlycoBlue, was air-dried in a laminar flow hood for about 30 min and resuspended in 100 µL of 10 mM Tris pH 8.0. The DNA was completely resuspended in a water bath at 65 °C for 5 min before being stored at -20 °C. For DNA purification, the EchoCLEAN DNA CleanUp column (BioEcho Life Sciences, Cologne, North Rhine-Westphalia, Germany) was used according to the manufacturer's instructions.

1.4 MOLECULAR DETECTION OF *PHAKOPSORA PACHYRHIZI* IN AEROBIOLOGY SAMPLES USING QPCR

The qPCR reactions were conducted in a 15 µL reaction volume in 96-well PCR plates with lids. Each reaction contained 2 µL of DNA sample, 7.5 µL of KAPA Probe Fast qPCR Master Mix (Biosystem, Foster City, California, USA), 5.425 µL of sterile distilled water containing the primers Ppm1 F and Ppa2 R, and the ASRP1_HEX probe labeled with 5′HEX and 3′BHQ1 for the detection of *Pp*, targeting a 141 bp amplicon as described by Frederick *et al*. (2002)), as well as the reference dye Rox (Invitrogen; 0.075 µL per reaction). The cycling protocol consisted of an initial step at 98 °C for 2 minutes, followed by 50 cycles of denaturation at 95 °C for 10 seconds and extension at 60 °C for 30 seconds. Each sample was analyzed in duplicate using the AriaMx Real-Time PCR System (Agilent, Santa Clara, California, USA). For the standard curve construction, each qPCR run included standard samples containing known amounts of target *Pp* DNA (0.01 pg; 0.1 pg; 1 pg; 10 pg; 100 pg; 1000 pg; 10,000 pg). Non-template controls (NTC, i.e., sterilized distilled water) were also included in each qPCR run. All data were processed using Agilent AriaMx software.

Table 1 - Primers and probes used for the q-PCR detection of *Phakopsora pachyrhizi* from fungal DNA extracted from airborne spore samples.

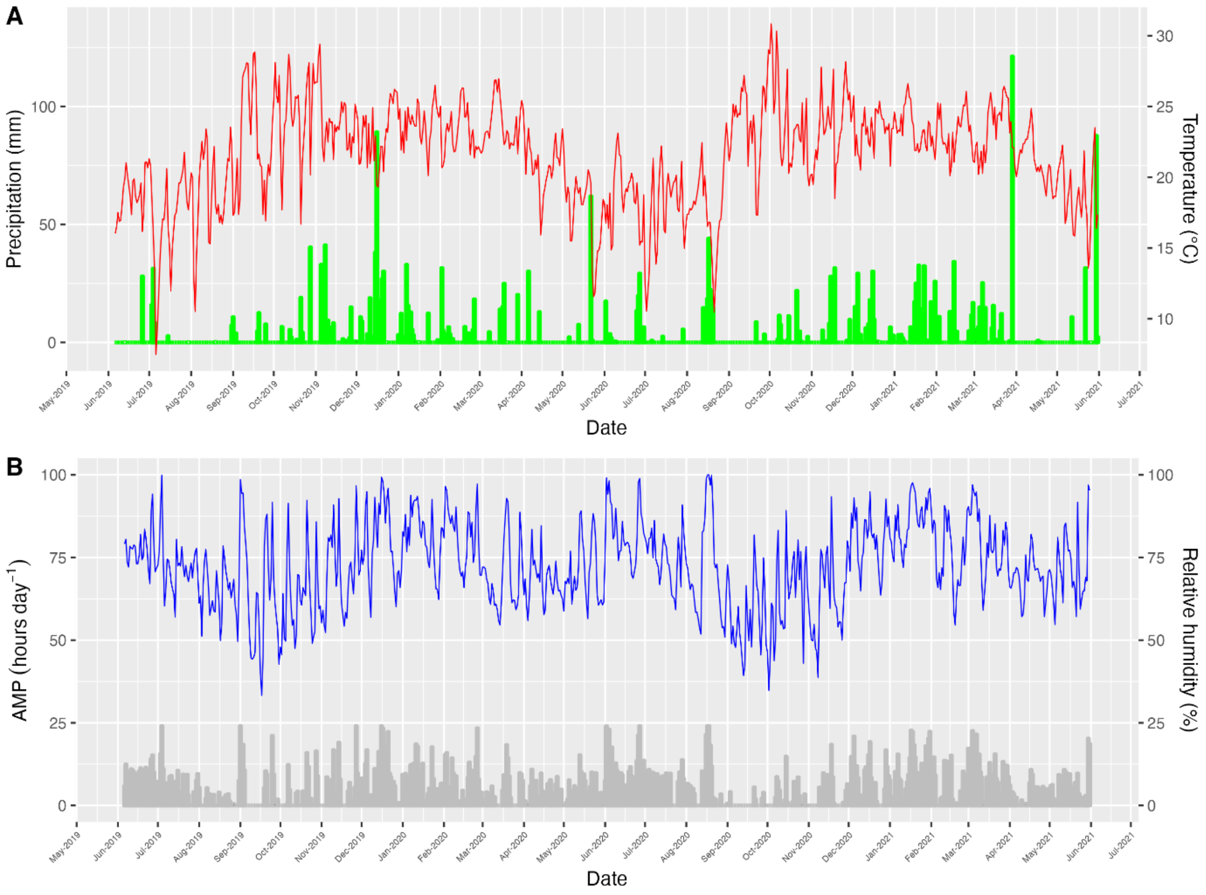
Oligonucleotide name	**Type**	**Oligonucleotide sequence and labeling (5′−3′)**
Gene ITS1 5.8S ITS2		
Ppm1	Forward primer	GCAGAATTCAGTGAATCATCAAG
Ppa2	Reverse primer	GCAACACTCAAAATCCAACAAT
ASRP1_HEX	Probe	TGAACGCACCTTGCACCTTTTGGT

Fonte: Próprio autor.

1.5 CANONICAL CORRELATION ANALYSIS BETWEEN CLIMATIC VARIABLES AND FUNGAL DNA QUANTITY OF THE TARGET GENE DETECTED IN AIRBORNE SPORE SAMPLES OF THE PATHOGEN BY QPCR

Meteorological data were collected at the IDR Paraná Experimental Station, located near the air sampling site. Over two years, daily recordings were made of maximum, minimum, and average temperatures (°C), rainfall amount (mm), daily duration of leaf wetness period (hours.day^{-1}), as well as maximum, minimum, and average relative humidity (%), and wind speed (m.s^{-1}) (Figure 2).

Figure 2 - Daily rainfall (A) (mm, red lines) and daily average temperature (°C, green bars), (B) leaf wetness period (hours per day, gray bars) and daily average relative humidity (% , blue lines) in Londrina, PR, at the IDR-Paraná Experimental Station, from June 2019 to June 2021



Fonte: Próprio autor.

To investigate the relationship between climatic variables and the amount of *Pp* DNA detected in airborne inoculum, we used daily rainfall data and leaf wetness period accumulated over 5, 10, 15, and 30 days before the detection event (dbde). This resulted in the variables of rainfall and leaf wetness period (LW) at 5, 10, 15, and 30 dbde. We also used daily relative humidity values, reflecting the optimal relative humidity index (ORHI, number of days with relative humidity ≥ 85%, considered ideal for fungal sporulation), accumulated over the periods of 5, 10, 15, and 30 days. Additionally, we calculated the optimal average temperature index (OTmI, number of days within the range of 21°C to 28°C, ideal for the development of soybean Asian rust) accumulated over the same periods.

Canonical correlation analyses were conducted for two-month data periods, covering the reproductive stage of soybeans defined based on the two main regional sowing windows (A1 and A2) in the state of Paraná and the sanitary void period (A3), as defined by the National Agricultural Zoning Program based on Climatic Risk for the State of Paraná (ZARC 2022). Furthermore, we included a soybean sanitary void period (June to September) to assess the correlations with climatic variables.

1.6 DESCRIPTIVE REPRESENTATION OF TIME SERIES OF QUANTITATIVE FUNGAL DNA DATA AND CANONICAL CORRELATION ANALYSIS

The time series of daily *Pp* DNA quantities detected in aerosol samples using qPCR were synthesized using R software packages (R Development Core Team 2022), including dplyr, lubridate, scales, gridExtra, ggthemes, and ggplot2, with the use of functions geom_line, geom_point, and facet_wrap. Canonical correlation analysis (CCA) ($p \leq 0.05$) between the climatic variables and the amount of *Pp* DNA was performed using R statistical and performance analysis packages, with the R Studio interface (R Development Core Team 2022).

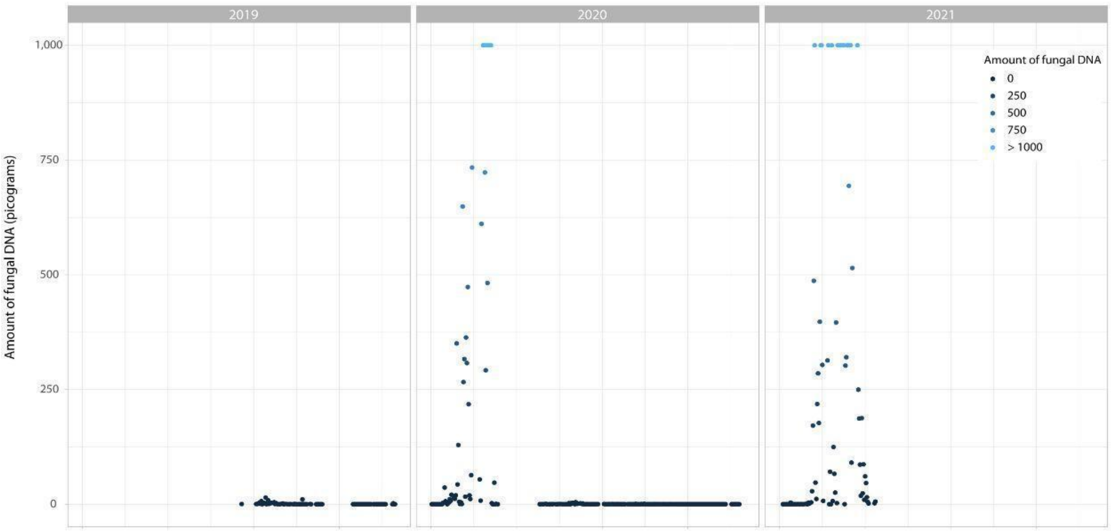
1.7 RESULTS

1.7.1 Detection of *Pp* in Aerosol Samples Using qPCR

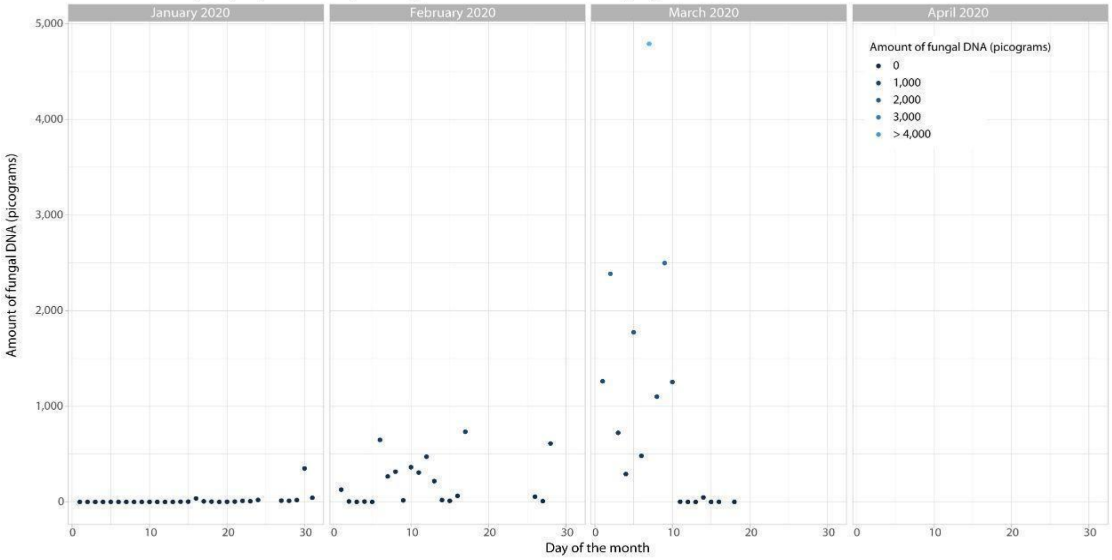
From July to September 2019, corresponding to the off-season (sanitary void) for soybeans in the Londrina region, the average daily amount of fungal DNA detected was 3.62 pg (± 3.41), likely originating from volunteer plants still present in the cultivation area. The highest peaks of *Pp* DNA were detected during the 2019/20 growing season (September 2019 to March 2020), with an average of 54.034 pg (± 894.70), and only a few days below the detection limit.

Figure 3 - Average daily quantities of *Phakopsora pachyrhizi* DNA detected by qPCR in aerosols sampled for 12 hours daily with a high-volume cyclonic air sampler in Londrina, Paraná, from July 5, 2019, to April 14, 2021.* *The average daily amount of target DNA was determined from 2 µL of the sample tested in duplicate

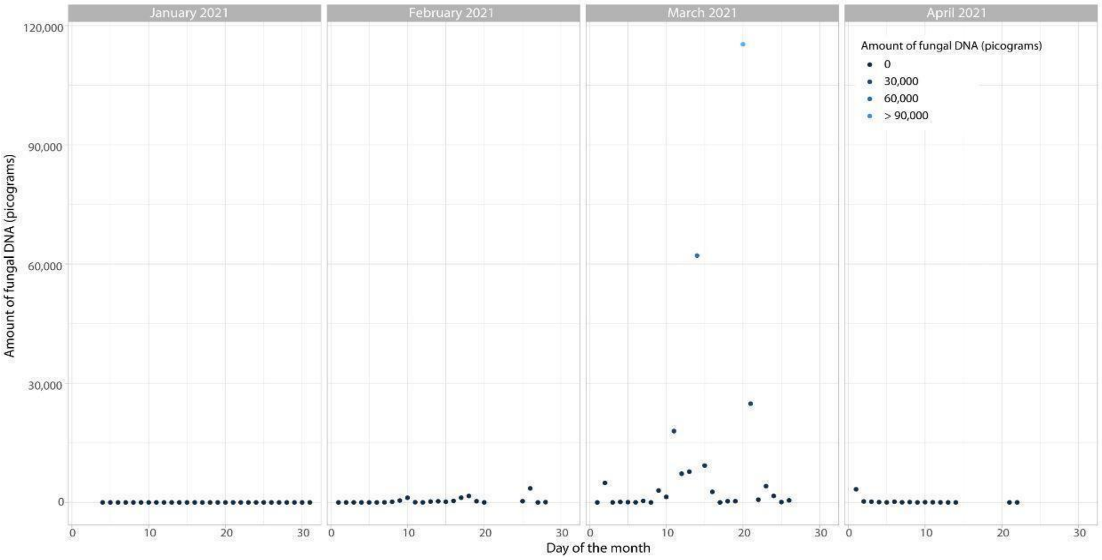
A. Detection of *Phakopsora pachyrhizi* in aerial spores samples – Londrina, Paraná by **cropping season**



B. Detection of *Phakopsora pachyrhizi* in aerial spores samples – Londrina, Paraná in **2020** cropping season



C. Detection of *Phakopsora pachyrhizi* in aerial spores samples – Londrina, Paraná in **2021** cropping season



Fonte: Próprio autor.

During the off-season of 2020 (April to September), the average amount of fungal DNA detected was 3.04 pg (± 0.76), lower than the previous period, indicating a significant reduction in the inoculum source. In the 2020/21 growing season (September 2020 to April 2021), the average values were considerably higher, with an average daily amount of 186.67 pg (± 17,742.49) of fungal DNA, with the highest peaks observed in the final months of the crop cycle.

Analyzing the 2020 growing season (Figure 3, B), we observed that fungal DNA quantities increased in February, when the crop was in the grain-filling stage, reaching an average value of 312.00 pg (± 1,030.07), during this period which coincided with the highest disease incidence. The highest detection peaks occurred in March, at the end of the crop cycle (harvest time), with notable values of 4,790.40 pg, 2,386.40 pg, and 2,498.33 pg detected in March 2020.

In the 2021 growing season (Figure 3, C), the highest concentrations were concentrated in March (at the grain maturation and harvest phase), with an average fungal DNA amount of 10,181.26 pg. Four isolated peaks exceeded previous values: 17,968.27 pg, 62,129.89 pg, 115,292.82 pg, and 24,850.49 pg, all occurring in March 2021.

1.5.2 Canonical Correspondence Analysis (CCA) between *Phakopsora pachyrhizi* DNA quantities in aerosol samples and meteorological variables

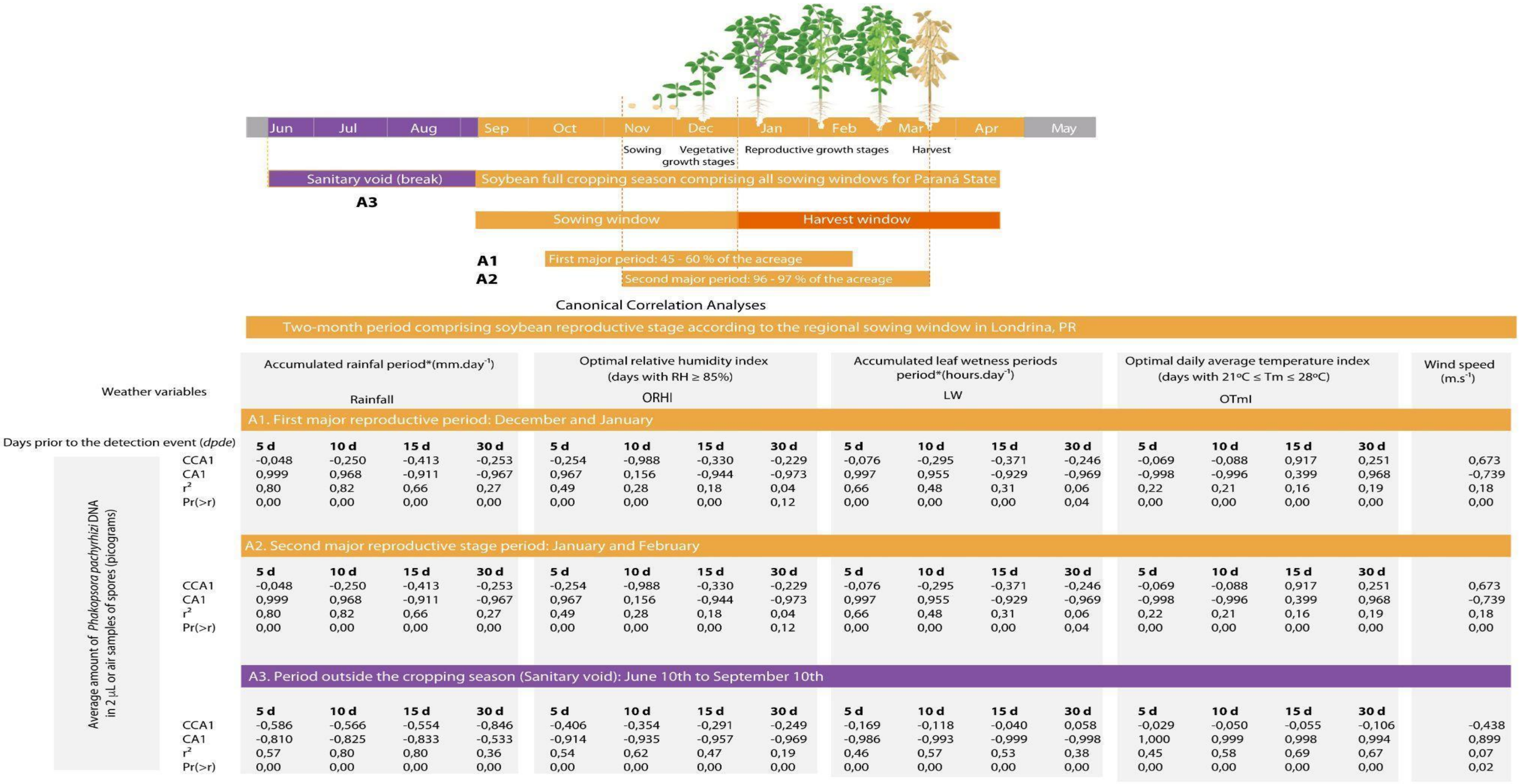
First Period: December to January (first sowing window)

Accumulated rainfall and the optimal relative humidity index showed significant negative correlations with the pathogen DNA over different time intervals, while wind speed at 2 meters showed a high positive correlation at 15 and 30 days. The R^2 correlations were especially high for accumulated periods of 5 to 15 days, suggesting that these variables explain a substantial portion of the variance, with p-values (< 0.05) indicating statistical significance.

During the reproductive stage of soybeans in the first sowing window (Figure 4, A1), analyses showed that environmental variables such as accumulated rainfall (5, 10, 15, and 30 days), optimal average temperature index (OTmI, 5, 10, 15, and 30 days), optimal relative humidity index (ORHI, 5, 10, and 15 days), and leaf wetness period (LW, 5 and 10 days) had significant associations with the prevalence of *Pp* fungal DNA. In contrast, OTmI over the last 15, 10, and 5 days, ORHI at 30 days, and wind speed (WS) did not show significant associations.

The analysis revealed that rainfall, relative humidity index, and leaf wetness period are critical variables for the variation in fungal DNA. The strongest associations were observed for accumulated rainfall over 15 days, OTmI over 5 days, ORHI over 15 days, and LW over 15 days, suggesting that these variables have the greatest influence on the prevalence of the inoculum.

Figure 4 - Canonical Correspondence Analysis (CCA) between the target DNA of *Pp* (pg) present in daily aerosol spore samples (2 µl tested) and meteorological variables accumulated 5, 10, 15, and 30 days before the spore release detection event (dbde). The considered variables were rainfall, optimal relative humidity index (ORHI, days with RH ≥ 85%), leaf wetness period (LW), optimal average temperature index (OTmI), and wind speed (WS) at 2 meters. Significance levels were considered with p-values < 0.05. The analyses were conducted for two-month periods, covering the reproductive stage of soybeans as defined based on the two main regional sowing windows (A1 and A2) in the state of Paraná and the sanitary void period (A3). Thus, CCA was performed separately for two-month periods of data, covering the reproductive stage of soybeans defined based on the two main regional sowing windows (A1 and A2) in the state of Paraná and the sanitary void period (A3) B.



Canonical Correlation Analyses

Two-month period comprising soybean reproductive stage according to the regional sowing window in Londrina, PR

Average amount of *Phakopsora pachyrhizi* DNA in 2 µL or air samples of spores (picograms)

Weather variables	Accumulated rainfal period*(mm.day^{-1}) Rainfall				Optimal relative humidity index (days with RH ≥ 85%) ORHI				Accumulated leaf wetness periods period*(hours.day^{-1}) LW				Optimal daily average temperature index (days with 21ºC ≤ Tm ≤ 28ºC) OTmI				Wind speed (m.s^{-1})
A1. First major reproductive period: December and January																	
Days prior to the detection event (*dpde*)	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	
CCA1	-0,048	-0,250	-0,413	-0,253	-0,254	-0,988	-0,330	-0,229	-0,076	-0,295	-0,371	-0,246	-0,069	-0,088	0,917	0,251	0,673
CA1	0,999	0,968	-0,911	-0,967	0,967	0,156	-0,944	-0,973	0,997	0,955	-0,929	-0,969	-0,998	-0,996	0,399	0,968	-0,739
r^2	0,80	0,82	0,66	0,27	0,49	0,28	0,18	0,04	0,66	0,48	0,31	0,06	0,22	0,21	0,16	0,19	0,18
Pr(>r)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,12	0,00	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00
A2. Second major reproductive stage period: January and February																	
	5 d	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	
CCA1	-0,048	-0,250	-0,413	-0,253	-0,254	-0,988	-0,330	-0,229	-0,076	-0,295	-0,371	-0,246	-0,069	-0,088	0,917	0,251	0,673
CA1	0,999	0,968	-0,911	-0,967	0,967	0,156	-0,944	-0,973	0,997	0,955	-0,929	-0,969	-0,998	-0,996	0,399	0,968	-0,739
r^2	0,80	0,82	0,66	0,27	0,49	0,28	0,18	0,04	0,66	0,48	0,31	0,06	0,22	0,21	0,16	0,19	0,18
Pr(>r)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,12	0,00	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00
A3. Period outside the cropping season (Sanitary void): June 10th to September 10th																	
	5 d	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	**5 d**	**10 d**	**15 d**	**30 d**	
CCA1	-0,586	-0,566	-0,554	-0,846	-0,406	-0,354	-0,291	-0,249	-0,169	-0,118	-0,040	0,058	-0,029	-0,050	-0,055	-0,106	-0,438
CA1	-0,810	-0,825	-0,833	-0,533	-0,914	-0,935	-0,957	-0,969	-0,986	-0,993	-0,999	-0,998	1,000	0,999	0,998	0,994	0,899
r^2	0,57	0,80	0,80	0,36	0,54	0,62	0,47	0,19	0,46	0,57	0,53	0,38	0,45	0,58	0,69	0,67	0,07
Pr(>r)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02

Fonte: Próprio autor.

Second Period: January to February (Second Sowing Window)

The patterns observed during this period were similar to those in December-January, indicating a continuity in the influence of climatic variables on inoculum dynamics across different sowing windows. The climatic variables maintained significant correlations (either positive or negative) depending on the time interval considered. The R^2 correlations remained strong, and the p-values indicated statistical significance.

In the second analysis period, corresponding to the second sowing window (Figure 4, A2), it was observed that accumulated rainfall (5, 10, and 15 days), OTmI (5, 10, and 15 days), ORHI (15 days), and LW (10 and 15 days) continued to have a significant effect on inoculum prevalence. Among these variables, rainfall had the most pronounced effect, especially over shorter periods (5 days), indicating its relevance in *Pp* inoculum dynamics.

Additionally, wind speed (WS) had a moderate impact on inoculum prevalence, but ORHI at 30 and 10 days and LW at 30 days did not show significant associations, reinforcing that rainfall and leaf wetness-related variables are more robust predictors.

1.5.2 Sanitary Void Period

During the sanitary void period (Figure 4, A3), there was a significant reduction in the presence of *Pp* inoculum, evidenced by the negative association between the optimal average temperature index (OTmI) and the relative humidity index across various accumulated periods (30, 15, 10, and 5 daed). This suggests that drier conditions and lower temperatures during the sanitary void period are associated with lower inoculum presence.

Additionally, wind speed showed a negative, though weaker, association with inoculum prevalence, suggesting that higher wind speeds do not favor the presence of the pathogen during this period. These results reinforce the effectiveness of the sanitary void period in reducing *Pp* inoculum.

1.6 DISCUSSIONS

This study aimed to monitor airborne populations of *Phakopsora pachyrhizi*, the causative agent of Asian soybean rust, in Londrina, a region of great importance for soybean production in Paraná. Using a high-volume cyclone and real-time quantitative PCR (qPCR) technique, we conducted continuous monitoring between 2019 and 2021. This approach not only quantified the presence of airborne *P. pachyrhizi* uredospores but also allowed for the analysis of the aerobiology of other pathogens, as demonstrated in the study by Vicentini *et al*. (2023).

The collection of spores from soybean cultivation areas, combined with qPCR detection, revealed the pathogen's dispersion dynamics and its relationship with climatic conditions. During the monitoring period, we detected the target DNA of *P. pachyrhizi* in 24.73% of the samples, with significant concentration peaks, especially in March 2020 and 2021. These findings indicate that Asian soybean rust has a high potential for airborne dispersion, particularly under favorable climatic conditions, corroborating the literature that highlights leaf wetness and temperature as key factors for the epidemic (Irwin 1999; Jeger 1999; Isard *et al*. 2007).

Analysis of the climatic variables revealed that rainfall and relative humidity strongly influenced the amount of detected inoculum. We observed that rainfall had a positive impact on spore release, especially when associated with periods of leaf wetness, which are critical for the pathogen's germination. Wind speed also showed a moderate effect, facilitating spore dispersion during certain periods. These correlations are crucial for adapting management strategies, allowing adjustments in fungicide use and outbreak prevention.

Furthermore, the statistical analysis indicated significant negative correlations between the presence of pathogen DNA and accumulated rainfall, suggesting that heavy rains may limit airborne dispersion in certain contexts. Conversely, high relative humidity combined with temperatures between 18-26°C favored spore release and viability, corroborating previous studies that identify these conditions as ideal for the Asian rust epidemic (Alves *et al*. 2007; Blum *et al*. 2015).

Comparing our data with the study by Moura (2024), we observed that despite both studies employing efficient methodologies, our research recorded significantly higher concentrations of *P. pachyrhizi* DNA. This suggests that the methodology used in our study may be more sensitive, providing a valuable tool for pathogen monitoring in intensive agriculture.

The implementation of an integrated monitoring system that combines predictions of pathogen airborne dispersion with meteorological data could be a promising approach to optimize Asian rust management. By combining real-time climatic condition data with information on pathogen dispersion, farmers can make more informed decisions, potentially reducing the need for chemical applications and minimizing resistance risks.

In summary, this study not only enhances the understanding of *P. pachyrhizi* dispersion dynamics but also underscores the importance of an integrated approach that considers multiple climatic variables and advanced detection techniques. The ability to predict pathogen dispersion based on climatic conditions can improve management effectiveness and contribute to more sustainable agricultural practices. Understanding the correlations observed during different periods is essential for developing more accurate predictive models, which can aid in proactive disease management. This version includes more details about climatic variables and uses more generic terminology for the monitoring system.

1.7 CONCLUSIONS

- Airborne monitoring of *Phakopsora pachyrhizi* populations in Londrina, Paraná, allowed for the quantification of pathogen DNA in 24.73% of the samples collected between 2019 and 2021.
- Sample collection was conducted using a high-volume cyclone, which captured airborne particles, including fungal spores, in soybean cultivation areas.
- Significant peaks in DNA detection occurred in March 2020 and 2021, coinciding with the critical soybean grain-filling period, indicating times of higher pathogen dispersion.
- Climatic variables such as rainfall, relative humidity, and wind speed showed important correlations with the presence of *P. pachyrhizi* DNA, suggesting that climatic conditions influence spore dispersion dynamics.

- Accumulated rainfall showed a significant negative correlation with the presence of pathogen DNA, while relative humidity and wind speed showed positive correlations during certain periods.
- Pathogen DNA detection was carried out using quantitative real-time PCR (qPCR), which proved to be effective and sensitive, offering a valuable tool for the airborne monitoring of *P. pachyrhizi*.
- The information obtained contributes to the understanding of *P. pachyrhizi* aerobiology and can guide more informed and efficient management practices.

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2 PREVALENCE OF THE SDHC I86F SUBSTITUTION ASSOCIATED WITH RESISTANCE TO SDHI FUNGICIDES IN POPULATIONS OF THE ASIAN SOYBEAN RUST PATHOGEN IN BRAZIL IN RECENT CROPPING SEASONS

ABSTRACT

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* (Pp), is the most important disease of soybean crops in Brazil. For over a decade, disease management has strategically focused on extensive, prophylactic applications of systemic, single-site chemical fungicides with high risk for resistance (especially QoIs and DMIs). Due to widespread resistance to QoIs and DMIs in the country, the efficacy of these site-specific fungicides has significantly decreased, resulting in yield losses below 20%. Since 2013, new succinate dehydrogenase inhibitor (SDHI) fungicides, also site-specific, have been included in the chemical management portfolio for ASR. However, during the 2015/2016 season, *Pp* isolates with a mutation in the *SdhC* gene were detected, resulting in the substitution of the amino acid SdhC-I86F at the target site, conferring resistance to SDHI fungicides. Subsequent surveys until 2019 indicate that SDHI resistance has become pervasive in the country. Our study aimed to determine, using specific real-time quantitative PCR (RT qPCR) detection of the SdhC-I86F mutation, the prevalence of this substitution in field populations of the ASR pathogen sampled during the 2020/21 and 2022/23 seasons in different Brazilian states. We obtained 51 *Pp* samples from Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Rio Grande do Sul, and São Paulo. We also determined the EC_{50} (fungicide concentration sufficient to reduce in vivo ASR severity by 50%) in samples from six distinct pathogen populations where the SdhC-I86F substitution was detected. The SdhC 86F mutant allele associated with SDHI resistance was detected in the majority *Pp* populations sampled. The six phenotyped populations showed reduced sensitivity to the fungicide fluxapyroxad. We conclude that the SdhC-I86F substitution remained prevalent in the country in recent cropping seasons.

Keywords: *Phakopsora pachyrhizi;* SdhC-I86F substitution; fluxapyroxad; succinate dehydrogenase inhibitors.

RESUMO

A ferrugem asiática da soja (FAS), causada pelo fungo *Phakopsora pachyrhizi* (*Pp*), é a doença mais importante das lavouras de soja no Brasil. Há mais de uma década, o manejo da doença tem se concentrado estrategicamente em aplicações extensivas e profiláticas de fungicidas químicos sistêmicos de site único com alto risco de resistência (especialmente QoIs e DMIs). Devido à resistência generalizada a QoIs e DMIs no país, a eficácia desses fungicidas de site específico diminuiu significativamente, resultando em perdas de produtividade abaixo de 20%. Desde 2013, novos fungicidas inibidores da succinato desidrogenase (SDHI), também de site específico, foram incluídos no portfólio de manejo químico para a ASR. No entanto, durante a temporada 2015/2016, foram detectados isolados de *Pp* com uma mutação no gene *SdhC*, resultando na substituição do aminoácido SdhC-I86F no local-alvo, conferindo resistência aos fungicidas SDHI. Pesquisas subsequentes até 2019 indicam que a resistência a SDHI se tornou prevalente no país. Nosso estudo teve como objetivo determinar, usando a detecção específica por PCR quantitativo em tempo real (RT qPCR) da mutação SdhC-I86F, a prevalência dessa substituição em populações de campo do patógeno da FAS amostradas durante as temporadas 2020/21 e 2022/23 em diferentes estados brasileiros. Obtivemos 51 amostras de *Pp* da Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Rio Grande do Sul e São Paulo. Também determinamos a EC_{50} (concentração de fungicida suficiente para reduzir a severidade da FAS *in vivo* em 50%) em amostras de seis populações distintas do patógeno onde a substituição SdhC-I86F foi detectada. O alelo mutante SdhC 86F associado à resistência a SDHI foi detectado na maioria das populações de *Pp* amostradas. As seis populações fenotipadas mostraram sensibilidade reduzida ao fungicida fluxapiroxade. Concluímos que a substituição SdhC-I86F permaneceu prevalente no país nas últimas temporadas de cultivo.

Palavras chaves: *Phakopsora pachyrhizi;* substituição SdhC-I86F; fluxapiroxade; inibidores da succinato desidrogenase.

2.1 INTRODUCTION

Since its introduction in Brazil in 2001, Asian soybean rust (ASR), caused by the biotrophic basidiomycetous *Phakopsora pachyrhizi* (*Pp*), has emerged as the most critical soybean disease in the country (Yorinori 2021a). On soybean crops not treated with fungicides, ASR can cause yield losses of up to 90% on susceptible varieties under favorable climatic conditions (Juliatti and Zambolim 2021). Crop losses have been recurrent over the years, ranging from a minimum of 363.5 thousand tons in 2011/2012 to a historical maximum of 4.6 million tons in 2003/2004, strongly impacting the Brazilian economy, which is based mainly on the export of commodities (Godoy *et al*. 2016; Juliatti and Zambolim 2021).

With a nationwide south-north distribution, ASR epidemics are widespread in Brazilian agroecosystems, where the fungus can survive year-round in volunteer soybean plants (Fanaro *et al*. 2011; Yorinori 2021b). To mitigate this, since 2007, federal and state regulations imposed a sanitary void (60-90 days between harvests, free from soybean cultivation) to restrict the occurrence of voluntary soybean plants at the end of the harvest and, consequently, to reduce the inoculum survival between growing seasons and the early-season inoculum (Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA) 2021a, b, 2022). Despite this measure, 573 outbreaks of ASR were reported in recent cropping seasons (Consórcio Antiferrugem 2023).

ASR significant dominant resistance genes (*Rpp1* to *Rpp7*) were mapped in the soybean genome. However, few resistant soybean cultivars are available (Li *et al*. 2012; Childs *et al*. 2018; Lin *et al*. 2022). Resistance has not been durable and stable, though, due to its rapid breakdown by selection and emergence of new compatible virulent fungal genotypes (Hartman *et al*. 2005; Yorinori *et al*. 2005; Akamatsu *et al*. 2013, 2017; Yorinori 2021c). Therefore, disease management has mainly relied on chemical control with sequential sprays of site-specific systemic fungicides, becoming a threat to the sustainability of the soybean agroecosystem in Brazil (Godoy *et al*. 2015, 2016). The effectiveness of site-specific fungicides and soybean yield response has steadily declined over the past decades (Godoy *et al*. 2016; Dalla Lana *et al*. 2018; Barro *et al*. 2021). This decline is attributed to continued use of the same or related single active ingredient fungicides and repeated use of co-formulations for at least four consecutive years (Barro *et al*. 2021). These fungicides included the single active ingredients azoxystrobin, cyproconazole, and tebuconazole and also the premixes azoxystrobin + cyproconazole, picoxystrobin + cyproconazole, and pyraclostrobin + epoxiconazole (Dalla Lana *et al*. 2018). This reduction in efficacy was linked to the emergence of resistance to quinone-outside inhibitors (QoIs, such as azoxystrobin) and demethylation inhibitors (DMIs, such as cyproconazole and tebuconazole), the two main classes of fungicides used for ASR management in Brazil (Schmitz *et al*. 2014; Müller *et al*. 2021).

Fungicide resistance emergence is due to high selection pressure on ASR pathogen populations from the intensive use of high-risk systemic fungicides through calendar-based sequential prophylactic sprays (Godoy *et al*. 2016; Dalla Lana *et al*. 2018; Barro *et al*. 2021). Despite decreased fungicide effectiveness (Godoy *et al*. 2016; Dalla Lana *et al*. 2018; Barro *et al*. 2021) and subsequent reports of fungicide resistance (Schmitz *et al*. 2014; Müller *et al*. 2021), ASR management remains heavily reliant on chemical control, costing up to US$2.2 billion per soybean cropping season in Brazil (Godoy *et al*. 2016; Yorinori 2021a, c; Ishikawa-Ishiwata and Furuya 2021).

Considering reports from countrywide field trials indicating a continuous decrease in fungicide effectiveness for controlling ASR over the last decades, the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), followed by Paraná Agribusiness Defense Agency (ADAPAR), suspended the recommendation of 63 mixtures of commercial fungicides for controlling the disease (Paraná Agribusiness Defense Agency (ADAPAR). 2015; Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA) 2016). Nonetheless, a MAPA Technical Committee recommended that these fungicides remained labeled for disease management programs targeting other pathogens, potentially overlooking that selection pressure towards ASR pathogen resistance remains high even if ASR is not the primary target of the spray (Technical Note 8/2017 (Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA). 2017)).

Contrary to this recommendation, the full suspension of fungicide labeling that has lost efficacy could be used as a strategy for managing fungicide resistance, especially if there is a fitness penalty for resistance (Parnell *et al*. 2005). Since resistant and sensitive lineages of the pathogen may coexist, the removal of the selection pressure would impact the pathogen´s population as the resistant lineages would be less fit than the wild type in the absence of the fungicide sprays (Parnell *et al*. 2005; Hawkins and Fraaije 2018). As an example, the efficacy of tebuconazole sprayed as single active ingredient has significantly decreased at 7.7 percentage points per year from 2005—2014 (Dalla Lana *et al*. 2018) reaching a minimum of ≈15% in 2013/2014, but in a more recent period after its removal (from 2015—2020) its efficacy has partially reversed consistently to ≈40% (Godoy *et al*. 2020). Efficacy of azoxystrobin was not reserved though (Dalla Lana *et al*. 2018; Godoy *et al*. 2020). In fact, DMI-resistant *P. pachyrhizi* isolates with *CYP51* mutations had competitive disadvantages relative to sensitive isolates with the wild-type CYP51, which explains these field observations for the DMI tebuconazole (Klosowski *et al*. 2016a). In contrast, the QoI-resistant isolates with the F129L mutation in the *CYTB* gene were as fit as the QoI-sensitive, wild-type *CYTB* isolate (Klosowski *et al*. 2016a).

The labeling of broad-spectrum protectant multi-site fungicides, such as the old copper- and/or dithiocarbamates-based formulations were sought as alternatives with some acceptable level of efficacy, once the available single site QoI and DMIs fungicides were no longer efficacious for ASR management, especially as single actives (Silva *et al*. 2015; Godoy *et al*. 2016; Juliatti *et al*. 2017; Yorinori 2021c). Though these protectant multi-site fungicides were available to the Brazilian soybean pesticide market even before the 1970s, until very recently no co-formulation with the high-risk systemic single-site fungicides have been labeled so far for ASR control (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023) as a broadly effective anti-resistance strategy (Mikaberidze *et al*. 2014; Corkley *et al*. 2022).

In 2013, new site-specific systemic succinate dehydrogenase inhibitors (SDHI fungicides) from the second-generation carboxamide group were labeled for the management of soybean diseases in Brazil (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023). SDHI fungicides specifically inhibit the mitochondrial respiration by targeting succinate dehydrogenase (Sdh enzyme or respiratory chain complex II). The Sdh enzyme is a heterotetramer with four nuclear-encoded subunits responsible for transferring electrons directly to ubiquinone in the respiratory chain. The strong binding of SDHI fungicides to the ubiquinone-binding site physically blocks access to the substrate, preventing the oxidation cycle of succinate, which is essential for the respiratory chain (Sierotzki and Scalliet 2013; Klaubauf *et al*. 2014). Structurally, the Sdh enzyme consists of two groups of subunits: a) The largest, which has the subunits SdhA and SdhB, which are peripheral and hydrophilic; b) The smallest, which consists of the hydrophobic subunits SdhC and SdhD integrated into the inner membrane of mitochondria (Ōmura and Shiomi 2007).

Probably because SDHI fungicides are considered a medium to high risk group of fungicides for resistance selection in plant pathogen populations (Simões *et al*. 2018; Borba 2019), they were formulated and labeled in co-formulations with other fungicides as an anti-resistance strategy [36]. However, these coformulations included QoI and DMI fungicides that were ineffective for controlling ASR, for which a reduction in sensitivity and resistance in *Pp* had already been detected (Klosowski *et al*. 2016a, b, 2018; Müller *et al*. 2021). It is not surprising that insensitivity to SDHIs has been reported in *Pp* isolates shortly after the labeling of these fungicides in Brazil (Simões *et al*. 2018; Müller *et al*. 2021).

In fact, either due to a concerted pattern of fungicide recommendation from similar mode of action all throughout the country over the years (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023), or due to the pathogen´s long distance gene flow (Twizeyimana *et al*. 2011), resistance to QoI and DMI fungicides became widespread in Brazil as it has been reported in GO, MT, MS, MG, SP, PR and RS (Klosowski *et al*. 2016b, 2018; Müller *et al*. 2021). For QoI-resistant strains, a non-synonymous mutation in the *cytB* gene resulting in the aminoacid substitution F129L in the cytochrome b is prevalent in the country (Klosowski *et al*. 2016b). Several mutations detected in the *CYP51* gene in DMI-resistant strains resulted in combinations of target site changes (e.g., F120L + Y131H; Y131F + K142R; Y131F + I475T) (Klosowski *et al*. 2016a, 2018; Müller *et al*. 2021). Overexpression of the *CYP51* gene may also be associated with reduced sensitivity to DMIs as reported for other rusts (Stammler *et al*. 2009). *Pp* isolates resistant to SDHI fungicides that emerged in 2015/2016 cropping season, soon after the introduction of these fungicides in Brazil, presented a mutation in the *SdhC* gene, resulting in the amino acid substitution SdhC-I86F in the target site (Müller *et al*. 2021). A selective advantage of SDHI resistant *Pp* isolates that carry this substitution is probably responsible for the increase in frequency and rapid spread in soybean fields across the country from 2015 - 2019 under fungicide pressure (Mello *et al*. 2021), acquiring similar relevance as the historical mutations for resistance to QoI and DMIs.

Therefore, the general objectives of this study were to survey geographical field populations of the ASR pathogen across different Brazilian States and to determine the prevalence of the mutation I86F in the *SdhC* gene in more recent populations of the *Pp*, using quantitative real-time for detection.

It would be essential, however, to further develop and implement automated and high-performance monitoring tools. These tools would allow for the assessment of inoculum levels of the ASR pathogen and the quantitative detection of resistant alleles from the Sdh target genes that confer resistance to the SDHI fungicides.

2.2 MATERIALS AND METHODS

2.2.1 Field populations sampling, urediniospores collection and dna extraction

The field population samples of the ASR pathogen were obtained from soybean farms in 2020/21 growing season, from several Brazilian states including Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Rio Grande do Sul and São Paulo, totaling 51 samples (Table 1, Additional file 1). In each farm, leaves from up to 50 infected plants were randomly collected following a transect sampling across the field, packed altogether in a paper bag and shipped by mail. Upon arrival, each composite soybean leaf sample was immediately processed for harvesting urediniospores by scraping the symptomatic leaves, with the aid of a brush, and transferring to 2.0 mL tubes containing 0.5 mL of 1 mm diameter glass beads. The composite urediniospore samples were stored in a -20°C freezer, for subsequent lyophilization and DNA extraction (Figure 1).

Table 1 - Geographic origin of *Phakopsora pachyrhizi* field population samples obtained from composite samples of soybean leaves from different Brazilian states.

Number of samples	**State**	**Counties**	**Harvest**
2	Goiás (GO)	Jataí	2020/21 and 2022/23
6	Mato Grosso (MT)	Lucas do Rio Verde	2020/2021
10	Mato Grosso do Sul (MS)	Chapadão do Sul, Bandeirantes	2020/21 and 2022/23
11	Minas Gerais (MG)	Lavras, Uberlândia	2020/21 and 2022/23
15	Paraná (PR)	Londrina, Mauá da Serra, Tamarana	2020/21 and 2022/23
2	Rio Grande do Sul (RS)	Passo Fundo	2020/21
4	São Paulo (SP)	Botucatu	2020/21
1	Bahia (BA)	Luis Eduardo Magalhães	2022/23

Fonte: Próprio autor.

Urediniospore samples were then lyophilized for 24 hours, bead-beatered in a fast-prep device for 20 seconds at speed 4 m s-1, and DNA extraction were conducted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's instructions, and quantified in a NanoDrop® 2000C spectrophotometer (Thermo Fisher Scientific, USA). The total DNA concentration obtained varied from 11.4 to 2,641.0 ng.mL^{-1}.

2.2.2 Real time quantitative pcr (rt qpcr) detection of the SdhC alleles 86I and 86F in populations of the asr pathogen

Prior to the quantitative detection of the *SdhC* 86I and 86F alleles, *P. pachyrhizi* partial *SdhC* gene (381 bp) was amplified by conventional PCR to generate amplicons that were used as template DNA for the standard curve quantitative calibration in the RT qPCR reactions.

DNA from two *P. pachyrhizi* isolates (PP.SP6 and PP.MG9) were selected for this initial step of the assay. PCR reactions were conducted in a final volume of 30 µL containing ultrapure water, 50 ng of total DNA, 0.1 µM of each primer (Table 2), 0.2 mM of each dNTP, 2 mM of MgCl 2 , 3.0 µL of 10 x buffer and 1 U of Taq DNA Polymerase (Sigma-Aldrich, San Luis, MO, USA). Amplifications were performed in a Mastercycler® Nexus thermocycler (Eppendorf®, Hamburg, Germany) using the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55°C of annealing temperature for 1 min [43], and 72 °C for 1 min; and final extension at 72 °C for 5 min.

Table 2 - Primers for PCR amplification of the *SdhC* partial gene from *Pp* used for the RT qPCR standard curve^1.

Primers	**Sequence (5’ - 3’)**
SdhC-5224 F	CGA AGG CTA CAT ACA CTT C
SdhC-5223 R	GCG TCT CAG AAT CCA TAG

Nota: ^{1}These primers amplify a *SdhC* fragment of 381 bp in length, according to Borba (Borba 2019).
Fonte: Próprio autor.

The positive amplification of the two PCR amplicons were checked by electrophoresis in a 1% agarose gel. PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and stored for further use. For the RT qPCR standard curves quantitative calibration for each one of the SdhC alleles, a serial dilution of a pool of the template DNA from the two isolates was prepared to obtain the following concentrations in a final reaction volume of 20 µL: 900; 450, 90, 9, 0.9 and 0.09 pg .mL^{-1}.

The RT qPCR reactions were prepared with a final reaction volume of 20 µL, containing 10 µL of 1X Master Mix iTaq Probe (Bio Rad, Hercules, CA, USA), 1 µL of forward primer at 300 nM, 1 µL of reverse primer at 300 nM, 1 µL of the probe at 150 nM (Table 3), 3 µL of ultrapure deionized water, and 4 µL of template DNA from the unknown sample. The reactions were carried out using the qPCR thermocycler CFX96 (Bio Rad, Hercules, CA, USA). The following amplification conditions were applied: 2 min at 95 °C; (2 - 5 sec at 95 °C, 15 – 30 sec at 60 °C) for 40 cycles. Each RT qPCR reaction was conducted in duplicate. Results were analyzed using the CFX Maestro software from Bio Rad.

Table 3. Primers and probe used for real time quantitative PCR detection and quantification of the I86F substitution in population samples of *Phakopsora pachyrhizi*. [1]

Primers and probe[2, 3]	**Sequence (5´- 3´)**	**Primers combination for allele specific detection**
Forward primers		
KES_2156_86F (A)	AAC AGC TAA CTT GGT ATT CCA GG**T**	SdhC 86F: A + C
KES_2155_86I (B)	AAC AGC TAA CTT GGT ATT CCA GG**A**	SdhC 86I: B + C
Reverse primer		
qPCR-sdhC R (C)	AAA CCA CCG GAT CAC TGA TGT	
Probe - sdhC [4]	6-FAM-AACGGGTTGCGCTCTTACAGGAGGTATTC-BHQ1	

Nota: [1] These primers amplify a SdhC fragment of 103 bp in length (Simões *et al*. 2018); [2] Forward primers according to Simões *et al*. (Simões *et al*. 2018); [3] Reverse primer according to ; identical to KES_2153 from Simões *et al*. (Simões *et al*. 2018); [4] FAM = 6-carboxyfluorescein; BHQ1 = black hole quencher 1. Fonte: Próprio autor.

This RT qPCR detection assay allowed us to quantify the prevalence of each of the SdhC gene alleles (encoding for the wild type SDHC variant 86I or the mutant SDHC 86F), thus determining their relative frequencies in populations of the ASR pathogen.

2.2.3 Sensitivity status of populations of the asr pathogen to the sdhi fungicide fluxapyroxad

Pp urediniospores from freshly collected infected soybean leaflets obtained from plants sampled in 2020/21 cropping season, were included in this assay (Figure 1). These composite population samples were collected in Rio Grande do Sul (PP.RS2.21), Paraná (PP.PR21.21 e PP.PR22.23), Mato Grosso do Sul (PP.MS12.23), Minas Gerais (PP.MG14.23) and Goiás (PP.GO2.23) as described in item 2.1. Infected leaflets were kept in a humid chamber for 24 hours, cut into pieces of approximately 0.5 x 0.5 cm, transferred to a 1% Tween solution in a 50 mL Falcon tube and shaken vigorously to release urediniospores from the pustules. Urediniospores were viable and active (evidenced by abundant germination in aqueous solution) for subsequent phenotyping fungicide resistance phenotype.

In order to multiply the inoculum of the pathogen, soybean plants in the phenological stage V3 (three nodes with fully developed unifoliate leaves (according to Fehr *et al*. (Fehr *et al*. 1971)) contained in 2 L pots were inoculated (Figure 1), using an airbrush coupled to a OneTools compressor model ON10 (Onetools GmbH & Co. KG, Mainz, Germany, with a final inoculum concentration adjusted to 1 × 10^5 urediniospores mL-1, after reading in a Neubauer chamber (Figure 1). The inoculated plants were transferred to trays in metallic structure cages wrapped in transparent plastic. Plants were kept in the dark for 24 hours and subsequently the cultivation conditions were 12 h photoperiod at 24ºC for 30 days, until abundant sporulation of *Pp* on soybean leaves (Figure 1).

Figure 1. Protocol for checking the sensitivity status of the Asian Soybean Rust pathogen to fungicides. **A**. Twenty four hours drying step for 24 h during the spore preservation process before storage at -20 ºC. **B**. Collection of *P. pachyrhizi* urediniospores using a brush. **C**. Tubes containing urediniospores of *P. pachyrhizi* collected from composite samples of soybean leaves infected with ASR for long term preservation and eventual reactivation for plant inoculation. **D**. Soybean plant growing in the healthy plant room for further inoculation with *P. pachyrhizi*. **E** and **F**. Preparing suspension of germinating reactivated urediniospores in water solution then filtering thru cheesecloth. **G**. *Phakopsora pachyrhizi* urediniospores viable for inoculation. **H**.

Soybean plants kept in metallic cages surrounded by transparent plastic, after inoculation, for multiplication and maintenance of pathogen inoculum. **I** and **J.** Fungicide application by the leaflet immersion method. **K**. Inoculation of the pathogen in spore suspension with the aid of an airbrush; inoculated leaflets held in Petri dishes containing moistened sterilized filter paper used in fungicide sensitivity assays. **L**. Inoculation and incubation room for *P. pachyrhizi* isolates, where soybean leaflets remained for 15 days at 24°C for 12 h of photoperiod to assess the severity of ASR in fungicide sensitivity experiments. **M**. Assessment of ASR disease severity in fungicide sensitivity assays beginning with delimiting the evaluation area, carried out with the aid of a hole punch. **N**. Use of a stereoscopic microscope to visualize lesions and uredias. **O.** Soybean rust pustules developed on inoculated leaflets observed under stereomicroscope. O. Monopod and photo box for assessment of ASR disease severity.

Fonte: Própria autora

Urediniospores harvested from freshly sporulating pustules in soybean leaves from actively growing plants were used as the inoculum source for the in planta fungicide sensitivity phenotyping assay (Figure 1). These urediniospores were collected by transferring the leaflets to an aqueous solution of Tween 20 (two drops per liter) in 15 mL Falcon tubes, followed by shaking. The urediniospore suspension obtained was then adjusted to 10^5 spores mL^{-1} using a Neubauer chamber. *In planta* sensitivity assay for SDHI fungicide were conducted according to the standard protocol proposed by FRAC (Scherb and Mehl 2006), with modifications, using healthy leaflets detached from leaves of soybean cv. ST 271 IPRO at stage V1 (fully developed leaves in unifoliate nodes, according to Fehr *et al*. (Fehr *et al*. 1971)) (Figure 1).

The leaflets collected were immersed for four seconds in fungicide solutions at the following concentrations of the SDHI fungicide fluxapyroxad: 0, 0.1, 0.3, 1, 3, 10, 30 and 100 µg mL^{-1} (Klosowski *et al*. 2018; Müller *et al*. 2021). Fungicide treated leaflets were transferred to Petri dishes containing sterilized filter paper disc moistened with sterilized distilled water (Figure 1). Each experimental plot was represented by a Petri dish containing detached healthy unifoliate leaves, for each fungicide concentration (Figure 1). After 24 hours of the fungicide treatment, soybean leaflets were inoculated with fungal inoculum suspensions adjusted to 1 x 10^5 urediniospores mL^{-1} to the point of runoff, using an airbrush coupled to a OneTools compressor model ON10. Experimental plots were kept for 24 h in the dark at 24 ^oC and subsequently transferred to a 12 h photoperiod chamber at 24 ^oC for 14 days until disease severity was assessed (Figure 1, M to P). The experimental design consisted of complete randomized blocks, with four replications per treatment, fungicide and dose. The entire experiment was repeated once.

At 14 days after inoculation, disease severity was estimated using the diagrammatic scale developed by Franceschi *et al*. (Franceschi *et al*. 2020). The number of $lesions.cm^{-2}$ and $uredias.cm^{-2}$ were quantified in predetermined areas of the leaves, with the aid of a stereoscopic microscope Zeiss model Stemi 2000 (Carl Zeiss AG, Oberkochen, Germany), according to Garcés-Fiallos and Forcelini (Garcés Fiallos and Forcelini 2013).

The concentration of fungicide sufficient to reduce the *in vivo* severity of ASR by 50% (EC_{50}) was calculated based on disease severity estimates. Other EC_{50} estimates were based on the number of lesions and uredia densities per cm^2 (Garcés Fiallos and

Forcelini 2013). The EC_{50} values for each isolate and each fungicide were estimated using the *R* package (R Development Core Team 2022) *ec50estimator* coupled with the *drc* library for analyses of dose response curves (Knezevic *et al*. 2007), from multiple isolates data sets. This package also allows for testing the hypothesis on the most fit model function (*fct*) for estimating the EC_{50} by a dose–response curve. Analysis of variance (ANOVA) was conducted to determine the significance of the effects of isolates by the *F* test (Bruce *et al*. 2020). The average EC_{50} from distinct populations of the pathogen were compared by the Tukey test (at 5% probability) using the *ExpDes Experimental Design* package (Knezevic *et al*. 2007) in *R* software (R Development Core Team 2022).

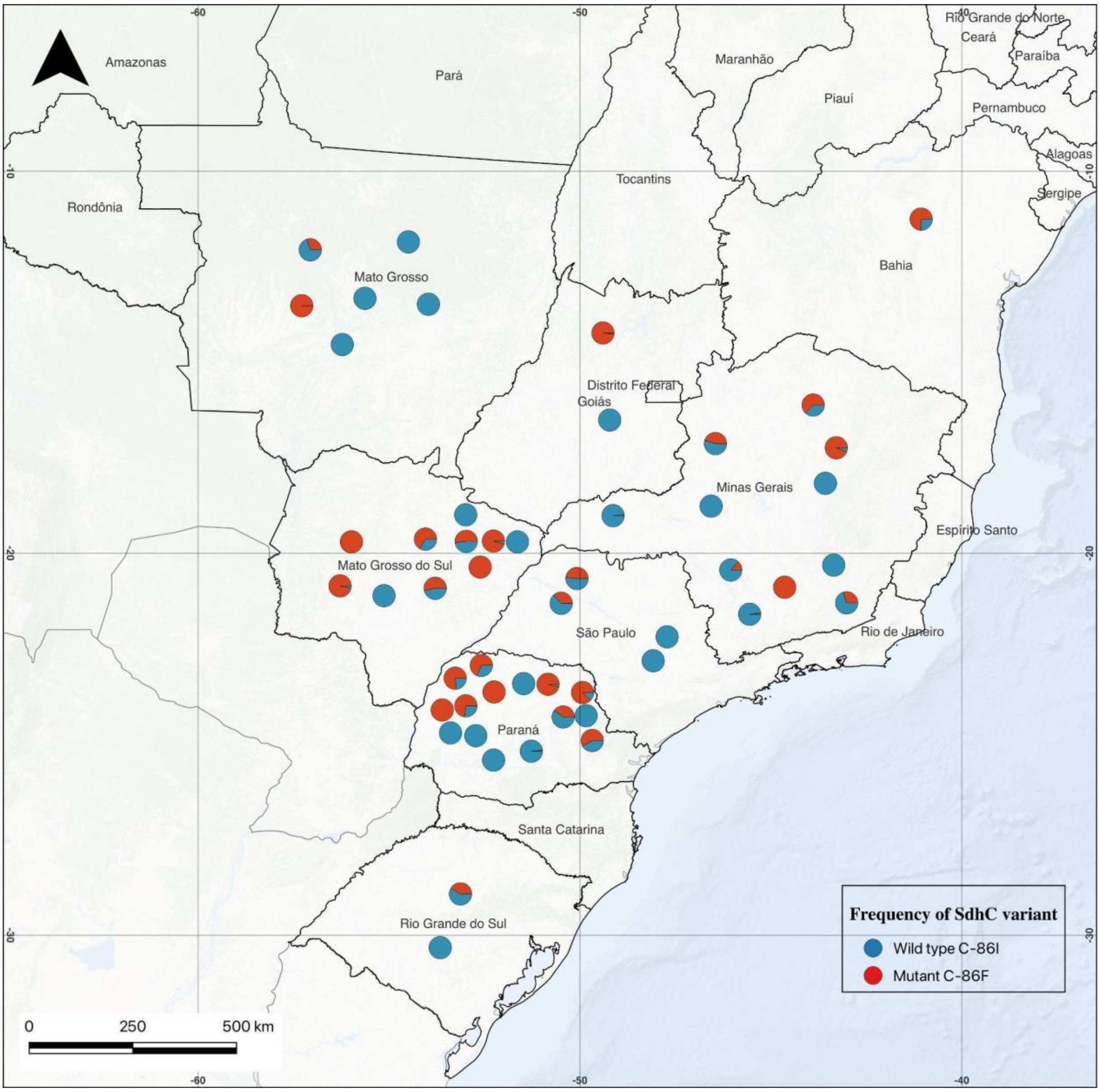
2.3 RESULTS

2.3.1 Prevalence of SdhC I86F substitution in populations of the asr pathogen

The mutant allele SdhC 86F associated with resistance to SDHI fungicides was detected in *Pp* populations from all eight Brazilian states sampled (Figure 2, Additional file 1). The average proportion of the SdhC 86F mutant allele detected in each state varied from 11.4% in SP (N=4), 19.6% in MT (N=6), 20,9% in RS (N=2), 31.7% in MG (N=11), 46.7% in PR (N=15), 49.9% in GO (N=2), 56.2% in MS (N=10), and 74.4 % in BA (N=1).

In MG we highlight three sample sites with a prevalence of the mutant allele SdhC 86F (from 62.7 to 100%), while in five other *Pp* population samples it was either not detected at all or its frequency was ≪2%. In MS, while the mutant SdhC 86F allele predominated in seven of the ten populations sampled, the wild-type SdhC 86I allele completely prevailed in three populations. On average, the frequency of the SdhC 86F allele was 56.2%, which was the second highest in comparison with the other states, in addition to ranging from 95.6 to a maximum of 100% in four populations. In contrast, in MT, in four out of the six populations sampled, the mutant allele SdhC 86F was not detected, whereas, in the two other *Pp* populations its frequency ranged from 17.5% to 99.8%. In PR, the SdhC 86F mutant allele was detected in 10 of the 15 populations sampled, with a mean frequency of 46.7%. In SP and RS, the average frequencies of the SdhC 86F allele (11.4 and 20.9, respectively) were much lower than the wild-type allele. The mutant allele was detected in only one population in both states. In the *Pp* population from Goiás (GO), similarly to RS, we only obtained two samplings. In one of them, the mutant allele 86F was not detected, while in the other, the frequency was 98.3%. In Bahia (BA), the frequency of the SdhC 86F allele was 74.4%, in a single population sampled in the state.

Figure 2 - Prevalence of the SdhC I86F substitution associated with resistance to SDHI fungicides in populations of the ASR pathogen *Phakopsora pachyrhizi* sampled from 51 sites in seven Brazilian states in recent cropping seasons (2020/21 or 2022/23).

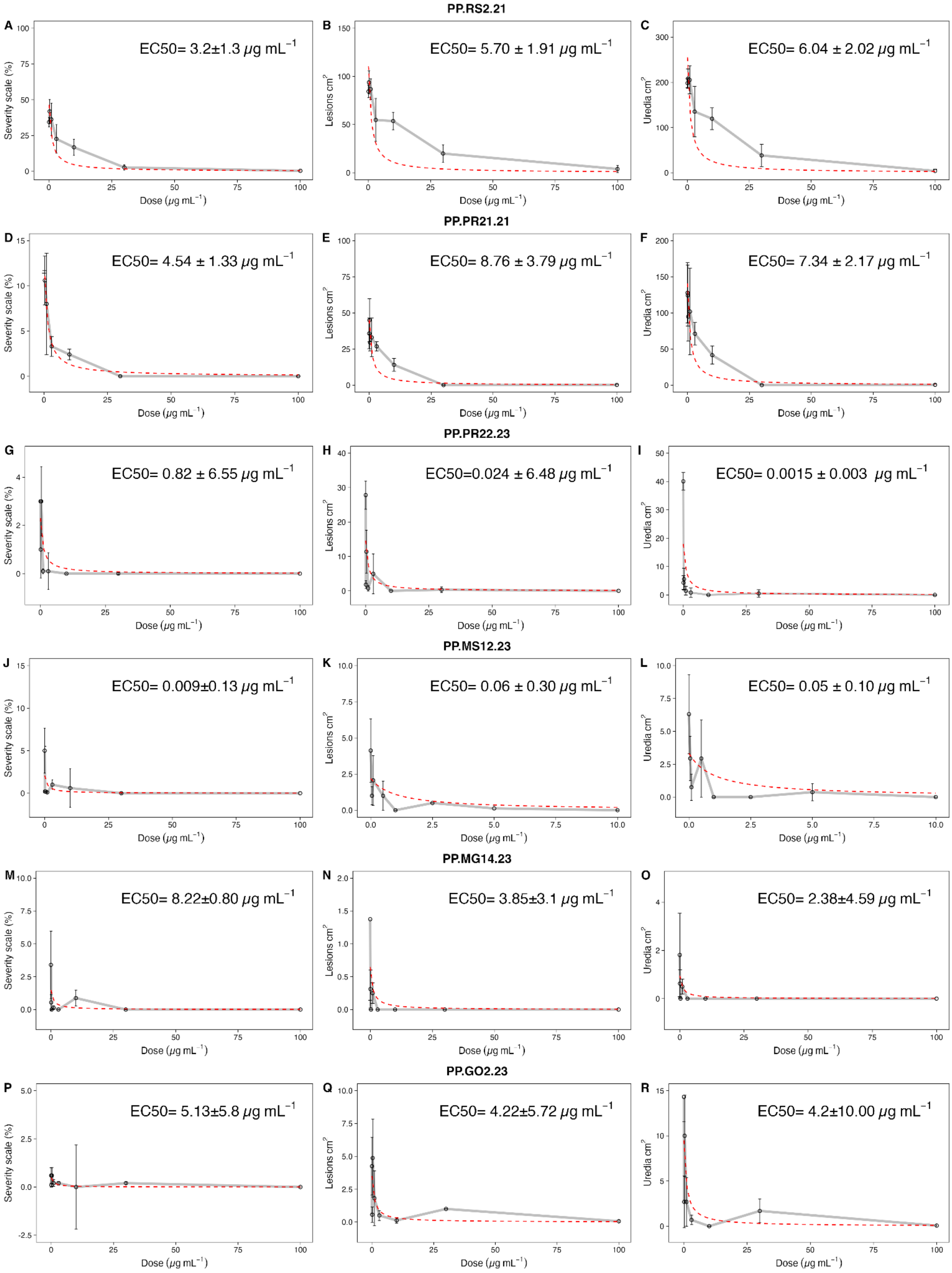


Fonte: Propria autora

2.3.2 Sensitivity status of populations of the asr pathogen to the sdhi fungicide fluxapyroxad

There was a decrease in disease severity in response to the increase in fluxapyroxad doses in both composite populations of *P. pachyrhizi*, with a similar pattern of reduced sensitivity to SDHI (Figure 3). The composite *Pp* population PP.RS2.21 had EC_{50} for fluxapyroxad = 3.2 ± 1.3 μg mL^{-1} for disease severity, 5.7 ± 1.91 μg mL^{-1} for lesions.cm^{-2}, and 6.04 ± 2.02 μg mL^{-1} for the number of uredia.cm^{-2} (Figure 3, top). In comparison, the *Pp* population PP.PR21.21 had similar EC_{50}s (4.54 ± 1.33 for disease severity, 8.76 ± 3.79 μg mL^{-1} for lesions.cm^{-2}, and 7.34 ± 2.17 μg mL^{-1} for the number of uredia.cm^{-2}). In the *Pp* population PP.PR22.23, the EC_{50} was 0.82 ± 6.55 μg.mL^{-1} for disease severity, 0.024 ± 6.48 μg.mL^{-1} for lesions.cm^{-2}, and 0.0015 ± 0.003 μg.mL^{-1} for the number of uredia.cm^{-2}. Meanwhile, the *Pp* population PP.MS12.23 showed the lowest EC_{50} values (0.009 ± 0.13 μg.mL^{-1} for disease severity, 0.06 ± 0.30 μg.mL^{-1} for lesions.cm^{-2}, and 0.05 ± 0.10 μg.mL^{-1} for the number of uredia.cm^{-2}. In the *Pp* population PP.MG14.23 the EC_{50} values were 8.22 ± 0.80 μg.mL^{-1} for disease severity, 3.85 ± 3.1 μg.mL^{-1} for lesions.cm^{-2}, and 2.38 ± 4.59 μg.mL^{-1} for the number of uredia.cm^{-2}. The composite *Pp* population PP.GO2.23 exhibited similar EC_{50} values (5.13 ± 5.8 for disease severity, 4.22 ± 5.72 μg.mL^{-1} for lesions.cm^{-2}, and 4.2 ± 10 μg.mL^{-1} for the number of uredia.cm^{-2}). Overall, the populations PP.PR22.23 and PP.MS12.23 showed the lowest EC_{50} values, which were significantly different from all the other populations by the Tukey test at 5% probability.

Figure 3 - *In vivo* sensitivity of composite population samples of *Phakopsora pachyrhizi* from Paraná (PP.PR21.21 and PP.PR22.23, Rio Grande do Sul (PP.RS2.21), Mato Grosso do Sul (PP.MS12.23), Minas Gerais (PP.MG14.23), and Goiás (PP.GO2.23) to the fungicide fluxapyroxad, and respective EC_{50} based on disease severity (A, D, G, J, M, P), number of lesions.cm^{-2} (B, E, H, K, N, Q), and uredia.cm^{-2} (C, F, I, L, O, R) in the soybean cultivar ST 271 IPRO. The red dotted line represents the data trend curve. EC_{50} values are presented with the respective standard error. *F* tests indicated significant differences among populations of the pathogen: $F_{\text{disease severity}}$ = 4.72, *p* = 0.00164; $F_{\text{number of lesions.cm\^{}-2}}$ = 4.65, *p* = 0.00183; $F_{\text{uredia.cm\^{}-2}}$ = 3.04, *p* = 0.0199.



Fonte: Propria autora

2.4 DISCUSSION

Resistance to QoI and DMI fungicides has already been widespread in Brazil for over a decade now and, as consequence, a significant decrease in the efficacy of these two fungicide groups has been reported (Klosowski *et al*. 2016a, b, 2018; Dalla Lana *et al*. 2018; Godoy *et al*. 2020; Müller *et al*. 2021). More recently, a consistent decline in control efficacy and yield response levels for the premixes azoxystrobin (QoI) + benzovindiflupyr (SDHI) (35.3% decreased efficacy; 550 kg.ha^{-1}) and picoxystrobin + benzovindiflupyr (15.5% decrease; 359.8 kg.ha^{-1}) detected in a meta-analysis spanning 177 fungicide trials in 46 locations across 10 states in Brazil, from 2015 to 2020 (Barro *et al*. 2021), were associated with resistance to both QoIs (Klosowski *et al*. 2016b) and SDHIs (Simões *et al*. 2018; Müller *et al*. 2021) in populations of *P. pachyrhizi*.

Our study surveyed recent geographical field populations of *P. pachyrhizi* across different Brazilian States and determined the prevalence of the substitution SdhC-I86F using real-time quantitative PCR for detection.

Historically, the first report of resistance to SDHI fungicides in populations of *Pp* from Brazil dates back to 2015/16 cropping season in Rio Grande do Sul (RS). This resistance was associated with a mutation in the *SdhC* gene leading to the amino acid substitution I86F in the corresponding SdhC subunit of succinate dehydrogenase protein (Simões *et al*. 2018). Virtually undetected before the labeling of SDHIs in Brazil in 2013 (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023), as from 2016/17 cropping season the SdhC I86F substitution began increasing in frequency in *Pp* populations from several distinct regions in the country (Simões *et al*. 2018).

A countrywide monitoring of *Pp* populations conducted by Mello and collaborators (Mello *et al*. 2021) indicated that resistance to SDHI fungicide became widespread in Brazil from 2015 to 2018. This uniform distribution in most of the Brazilian soybean-growing regions has probably been reached by recurrent and simultaneous fungicide selection pressure (Mello *et al*. 2021) or by efficient gene flow (Twizeyimana *et al*. 2011). Besides an increase in the mean EC_{50} for the SDHI-resistant *Pp* populations, the prevalence of the substitution SDHC-I86F increased from seven percent in 2015/16 to 90.4% in 2018/19 soybean cropping seasons, while the wild-type populations with no mutation represented only 9.6% of the total sample (Mello *et al*. 2021).

Concerning the decreased sensitivity to SDHIs, while in 2015/16 the SDHI-resistant *Pp* populations from RS with ≈50% SdhC-I86F had fluxapyroxad EC_{50} = 9.5 ± 1.7 (Simões *et al*. 2018), the most current survey from 2018/19 indicated mean EC_{50} above 10 mg L^{-1} in 15 out of 770 (1.9%) *Pp* populations sampled (Mello *et al*. 2021). In contrast, the fluxapyroxad-sensitive baseline *Pp* populations from 2015/16 sampling with 0% SdC-I86F had EC_{50} = 2.3 ± 0.9 (Simões *et al*. 2018).

In our survey from more recent cropping seasons (2020/21 and 2022/23), with a much narrower sample size of N = 51 populations, the mutant allele SdhC 86F associated with SDHI-resistance was detected in *Pp* populations from all Brazilian states sampled (Figure 2). The prevalence of populations with the SdhC 86F mutant allele varied from 19,6% in MT (N=6), 11,4% in SP (N=4), 46,7% in PR (N=15), 31,7% in MG (N=11), and 56,2% in MS (N=10). In addition, the populations chosen from our phenotypic study had EC_{50} for fluxapyroxad ranging from a minimum of 0.005 ± 0.10 μg mL^{-1} to a maximum of 8.76 ± 3.79 μg mL^{-1}, depending on the variable analyzed (Figure 3), falling either within the reduced sensitivity or the resistance category.

The highest within-population frequency of the SdhC-I86F mutation described so far across different Brazilian states sampled between 2015 and 2018 has apparently reached its stability at ≈50% maximum frequency (Simões *et al*. 2018; Mello *et al*. 2021; Müller *et al*. 2021). Considering the dikaryotic condition of *Pp* urediniospores (i.e. each urediniospore has two independent nuclei), this means 100% of the individuals would be resistant to SDHIs as heterozygous H strains (50% C-86F : I86 50%), with only one of the nuclei carrying the mutation (Mello *et al*. 2021). This has been described as a signal for fitness penalty associated with the SdhC-I86F substitution, resulting in the apparent absence of the homozygous R strain (100% C-88F) (Mello *et al*. 2021; Claus *et al*. 2022). Therefore, under a fitness penalty scenario, a resistant heterozygous H strain (50% C-86F:I86 50%) would be more fit than the resistant homozygous R strain (100% C-88F) in the absence of the fungicide selection pressure. In predominantly clonal populations of *P. pachyrhizi* lacking sexual recombination, heterozygosity has been described as considerably lower, though. The levels of observed heterozygosity were much lower than expected for the majority of neutral loci, with proportions ranging from as low as 0.038 to as high as high 1.0 and deviating from HWE (Anderson *et al*. 2008; Twizeyimana *et al*. 2011).

Against the odds that homozygosity for resistance to SDHI in the dikaryotic *Pp* would be virtually non-existent, a viable homozygous *Pp* R strain 100% SdhC-86F obtained under lab conditions has been described (Borba 2019). The homozygous R strain (100% 86F) was more resistant to SDHI fungicides and more aggressive than the heterozygous H strains, and had a competitive advantage under fungicide selective pressure. However, there was indeed a fitness cost for the homozygous R strain in competition with the wild type under no fungicide pressure. Without a homozygous R strain for comparison, Claus and collaborators (Claus *et al*. 2022) also detected fitness penalties associated with the SdhC-I86F substitution, including higher sensitivity to osmotic and oxidative stress under lab conditions, but not regarding the germination of spores under UV radiation (Claus *et al*. 2022). However, when not in competition with wild type strains, the populations with the C-86F substitution at 50% frequency survived after 36 generations in the absence of fungicide, indicating high stability of the C-86F substitution (Claus *et al*. 2022).

Up to date, field populations of *Pp* carrying 100% SdhC-I86F have not been identified (Simões *et al*. 2018; Mello *et al*. 2021). In our study, however, the frequency of the C-I86F was considerably higher than 50% in 16 populations (38.1% of the total) sampled in 2021/22. In these field populations, the average frequency of the C-I86F mutants was 82,4 ± 18.8. Three of these populations had the frequency of the C-I86F mutants fixed at 100%, pointing to the occurrence of the homozygous R strain (100% C-88F).

Though the 100% C-I86F strain was obtained under lab conditions by selecting survival uredinia after continuous exposure to high doses of SDHI fungicide (Borba 2019), clearly this selection also could happen in field conditions under similar circumstances, since fungicide pressure has been persisted since 2015/16 and SDHI resistant *Pp* populations are now prevalent in the entire country (Mello *et al*. 2021). And if there was initially a significant fitness cost for carrying C-I86F until 2019, it has probably been compensated by other genetic changes in the fungus occurring afterwards. For instance, a F129L mutation at *cytB* in homozygosity has been detected in the R strain 100% C-88F at the *sdhC* (Borba 2019), conferring resistance to both QoI and SDHI fungicides. In addition, though the SdhCI86F has been the main mechanism of resistance since the onset of selection and adaptation of resistant *Pp* populations to the SDHIs, other mutations in the *sdhC* gene leading to the substitution SdhC-N88S/D, -H154R, and -G92R have also been identified, at a low frequency, with limited knowledge of their ecological importance yet (Mello *et al*. 2021).

This current widespread distribution of resistance to SDHI fungicides in *Pp* populations from soybean fields all across Brazil is rather serious and should be addressed as another strategic failure in developing and deploying high-risk single-site agricultural fungicides properly, despite the abundant knowledge derived from the historical emergence and spread of QoI and DMI resistance in the country's soybean fields (Schmitz *et al*. 2014; Klosowski *et al*. 2016b, 2018).

Under such a scenario, the adoption of evolutionary-smart anti-resistance strategies grounded on significantly reducing the fungicide selection pressure over the pathogen´s populations is warranted to prolong the life of fungicides (Mikaberidze *et al*. 2014; Corkley *et al*. 2022, Ceresini *et al*. 2024). Perhaps distinct from other approaches focused on individual actions, these strategies, either proactive or reactive, are focused on institutional actions mostly, either from the public or private sectors involved, including public policies. Individual's actions strategies against fungicide resistance, legally included in fungicide labels since 2012 (Ministry of Agriculture, Livestock and Supply (MAPA) / Coordination of Pesticides and Related Products 2012), unfortunately have been failing, since the emergence and spread of resistance to new fungicide molecules has been a recurrent phenomenon in Brazil (Müller *et al*. 2021; Vicentini *et al*. 2022a).

The institutional strategies proposed (Ceresini *et al*. 2024) aim to extend the effectiveness of fungicides in agriculture and reduce the rate of evolution of ASR pathogen towards fungicide resistance:

a) Creation of a national network dedicated to fungicide testing to ensure consistent, large-scale, meta-analytic evaluation of fungicide efficacy and soybean yield responses under disease-favorable conditions over various times and locations. This network will monitor early negative trends indicative of resistance emergence and provide rapid, open access to the relevant data (Dalla Lana *et al*. 2018);

b) Development of a capacity-building program and network for the early detection of fungicide resistance and increased pathogen dispersal. This will involve ongoing large-scale monitoring of *Pp* populations, data analysis, interpretation, and consistent public dissemination of pertinent information (Ceresini *et al*. 2024);

c) Restricting the labeling of single-active ingredient formulations of high-risk single-site SDHI fungicides or their mixtures with other high-risk fungicides that have a history of resistance, such as QoIs or DMIs (Mikaberidze *et al*. 2014);

d) Favoring the preferential labeling of premixes that combine new single-site actives such as the SDHIs with multi-site fungicides like mancozeb (Godoy *et al*. 2016; Netto *et al*. 2020) or copper-based actives (Juliatti *et al*. 2017), due to their lower risk for the emergence of fungicide resistance. According to the MAPA database, there are currently 410 fungicide formulations approved for agricultural use in Brazil (MAPA – Ministério da Agricultura Pecuária e Abastecimento - Brazil 2023), with 172 of these being co-formulations. Of these, 26 commercial fungicide mixtures are combinations of single-site at-risk fungicides with low-risk multi-site fungicides, including double or triple co-formulations (Ceresini *et al*. 2024);

e) Ongoing reassessment of fungicides labeled for ASR control to detect any decrease in efficacy or resistance, enabling swift action from federal and state pesticide regulatory agencies (Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA) 2017);

f)Technical recommendations to discourage prophylactic, calendar-based, sequential applications of high-risk, single-site fungicides (specifically SDHIs) in environments conducive to disease. Instead, the use of premixes of new SDHIs with multi-site fungicides should be recommended (Brent and Hollomon 2007; Fraaije *et al*. 2012);

g) Advising through fungicide labels and extension services on the integration of diverse disease management strategies beyond fungicide applications. This includes adherence to mandatory off-season soybean-free cropping policies (Ministry of Agriculture, Livestock and Supply (MAPA) / Secretariat of Agricultural Defense (SDA) 2022), early sowing to avoid conditions favorable for ASR incidence (Koga *et al*. 2014; Dias *et al*. 2014), and the use of ASR-resistant cultivars, especially those with complete resistance, to eliminate the need for fungicide sprays (Hartman *et al*. 2005; Childs *et al*. 2018; Yorinori 2021c), and others.

We finally propose, as a smart anti-resistance strategy, the testing and implementation of an aerobiology-based warning system for predicting ASR epidemics and helping minimize fungicide sprays. This system implies the direct and prior detection of airborne inoculum levels, in combination with molecular detection of the pathogen and fungicide resistance alleles, and can be useful in providing more accurate predictions of the risks of severe ASR epidemics (Del Ponte *et al*. 2006; Nascimento *et al*. 2012; Danelli and Reis 2016; Minchio *et al*. 2018; Beruski *et al*. 2019) and the spread of fungicide resistance, preferentially before it occurs, as documented for other important pathosystems (Luo *et al*. 2007; Van der Heyden *et al*. 2021; Vicentini *et al*. 2022b, 2023).

For instance, if high levels of *Pp* airborne inoculum coincides with the soybean reproductive stage, considered the plant's most susceptible physiological phase (Young *et al*. 2011; Xavier *et al*. 2017), it can result in severe leaf infection and high yield losses (Nascimento *et al*. 2012). Therefore, the automated monitoring of *Pp* inoculum from air samples both within cropping fields, in the offseason, and at regional scale, can be a very crucial step for the development of an aerobiology-based forecasting model for ASR epidemics (Nascimento *et al*. 2012). The application of such an inoculum-based model could prevent major crop yield losses by ensuring that timely and appropriate disease management practices are adopted, allowing reduction of unnecessary fungicide sprays, alleviation of selection pressure for resistance, and decreasing the environmental impact (West and Kimber 2015; West *et al*. 2017).

In light of the adoption of the anti-resistance strategies proposed here, a shift towards decrease in the frequency of the substitution SdhC-I86F in *Pp* populations is plausible considering the evidence of fitness penalty associated with the resistance (Borba 2019; Claus *et al*. 2022).

2.5 CONCLUSIONS

The SdhC-I86F substitution continued to be prevalent in the 2020/21 cropping season survey.

The SdhC 86F mutant allele associated with resistance to SDHI was detected in *Pp* populations from all eight Brazilian states sampled.

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