University of São Paulo
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Does the I86F mutation of succinate dehydrogenase subunit c increase fungicide resistance and have a fitness in Asian Soybean Rust (*Phakopsora pachyrhizi*)?

Jackeline Pedriana Borba

Dissertation presented to obtain the degree of Master in Science. Area: Genetics and Plant Breeding

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To my family,

Silvana e Jackson

which I love unconditionally, you are my foundation.

With love I dedicate this work

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"E tudo quanto fizerdes, fazei-o de todo o coração, como ao Senhor, e não aos homens"
(Colossenses 3:23)

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RESUMO

A mutação I86F na subunidade c da enzima succinato desidrogenase aumenta a resistência à fungicidas e tem um custo adaptativo na Ferrugem Asiática da Soja (*Phakopsora pachyrhizi*)?

A Ferrugem Asiática da Soja (ASR) é a doença economicamente mais importante em todo o mundo, e pode causar perdas de rendimento de até 80%. O Brasil é o segundo maior produtor e o maior exportador de soja (CONAB, 2018), portanto, restrições à produção tem um impacto negativo na economia nacional. O manejo da doença depende principalmente da aplicação de fungicidas, entretanto, redução de sensibilidade para todos os fungicidas já foi reportada. Dentre os vinte ingredientes ativos que compõem os fungicidas inibidores da succinato desidrogenase (SDHI) listados no Comitê de Ação de Resistência a Fungicidas (FRAC), apenas três deles são rotulados para o controle da doença da ferrugem da soja. A disponibilidade de poucos grupos químicos para o controle da doença e o tempo para descoberta, desenvolvimento e registro de novos fungicidas tornam o manejo da resistência a fungicida crítico para a produção de soja. A estrutura genética populacional é importante para entender e prever o potencial de populações de patógenos para evoluir e se adaptar em ecossistemas agrícolas. Três isolados contrastantes para sensibilidade à SDHI – 100% I86 Selvagem (S); 50% I86/50% 86F Heterocariótico (H); 100% 86F Resistente (R) - foram obtidos a partir do isolamento de monolesões que sobreviveram a altas doses de carboxamida. O nível de resistência foi determinado por DL_{50} em ensaio de folhas destacadas. Frequências mais altas de mutações I86F foram associadas a resistência a maiores concentrações de fungicida. Benzovindiflupir e Fluxapiroxade apresentaram resistência cruzada entre si, mas os valores de DL_{50} para os isolados mutantes aumentaram menos para Fluxapiroxade do que para Benzovindiflupir. Os isolados mutantes apresentaram um desenvolvimento inicial mais rápido em comparação com o isolado sensível, com germinação e desenvolvimento de apressório mais rápidos, aumentando a chance de sucesso na infecção. Período mais curto de latênca e maior severidade foram observados para o isolado heterocariótico (H) em comparação com os isolados 86F (R) e selvagem (S). O custo adaptativo comparativo dos mutantes foi medido misturando os isolados resistentes e sensíveis e medindo a flutuação na frequência de 86F durante cinco ciclos de doença. Apesar do desenvolvimento inicial mais rápido e maior virulência, o isolado resistente (R) mostrou uma desvantagem competitiva quando foi misturado ao isolado selvagem (S) e uma redução na frequência do alelo 86F foi observada. A frequência do alelo 86F parece estável durante cinco ciclos consecutivos da doença quando cultivado sozinho em folhas de soja não tratadas com fungicida.

Palavras-chave: Ferrugem asiática da soja, SDHI fungicidas, Custo adaptativo, Manejo de resistência à fungicidas

ABSTRACT

Does the I86F mutation of succinate dehydrogenase subunit c increase fungicide resistance and have a fitness in Asian Soybean Rust (*Phakopsora pachyrhizi*)?

Asian Soybean Rust (ASR) is the most economically important soybean disease worldwide, and can cause yield losses of up to 80%. Brazil is the second largest producer and the most important soybean exporter (CONAB, 2018), therefore any constraints to soybean production have a negative impact on the national economy. ASR management relies mainly in fungicide application, but lower sensitivity for all the current mode of action fungicides has been reported. Of twenty active ingredients that comprise the SDHI fungicides, only three of these are labeled for soybean rust disease control. The limited availability of chemical groups for ASR disease control and the resourcing time to discovery, development and registration of new fungicides makes the fungicide resistance management critical for soybean crop production. Genetic structure is of importance to understand and predict the potential for pathogen populations to evolve and adapt in agricultural ecosystems. Three contrasting strains for SDHI sensitivity – 100% I86 susceptible (S); 50% I86/50% 86F resistant (R); 100% 86F resistant – were obtained by selecting survival uredinia at high carboxamide doses. The level of resistance was determined by EC_{50} in detached leaf assay. Higher I86F frequencies were associated with higher SDHI concentrations. Benzovindiflupyr and Fluxapyroxad are cross resistant between each other, but EC_{50} values for mutants strains increased less for Fluxaproxad than for Benzovindiflupyr. Mutant strains showed a faster initial development compared with the wild-type strain, with faster germination and appressorium development, increasing the chance of successful infection. Shorter latent period and higher disease severity was observed for the heterokaryotic (H) strain when compared with both 86F (R) and I86 (S) strains. Despite the faster initial development and virulence, resistant (R) strains showed a competitive disadvantageous when mixed with the wild type (S) strains. A decrease in 86F frequency was observed during five disease cycles. The frequency of 86F allele is stable in pure culture over five disease cycles in non-treated soybean leaves.

Keywords: Asian soybean rust, SDHI fungicides, Fitness cost, Fungicide resistance management

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1. INTRODUCTION

Asian Soybean Rust (ASR) is the most economically significant soybean disease worldwide and can cause yield losses of up to 80%. Brazil is the second-largest producer and the most important soybean exporter (CONAB, 2018). Therefore, any constraints to soybean production have a negative impact on the national economy. The basidiomycete *Phakopsora pachyrhizi* causes ASR, a biotrophic pathogen with a high destructive power due to its capacity to cause several infection cycles within the same season and its high reproductive rate.

ASR management relies mainly on fungicide application, however, showing reports of lower sensibility for all the current mode of action fungicides. Field resistance was first reported for Demethylation Inhibitor fungicides (DMIs) in 2014 (Schmitz et al., 2014), and Quinone Outside Inhibitor fungicides (QoI) in the following year (Klosowski et al., 2015). The first report of reduced Succinate Dehydrogenase Inhibitor fungicide (SDHI) performance appeared in the season 2015/16, increasing frequency in 2016/2017 (Simões et al., 2017). A point mutation in the succinate dehydrogenase subunit c gene sequence resulted in isoleucine (I) to phenylalanine (F) residue substitution in the position 86 of the protein sequence. The highest frequency of 86F found was around 50% (Simões et al., 2017), which suggests a fitness cost of the mutated enzyme.

Nowadays, twenty active ingredients comprise the SDHI fungicides, but only three labeled for soybean rust disease control. All current fungicides registered in the Agrofit (2019) include Fluxapyroxad, Benzovindiflupyr, or Bixafen as active ingredients. The limited availability of chemical groups for ASR disease control and the resourcing time to discovery, development, and registration of new fungicides makes the fungicide resistance management critical for soybean crop production.

Mutations leading to fungicide resistance may have deleterious pleiotropic effects, such as weakened virulence, competitiveness, survival, and reproductive success. Resistance may also be associated with metabolic costs, reducing the efficiency of important physiological and biochemical processes for the pathogen, leading to lower fitness. For optimized resistance management strategies, it is of importance to characterize the fitness costs in SDH mutated strains. Whether there is a competitive disadvantage of the mutant strain compared with the sensitive isolate, the removal of the fungicide pressure should result in the decline of resistant strains in field populations.

In order to assess the impact of SDHI fungicide resistance in *Phakopsora pachyrhizi*, resistant strains were obtained in the laboratory. A mutant with two mutated nuclei and 100%

86F (R) was generated by single lesion isolation of uredinia at high carboxamide selection pressure. The resistance level and the fit of the mutant were compared to wild type (S) and heterokaryotic (H) strains. Forecasting the behavior of these mutants is important for an effective disease management program.

2. GENERAL OBJECTIVE

The objective of this study is to obtain three strains differing about the number of mutated alleles carrying the I86F substitution in the succinate dehydrogenase subunit c enzyme sequence and evaluate if there is a difference in SDHI fungicide sensibility among them. A strain with two mutated alleles was obtained in laboratory conditions by increasing the SDHI fungicide dose exponentially and selecting the survival uredinia to understand whether there is any fitness cost associated with the Isoleucine–Phenylalanine substitution. If this is the case, the mutant would be less adapted to a free-fungicide environment. Forecasting the behavior of these mutants is essential for an effective disease management program.

2.1. Specific objectives

1. To obtain contrasting populations for SDHI sensitivity;
2. To determine if monouredinial strains with 100% 86F have a higher resistance level compared with heterokaryotic strains;
3. To determine if monouredinial strains with 100% 86F shows any fitness cost compared to wild type and heterokaryotic strains;
4. To evaluate the mutation stability;
5. To evaluate the fitness of the mutant resistant strain in competitive assays.

3. LITERATURE REVIEW

3.1. The soybean commercial importance and Asian Soybean Rust (ASR)

Soybean (*Glycine max* L.) is one of the most valuable and versatile leguminous crops. It is used as both food and feed source for its protein and oil content. Brazil is the second-largest producer and the most important soybean exporter. In the 2016/2017 crop season, 114 million tons of soybeans were produced, and about 63 million tons exported (CONAB, 2017). According to Agrostat (2017), in 2017, these exports were worth about 25.4 billion U.S dollars. Thus, any constraints to soybean production harm the national economy. Among the diseases that threaten soybean crop production, Asian Soybean Rust (ASR) is the most economically important worldwide. It affects all production areas in Brazil and is also a concern in the United States, the world's top producer. In Brazil, the environment is conducive for year-round *P. pachyrhizi* survival, increasing the risk of ASR. In the USA the environmental condition is less conducive to disease development due to low temperatures during the winter, and the beginning of the epidemic relies mainly on the long-distance dispersal of the inoculum from a source area.

3.2. *Phakopsora pachyrhizi* biology

The basidiomycete fungi *P. pachyrhizi* Syd causes ASR. This pathogen is an obligate parasite, and has a wide host range, infecting over 95 species of plants (Slaminko et al., 2008).

The complete life cycle of *P. pachyrhizi* is still unknown (Goellner et al., 2010). It produces two known types of spores: urediniospores and teliospores. Urediniospores (n+n, haploid, and dikaryotic) are asexual spores and the predominant form, responsible for multiple cycles of infection within a growing season. Teliospores (2n, diploid, and monokaryotic) are the sexual phase and do not infect plants. Germination of teliospores was only observed in laboratory conditions (Saksirirat & Hoppe, 1991; Jorge et al., 2015).

Urediniospores are globose to ovate or ellipsoid, hyaline to yellowish-brown, and about 18–38 μm long by 13–29 μm wide (Vitall et al., 2011). They are formed on short stalks within a uredium about 7–10 days after inoculation and released from uredia through an ostiole. Urediniospores germinate on the host under humid conditions, forming infective hyphae, which grow over the host surface and triggers the appressoria formation. The appressorium is globose, non-ornamented, and has approximately the same size as a urediniospore (Figure 1 A-C). It penetrates leaves through the cuticle rather than stomatal opening, different from most rust

fungus (Goellner et al., 2010). The appressoria form a funnel-shaped structure to penetrate the host tissue, termed the appressorial cone (Koch & Hoppe, 1988).

The resulted infectious hyphae develop within the intercellular space, producing branches called haustoria. The haustoria penetrate the host cell wall and form a tight connection with the host cell without destroying its plasma membrane, supporting sustained nutrient absorption from the living plant.

During the sporulation stage, the plant cuticle ruptures, releasing urediniospores into the environment and wind-spread (Tremblay et al., 2013). Urediniospore production can extend up to three weeks from the generation. (Tremblay et al., 2013). Figure 1-D shows the complete life cycle of *P. pachyrhizi*.

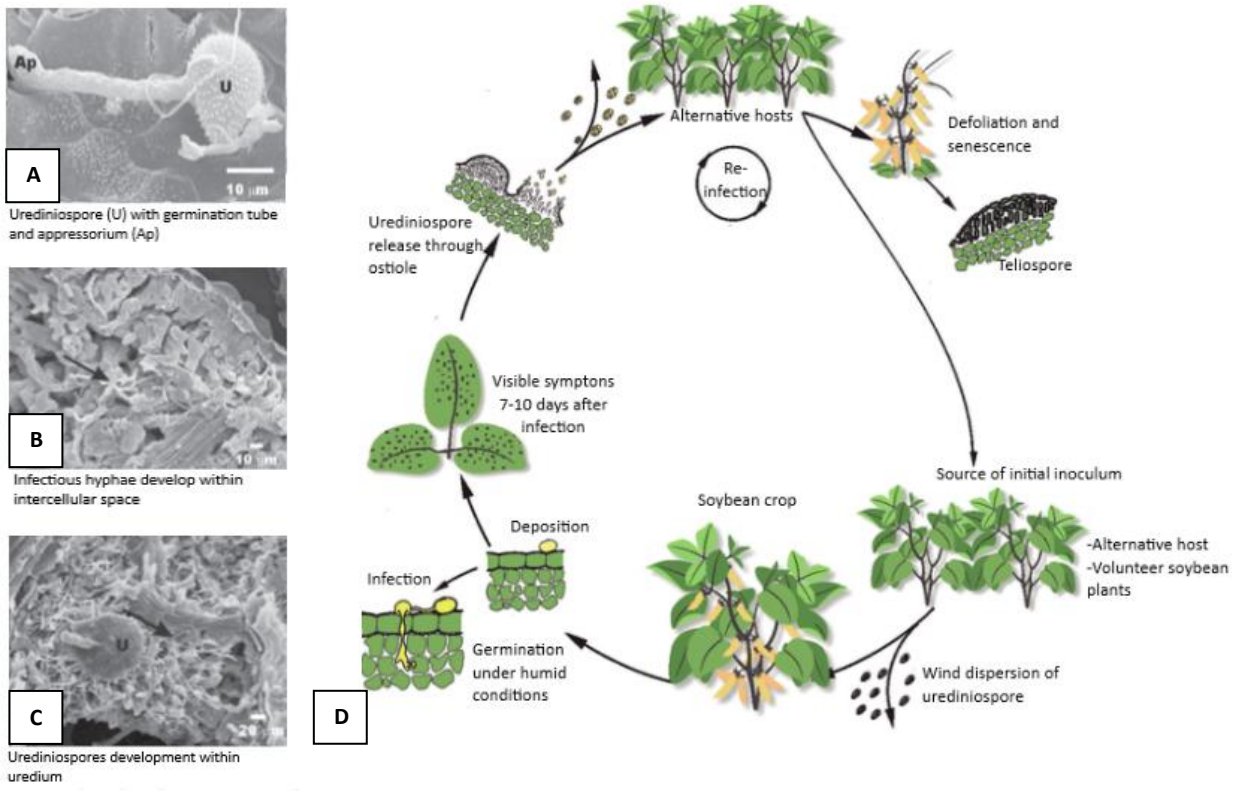

Figure 1. Asian Soybean Rust disease cycle. A) Germinated urediniospores. B) Infectious hyphae development within intercellular space. C) Urediniospores development within uredium. D) ASR complete life cycle. Adapted from: Reis et al. (2006). Soybean diseases I: Asian Soybean Rust. Universidade de Passo Fundo, Passo Fundo; and Magnani et al. (2007). Events of *Phakopsora pachyrhizi* pre-penetration, penetration and colonization processes in soybean leaflets.

3.3. Disease epidemiology

The infection begins with the arrival of airborne inoculum from a source area. Urediniospores are wind spread to long distances, and the infection is dependent on

environmental conditions. *P. pachyrhizi* can survive year-around in alternative hosts and volunteer soybean, increasing the ASR risk in the next soybean crop season. According to Ono et al. (1992), *P. pachyrhizi* can sporulate on 31 species in 17 genera of leguminous plants. Temperatures between 16°C and 28°C are optimum, but the infection can occur in lower temperatures (between 8 and 28°C). A minimum period of 6 hours of wetness is fundamental for urediniospores germination and infection (Rupe & Sconyers, 2008).

Usually, rust lesions are not seen until flowering, because the inoculum level is insufficient early in the season. Disease symptoms first appear on the adaxial side of the leaf as small brownish to dark brown lesions, typically visible seven days after infection. The first lesions often appear toward the base of the leaflet near the petiole and leaf veins. This part of the leaflet retains dew longer favoring the infection (Rupe & Sconyers, 2008).

ASR is a polycyclic disease, with a high potential of damage. A massive number of urediniospores are produced and wind-dispersed within a single growing season. ASR leads to defoliation and early senescence resulting in up to 80% yield losses (Hartman et al., 2015).

3.4. Genetic variability of *Phakopsora pachyrhizi*

Understanding the genetic structure of a pathogen population is essential to predict evolution and adaptation.

P. pachyrhizi is only known to reproduce asexually through urediniospores, which suggests low genetic diversity. However, Twizeyimana et al. (2011), studying 116 isolates in four different agroecological zones in Nigeria, showed high levels of genetic diversity in field populations. The diversity might be explained by gene flow, as spores disperse promptly by wind over long distances.

Comparing the soybean rust pathogenicity of 59 populations from Brazil, Argentina, and Paraguay, Akamatsu et al. (2013), showed a different virulence pattern among and within countries. The evaluation was done using 16 soybean lines containing different resistant genes (also called differentials) by measuring the lesion appearance, sporulation level, and the number of uredinia per lesion. They also observed differences between three crop seasons, indicating that virulece varies temporally and geographically.

P. pachyrhizi urediniospores are dikaryotic, containing a pair of nuclei. Upon germination, the nuclei travel through the germ tube into the developing appressorium and undergo a single mitotic division leading to four nuclei, which are present in a fully developed appressorium (Koch & Hoppe, 1988). During germ tube formation, hyphal of different

individuals grow towards each other and fuse. The nuclei from each strain migrate within the fused germ tube network to form a heterokaryon (Vittal et al., 2011). Heterokaryosis occasionally follows nuclear fusion, recombination, and reassortment of chromosomes, and it is a necessary mechanism for increasing genotypic diversity.

While uncommon throughout most of the life kingdoms, heterokaryosis is a hallmark of Fungi. It is an integral part of the parasexual cycle used to introduce and maintain genetic variation in populations. In Basidiomycota, the heterokaryotic phase is the dominant growth phase and required to complete its life cycle.

The heterokaryosis allows an individual nucleus to interact with another genetically different nucleus without undergoing karyogamy (James et al., 2008). They behave as diploids concerning dominance and recessivity of traits and trans-acting gene regulation (James et al., 2008). Each mutant allele can be partially/completely dominant or recessive to the wild-type allele, and when mutant and wild-type alleles of the same gene combine in the same fungal cell or hyphae, the phenotype may be fungicide resistant or fungicide sensitive (Angelini et al., 2015).

Heterokaryons are expected to have increased adaptive potential relative to homokaryons due to the genetic variation between the two nuclei (Clark & Anderson, 2004). Heterokaryons harboring both fungicide-resistance and fungicide-sensitive nuclei may be able to grow in the presence or absence of fungicides (Angelini et al., 2015).

According to Simões et al. (2017), strains of *P. pachyrhizi* with a lower sensitivity to SDHI fungicides selected from uredinia of SDHI treated leaves resulted in around 50% of I86F mutants, which suggests that selected strains contain one nucleus with I86 and one with 86F. The heterokaryotic state would be ideal for an optimal survival with and without SDHI selection pressure.

3.5. Disease control

Control of ASR relies mainly on fungicides. Per season, at least three fungicide applications are needed, and according to Godoy et al. (2016), the costs are estimated at more than US$ 2 billion per year. Thus, it is essential to monitor the efficacy of fungicides used in disease management programs, and to adopt practices recommended by the Fungicide Resistance Action Committee (FRAC), such as limit the number of applications of the same mode of action fungicide, the number of sequential applications, and time of application with respect to disease development.

Cultural practices known to reduce disease development are also strongly recommended by the FRAC to control ASR, such as planting early ripening varieties, monitoring the fields, introducing soybean-free growth periods (60–90 days) in the threatened areas, eradicate alternative and alternate host, and crop rotation. Wherever feasible, several strategies should be used together. Integrated Pest Management (IPM) is the best way to prolong the effectiveness of fungicides due to resistance problems and to limit crop losses.

By planting early, the crop will have more time to develop before pathogen inoculum is active or present. As in Brazil, the ambient conditions are conducive for year-round survival of the pathogen, the Ministério da Agricultura, Pecuária e Abastecimento, implemented, in 2007, the National Program for Asian Soybean Rust Control. According to this program, all states should evaluate the need to implement or not the soybean-free period with a minimum of 60 days in the off-season. It is expected that this free-host period will break the disease cycle, reduce inoculum, and delay the beginning of the epidemic in the regular crop season.

Another essential practice is the sowing calendar, which restricts the soybean sowing date. The objective is to reduce inoculum and consequently reduce the number of fungicide spray during the season. It reduces the fungicide selection pressure and avoids the increase in the frequency of resistant isolates to DMI, QoI, and SDHI fungicides in field populations.

Fungicide resistance in fungi spreads very quickly due to the rate of pathogen development. The number of disease cycles per season, the total of spores released, and the time to complete a cycle are determinants for *P. pachyrhizi* reproductive success and ASR resistance management. The existence of alternative hosts as an over seasoning refuge and a local source for initial inoculum for the next susceptible crop is significant for disease epidemiology and disease management. Pathogens that undergo regular, severe reductions in population size, e.g. as a result of annual climatic extremes that kill the majority of individuals, crop rotations or a free-host period are less diverse as a result of genetic drift and slower to adapt than populations that maintain a high population size year-round.

Sensitivity monitoring programs are carried out since 2005, four years after the pathogen introduction in Brazil, in 2001. The objective of the program is to evaluate the sensitivity shift of field samples across soybean production areas for the prime fungicides mode of action. The collected data is present at annual meetings with the FRAC, and is a guideline for resistance management strategies to prolong the effectiveness of fungicides and to limit crop losses.

3.6. ASR chemical control

Fungicides have been used for over 200 years to protect plants against fungi diseases. The use of synthetic fertilizer and pesticides has become an integral part of modern agriculture to maximize yield and achieve the production of high-quality crops. About 50 years ago, site-specific fungicides, mostly with systemic activity were introduced in order to improve chemical protection. However, only a few years later, loss of fungicide efficacy was observed in several plant pathogens in the field, mainly due to mutations leading to resistance.

Sterol Demethylation inhibitors (DMIs) fungicides inhibit the C14 demethylation step within fungal sterol biosynthesis. All chemical groups of this fungicide class are cross-resistant with each other. Schimtz et al. (2013) identified a sensitivity loss towards DMIs in 2009/2010 *P. pachyrhizi* strains collected in Brazil. It was associated with mutations at codons 120, 131, 142, 145, and 475 of cyp 51 gene (Schimtz et al., 2013). Besides mutations within the cyp 51 gene, other mechanisms causing sensitivity changes to DMIs have been reported, such as cyp51-overexpression. Studying the fitness of DMIs sensitive isolates, Klosowski et al. (2015) showed that the frequency of isolates with cyp 51 mutations decreased in the mixtures with the wild-type isolate, but was stable in isolates cultured alone on detached soybean leaves over four disease cycles.

Quinone outside inhibitor (QoI - "strobilurins") fungicides inhibit electron transfer at the Qo site in mitochondrial complex III, preventing the transfer of electrons and consequently ATP production, leading to an energy deficiency in fungal cells. This class of fungicide introduced in 1996 is widely used for many plant pathogens. Within two years after its introduction, loss of sensitivity was observed against powdery mildew in Europe, followed by resistance in a wide range of target pathogens, including ascomycetes, basidiomycetes, and oomycetes classes (Brent and Hollomon, 2007).

The resistance level among pathogens varies significantly. According to the FRAC, there are three point mutations at cytochrome b gene responsible for QoI resistance resulting in changes from glycine to alanine at position 143 (G143A), change from phenylalanine to leucine at position 129 (F129L), and change from glycine to arginine at position 137 (G137R) of the encoded protein. The G143A single mutation in cytochrome bc-1 protein is the most common in QoI-resistant pathogens and confers a high resistance level than the F129L and G137R mutations (Brent and Hollomon, 2007). However, in *P. pachyrhizi* and other rust pathogens (*Puccinia* spp., *Uromyces appendiculatus*, *Hemileia vastatrix*), the amino acid substitution of glycine to alanine at position 143 has not been observed despite significant use of QoI. There is an intron

just after the triplet that encodes for glycine, and the substitution for alanine strong affects the splicing process, leading to a deficient cytochrome b.

Schimtz et al. (2013) found no loss of sensitivity against QoI in isolates from different regions of Brazil in the 2009/2010 crop season (Table 1). Three seasons later, the F129L mutation was identified at high frequency in field isolates from Mato Grosso e Paraná (Klosowski et al., 2015). According to Klosowski et al. (2015) a heteroplasmy of the cyt b gene has not been found in *P. pachyrhizi*, which means that no or all cyt b genes in a monouredinial isolate are mutated. Studying the competitive fitness of isolates with lower QoI sensitivity compared to wild-type isolates, Klosowski et al. (2015) found out that isolates with the F129L mutation in the cyt b protein competed equally well with a QoI-sensitive, wild-type cyt b isolate.

SDHIs fungicides bind strongly to the ubiquinone-binding (Qp) site; upon binding, they physically block access to the substrate, which consequently prevents further cycling of succinate oxidation. In addition to its function as a dehydrogenase (oxidation of succinate to fumarate) in the respiratory system in complex II, the enzyme plays an important role in the tricarboxylic acid cycle (TCA).

Table 1. Fungicide resistance status and its corresponding amino acid substitution

Fungicide group	Year of mutation detection	Amino acid substitution	Target Genes
QoI	2015	F129L	cyt b
DMI	2010	F120L + Y131H Y131F + K142R Y131 + I145T	cyp 51
SDHI	2015	C-I86F	sdh c

The first type of SDHI fungicides (Carboxin) introduced in agriculture in earlies 1960 was limited to a few pathogens, mostly rusts and *Rhizoctonia* in seed treatments (Avenot et al., 2010; Sierotzki & Scalliet, 2013). Newly SDHI fungicide active ingredients are characterized by a broader spectrum of fungal activity on various crops due to its mode of action and specificity. The specificity improved disease control but is generally associated with the selection of resistance in field populations. The FRAC lists the SDHI fungicides as a medium to high risk of selecting resistance in fungal pathogen populations based primarily on single point mutations in the gene encoding the target succinate dehydrogenase enzyme.

They comprise ten chemical groups, including phenyl-benzamides and pyridinyl-ethylbenzamides as well as furan-, oxathiin-, thiazole-, pyrazole- and pyridine-carboxamides. Now, twenty active ingredients comprise the SDHI fungicides, but only three of these are labeled for soybean rust disease control (Table 2).

Thirteen SDHI fungicides currently registered in the Agrofit database (2019) include either Fluxapiroxade, Benzovindiflupyr, or Bixafen as active ingredients (Table 3). The limited availability of chemical groups for ASR disease control and the resourcing time to discovery, development, and registration of new fungicides makes the fungicide resistance management critical for soybean crop production.

Table 2. Chemical group and respective common name of SDHI fungicides (FRAC, 2019).

Code	Target site of action	Group name	Chemical group	Common name
7	Complex II	SDHI (**s**uccinate **de**h**y**drogenase **i**nhibitor)	Phenyl-benzamides	Benodanil Flutolanil Mepronil
			phenyl-oxo-ethyl thiophene amide	Isofetamid
			Pyridinyl-ethyl-benzamide	Fluopyram
			Furan-carboxamides	Fenfuram
			Oxathiin-carboxamides	Carboxin Oxycarboxin
			Thiazole-carboxamides	Thifluzamide
			Pyrazole-carboxamides	Benzovindiflupyr Bixafen Fluxapyroxad
				Furametpyr Isopyrazam Penflufen Penthiopyrad Sedaxane
			Pyridine-carboxamides	Boscalid
			N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides	pydiflumetofen
			Pyrazine-carboxamides	pyraziflumid

Table 3. Fungicides and its chemical group recommended for ASR disease control (Agrofit, 2019)

Common Name	Chemical Name	Chemical Group	Registration Holder
Ativum	epoxiconazole (triazol) + fluxapyroxad (carboxamide)+ pyraclostrobin (strobilurin)	Fluxapyroxad	Basf S.A.
Cuantiva	fluxapyroxad (carboxamide) + piraclostrobina (strobilurin)	Fluxapyroxad	Basf S.A.
Denaxo	epoxiconazole (triazol) + fluxapyroxad (carboxamide) + pyraclostrobin (strobilurin)	Fluxapyroxad	Basf S.A.
Desali	azoxystrobin (strobilurin) + benzovindiflupyr (pirazol carboxamide)	Benzovindiflupyr	Syngenta Proteção de Cultivos Ltda.
Elatus	azoxystrobin (strobilurin) + benzovindiflupyr (pirazol carboxamida)	Benzovindiflupyr	Syngenta Proteção de Cultivos Ltda.
Elatus Trio	azoxystrobina (strobilurin) + azoxystrobina (strobilurin) + benzovindiflupyr (pirazol carboxamide) + benzovindiflupyr (pirazol carboxamide) + difenoconazole (triazol) + difenoconazole (triazol)	Benzovindiflupyr	Syngenta Proteção de Cultivos Ltda.
Fox XPRO	Bixafem (carboxamide) + Protioconazole (Triazolinthione) + trifloxystrobin (strobilurin)	Bixafem	Bayer S.A.
Orkestra SC	fluxapyroxad (carboxamide) + pyraclostrobin (strobilurin)	Fluxapyroxad	Basf S.A.
Sesitra	epoxiconazole (triazol) + fluxapyroxad (carboxamide) + pyraclostrobin (strobilurin)	Fluxapyroxad	Basf S.A.
Tivaro	epoxiconazol (triazol) + fluxapyroxad (carboxamide) + piraclostrobina (strobilurin)	Fluxapyroxad	Basf S.A.
Veldara	fluxapyroxad (carboxamide) + pyraclostrobin (strobilurin)	Fluxapyroxad	Basf S.A.
Vessarya	benzovindiflupyr (pirazol carboxamide) + picoxystrobin (strobilurin)	Benzovindiflupyr	Du Pont do Brasil S.A.

3.7. Complex II (succinate:Q oxidoredutase)

Complex II differs from the other major complexes of the mitochondrial respiratory chain in several important ways. First, all proteins of complex II are nuclear encoded, whereas mtDNA encodes a number of the protein components of complexes I, III, IV and ATP synthase. Secondly, complex II is a direct enzymatic component of the TCA cycle.

Complex II is the smallest complex in the respiratory chain and transfers the electrons derived from succinate directly to the ubiquinone pool (Avenot et al., 2010). The mitochondrial SDH complex is composed of a membrane-peripheral domain and a membrane-anchor domain. The peripheral domain forms the soluble part of the complex and possesses the succinate dehydrogenase activity. It consists of two hydrophilic subunits SDHA and SDHB. SDHA is a flavoprotein (Fp) whose covalent FAD co-factor of the enzyme is part of the catalytic site, whereas SDHB is an iron-sulfur protein (Ip) containing three different iron-sulfur clusters [2Fee2S], [4Fee4S] and [3Fee4S] for electron transfer between the FAD and the membrane quinone (Avenot et al., 2010).

3.8. Carboxamide resistance - I86F substitution at succinate dehydrogenase subunit c (sdh c) gene

P. pachyrhizi strains with lower SDHI sensitivity have been found and reported for the first time in the season 2015-2016. Survival uredinia after SDHI treatments were found where previous populations were completely inhibited and its resistance mechanisms analyzed (Simões et al., 2017). Simões et al. (2017), investigated samples of past seasons (2008-2009, 2009-2010 and 2014-2015), for the most important production areas in Brazil, and found no detectable I86F mutation by the species-specific real-time PCR methodology developed in the study.

The insensitivity to SDHI fungicides is caused by a point mutation in the gene encoding succinate dehydrogenase subunit c. A nucleotide substitution at codon 86 results in an isoleucine to phenylalanine amino acid substitution. No relevant mutation was detected in the sdh b and sdh d genes, which could be assigned to SDHI-resistant isolates (Simões et al., 2017).

DNA alignment of the sdh genes among different fungi species showed that it is not a conserved position, and some species such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* with codons coding for alanine or glycine had been detected in the past (Simões et al., 2017). Thus far, no mechanism apart from mutations in sdh genes has been reported to be responsible for resistance in field strains (Sierotzki & Scalliet, 2013).

In order to forecast potential resistance problems, a 100% 86F isolate was obtained in the laboratory for this study by selecting survival uredinia of population exposed to high carboxamide doses. Importantly, it has not been found such high frequency field population within the resistance monitoring programs (data not shown).

4. METHODOLOGY

All experiments were conducted at FMC Innovation Center, located in Paulínia-SP, Brazil.

4.1. Obtaining the soybean leaves

Soybean cultivar BMX Potência was grown in NP6 (0,090 L) pots, in a mixture 1:1 of soil and rice husk substrate, and cultivated in the greenhouse. Irrigation was manually done twice a day. Unifoliate leaves with 14-20 days were used for pathogen propagation and detached leaf assays.

4.2. Obtaining the populations of study

The selection of spontaneous mutants was performed under high fungicide selection pressure. An initial population with about 50% of mutant cells was submitted to high doses of SDHI fungicide (Elatus® 300 g/kg Azoxystrobin + 150 g/kg Benzovindiflupyr), and the survival uredinia at 45 ppm were selected and multiplied *in planta* in growth chambers. The growth chambers were adjusted to 90% humidity, 12h of 40uM light, and 12h darkness at 22°C.

The frequency of the isolates was measured by Next Generation Sequencing (NGS) at FMC Stine Research Center and a population with 100% 86F frequency was selected and multiplied *in planta* within growth chambers for further studies. To maintain the frequency stable, a maintenance dose of Elatus® was applied to unifoliate soybean leaves with 14 days using a Generation III research sprayer.

As field samples contain a pool of individuals of *P. pachyrhizi* with an unknown level of genetic relatedness, single uredinium were isolated, and the resulted individuals treated as a strain. To handle all the single-urediniospore strains free of cross-contamination, they were kept in Petri dishes in detached soybean leaves.

To obtain the monouredinial strains, a suspension of 10^2 spores was prepared from each one of the three initial populations (about 50% 86F, 100% 86F and wild type - I86), and applied by airbrush to unifoliate soybean leaves placed in water agar (0,01%) Petri dishes. After 7 days, the resulting uredinia were cut before spores released and transferred to a small Petri dish (Figure 2). Ten to fourteen uredinium from each population were saved and regarded as a strain.

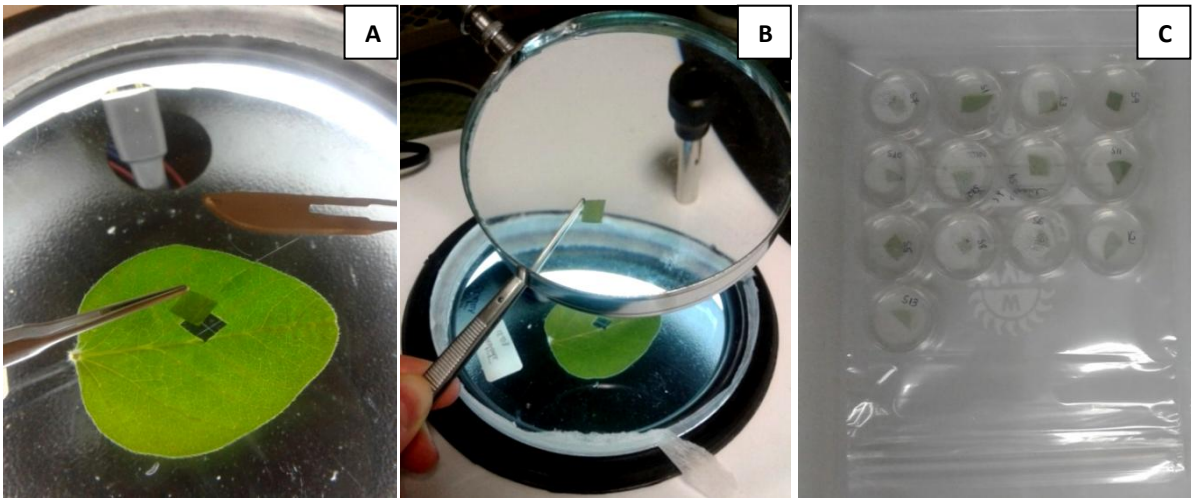

Figure 2. Obtaining the population of the study. A-B) A single uredinium was cut with a blaster, before urediniospore release. C) To avoid cross-contamination, each uredinium was kept in a small petri-dish and incubated within a growth chamber until spore release at 22°C, with a cycle of 12h of 40uM light.

After 9 days, sporulating uredinium were transferred to a microtube with 0.3 mL of 0.01% tween 20 in water, which were then agitated thoroughly with a vortex. The suspension was propagated to new leaves as small droplets (Figure 3). After inoculation, all plates were incubated overnight in the dark and then further incubated for 18 days with a cycle of 12h of 40uM light and 12h darkness at 22°C. The mutant strains were propagated in leaves treated with a maintenance dose of Elatus®, in order to avoid a shift in mutation frequency.

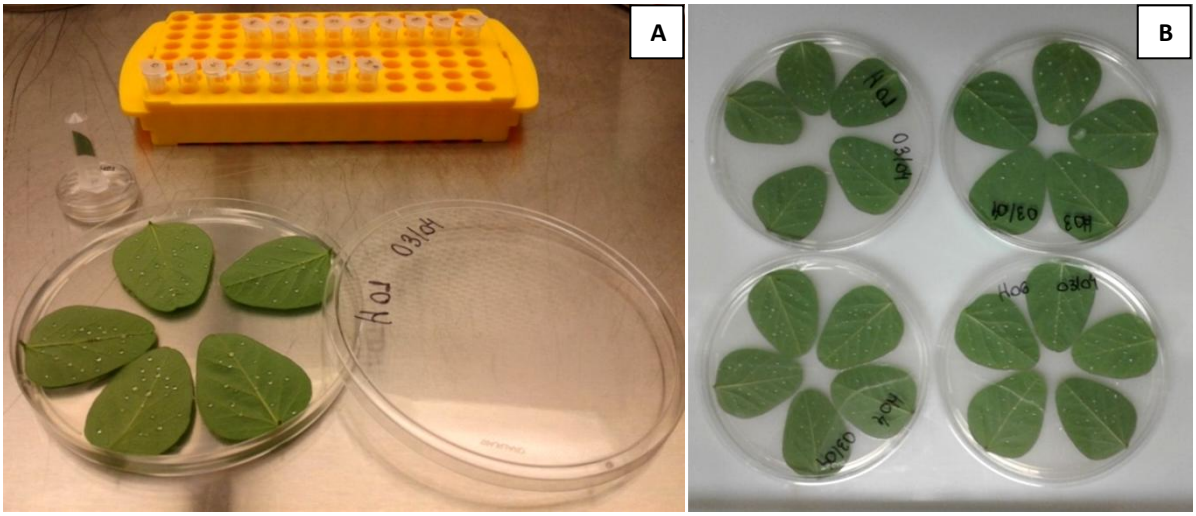

Figure 3. Obtaining the population of study. A) Sporulating uredinia were transferred to a microtube with 0.3 mL of 0.01% tween 20 in water and agitated thoroughly with a vortex. B) The suspension was propagated to new leaves as small droplets using a pipette.

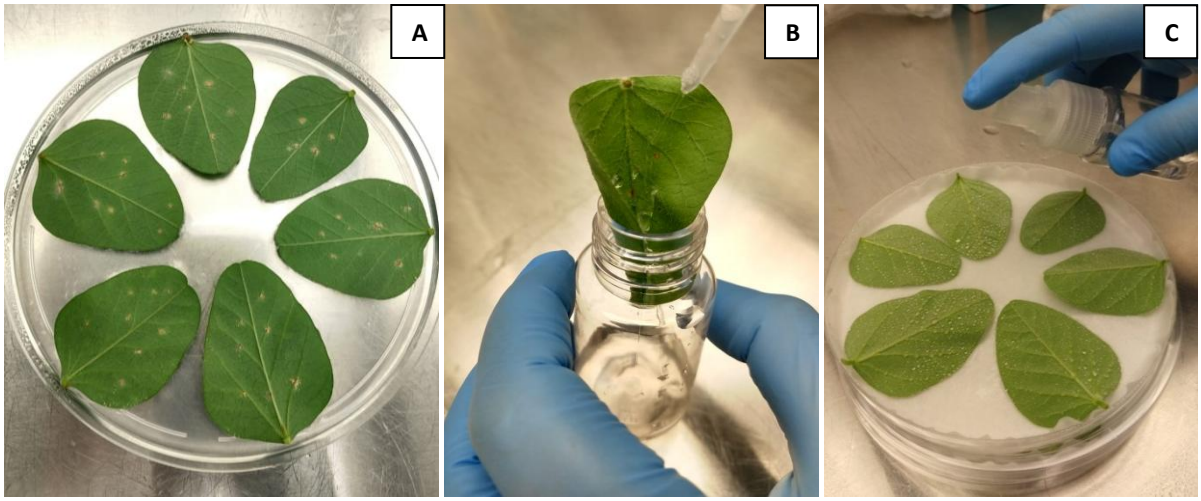

Figure 4. Propagating the populations of study. A) After 17 days incubation period, the uredinia were releasing urediniospores. B) The urediniospores suspension was done in 0,01% tween 20 water (v/v). C) The urediniospore suspension was propagated in new soybean leaves kept within Petri dishes using a manual airbrush. Mutant strains were propagated in leaves treated with a maintenance dose of Elatus®.

After 18 days, all the strains were propagated to new leaves in order to obtain enough spores for DNA extraction (Figure 4). As it is not possible to obtain *in vitro* cultures of *P. pachyrhizi,* each fourteen to twenty days all the strains were inoculated into new soybean leaves following the same procedure described above.

4.3. DNA Extraction, PCR, and sequencing

About 2 mg of spores were collected from each strain using a vacuum pump (Figure 5). DNA was extracted using the DNeasy Plant Mini Kit® (Qiagen), following the manufacturer's instructions. PCR reactions were performed using Q5® High-Fidelity DNA Polymerase. The sdh c gene sequence was amplified with the primers FW 5224 and RV 5223 (Table 4). Thermocycling conditions were initial heating at 98°C for 15s, 35 cycles at 98°C for 10s, 56°C for 15s and 72° C for 45s, followed by a final amplification step at 72°C for 1 min.

Table 4. Primers sequence and predicted amplicon size for sdh c gene amplification.

	Primers sequence		Amplicon size (bp)
5224	CGAAGGCTACATACACTTC	Foward	381
5223	GCGTCTCAGAATCCATAG	Reverse	

Figure 5. Spore collection for DNA extraction using a vacuum pump. About 2 mg of spores of each strain were collected in a 2mL vial tube and frozen at -80C until DNA extraction.

PCR products of all strains of each three populations were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega®). Sequencing was performed at CEBTEC-ESALQ using Sanger Technology. The chromatograms were analyzed with the software Chromas®.

4.3.1. Minor Variant Finder (MVF) Software for Sanger analysis

The single nucleotide polymorphisms (SNPs) were analyzed using the Minor Variant Finder (Thermofisher®), which allowed the calling of minor variants at a detection level as low as 5% (Schreiber et al., 2016). Using the same approach qualitative data was transformed in quantitative information for further analysis.

4.4. Dose-response curve

SDHI sensitivity was tested against two carboxamides - Benzovindiflupyr and Fluxapyroxad - in detached leaf assays. A dilution series ranging from 0,001, 0,003 to 30 and 100 ppm were prepared for each fungicide (Table 5). From each concentration, 6 leaves were treated. Two weeks after inoculation, infected leaf area (severity %) was evaluated by visual scoring, using a diagrammatic scale (Figure 6). The EC (Effective Concentration) 50 and 95% were calculated based on disease control.

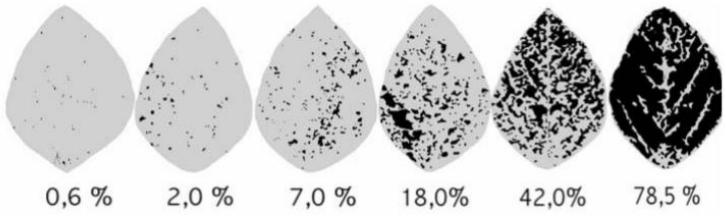

Figure 6. Diagrammatic scale for assessment of soybean rust severity (percentage of diseased leaf area). The representation of the symptoms includes tissues that became necrotic because of uredinia, chlorotic halos, and coalesced lesions. Source: Godoy et al., (2006).

Table 5. Dose response curve for sensitivity test. Fluxapyroxad and Benzovindiflupyr doses to determine EC_{50} and EC_{95} of the three strains.

Fungicide	Doses (ppm)										
Benzovindiflupyr	0,001	0,003	0,01	0,03	0,1	0,3	1	3	10	30	100
Fluxapyroxad	0,001	0,003	0,01	0,03	0,1	0,3	1	3	10	30	100

4.5. Detached leaf assay

A susceptible soybean BMX Potência variety was used. Detached leaf assay was conducted as recommended by FRAC. Unifoliate leaves of similar size were cut at the moment of the assay. For each treatment, 6 leaves were dipped into fungicide amended with water (1:1 w/w) (single formulated product) for four seconds. After the leaves dried, they were top side down on agar-water (0,01%) within a Petri dish (Ø140mm). On the next day, spore suspension of *P. pachyrhizi* isolates in 0.01% Tween 20 water (v/v) were applied to the abaxial surface of leaves with an Arprex airbrush, using 2PSI pressure. The spores were counted using a haemocytometer, and the concentration adjusted to 1.0×10^5 spores/mL (Figure 7). Subsequently, the plates were incubated in a humid chamber in the dark for 24h followed by 12h of light at 22°C. The assay followed a completely randomized design and the statistical model was built as:

$$y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where y_{ij} represent the observed response of the j^{th} replicat of i^{th} treatment, μ is a overal mean, T_i is the treatment effect and ε_{ij} is the random error term.

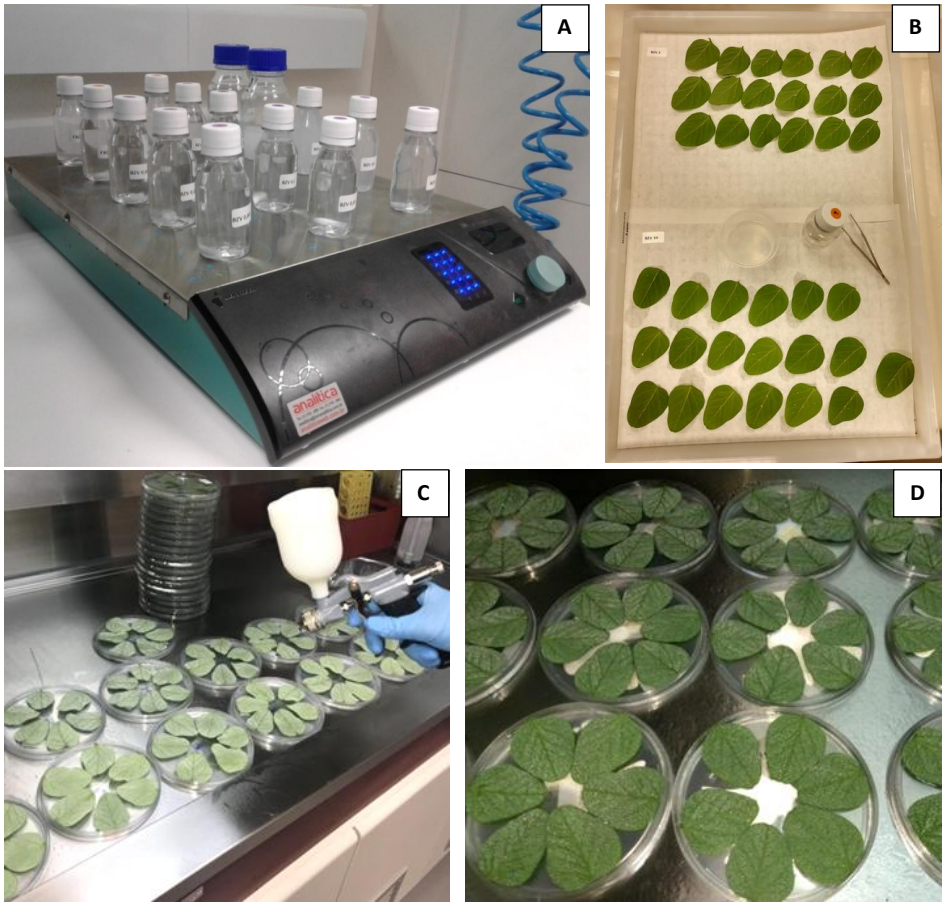

Figure 7. Detached leaf assay. A) Serial dilution of Benzovindiflupyr and Fluxapyroxad fungicides, ranging from 0,001 to 100 ppm. B) The leaves were dip into fungicide amended water treatments for four seconds, and top side down in agar water (0,01%) Petri dishes after dried. C) The inoculation was done with airbrush Arprex® using 2 PSI pressure. D) Detail of inoculation, size and distribution of droplets.

4.6. Biology characterization of the three strains

Three biological parameters to compare the biology among the strains were measured to understand the biology of resistant strains compared to the original sensitive population: time required for germination and appressorium development, latent period *in planta*, and virulence/severity *in planta*. Statistical analyses were performed using R version 3.5.1.

4.6.1. Germination and appressorium development

The time for germination and appressorium development was measured during an 8 hours experiment. A urediniospore suspension with 0,01% Tween/water was prepared for the three different strains, with three replicates each. 100 µL of suspension was added to each well of a 96 wells Elisa microplate covered with an aluminum foil. At every hour the percentage of germinated urediniospores and appressorium development out of one hundred urediniospores

were measured using a manual counter (Figure 8). The assay followed a completely randomized design.

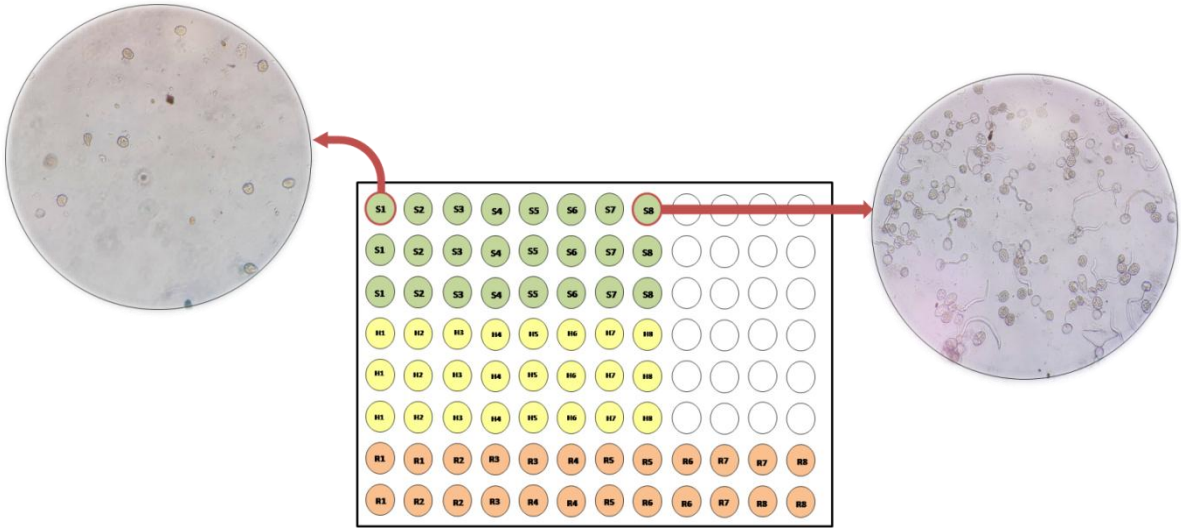

Figure 8. Biological parameters. Germination and appressorium development time-course. Urediniospore from wild-type (S), Heterokaryon (H) and Resistant (R) strains were incubated at 22°C in the dark and examined every hour during 8 hours.

4.6.2. Latent period *in planta*

The latent period, which is the time between infection and the onset of sporulation, were evaluated *in planta*. Twelve plants cultivated in NP6 pots were used for each strain. The spores were counted using a haemocytometer, and the concentration adjusted to 1.0×10^5 viable spores/mL. After inoculation, the plants were kept within a dew chamber for 24h at 22°C. After, the soybean plants were incubated within growth chambers, maintained with 12h of artificial light and 12h darkness for 14 days (Figure 9). The latent period were assessed in three different temperatures (20°C, 25°C and 30°C) in order to evaluate if the mutant strains have any penalty when incubated at suboptimal conditions. The experiment followed a factorial 3x2 design (3 strains by 2 temperatures) evaluated in a randomized complete block design (RBC) with three blocks (the tray was treated as block). The statistical model was built as:

$$y_{ij} = \mu + T_i + \beta_j + \varepsilon_{ij}$$

Where y_{ij} represent the observed response of the j^{th} replicat of i^{th} treatment, μ is a overal mean, T_i is the treatment effect, β_j are block effects and ε_{ij} is the random error term. The treatment sums of square were partitioned into three components: $SS_{Trt} = SS_S + SS_T + SS_{SXT}$

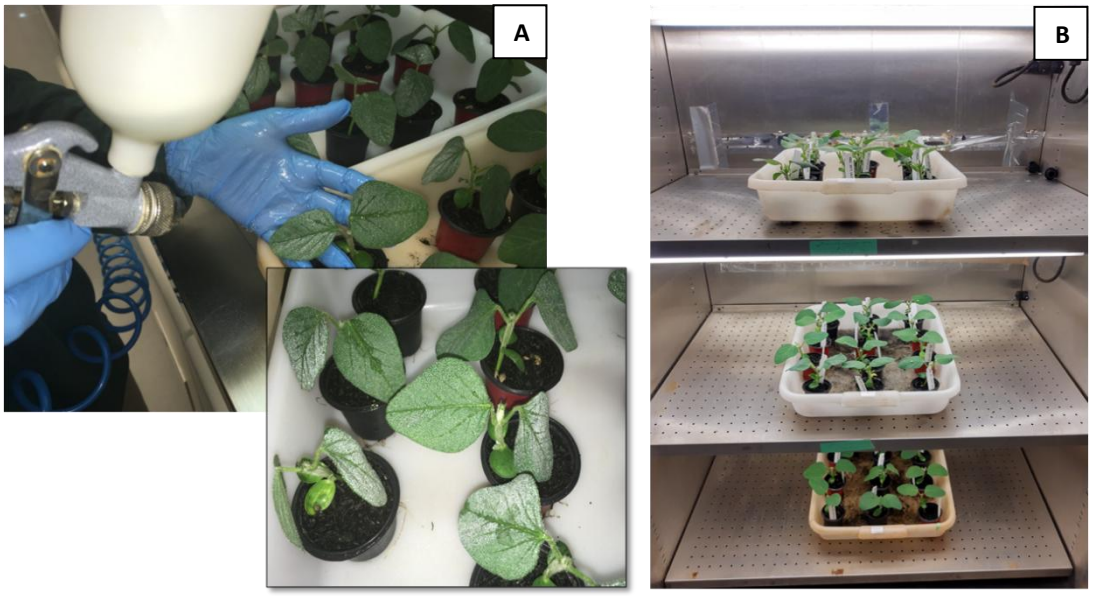

Figure 9. A) Inoculation of soybean plants was done with airbrush Arprex® using 2PSI pressure. After inoculation, the plants were kept within a dew chamber for 24h. B) Plants were incubated within growth chambers in three different temperatures (20°C, 25°C and 30°C) during 14 days.

4.6.3. Virulence/severity *in planta*

Disease severity after 8, 10 and 14 days was measured based on visual inspection using the diagrammatic scale (Godoy et al., 2006). Twelve plants cultivated in NP6 pots were assessed for each strain. The spores were counted using a haemocytometer, and the concentration adjusted to 1.0×10^5 viable spores/mL. After inoculation, the plants were kept within a dew chamber for 24h at 22°C. After, the soybean plants were incubated within growth chambers, maintained with 12h of artificial light and 12h darkness for 14 days. The virulence was determined in three different temperatures (20°C, 25°C and 30°C). The experiment was conducted the same way was mentioned for the Latent Period assay.

4.7. Competition assay

Resistant and wild type strains were mixed in equal proportions (50% I86: 50% 86F) and cultivated for five consecutive generations on untreated soybean leaves placed in Petri dishes. The results of three independent counts in a hemocytometer was used to adjust urediniospores concentration. As the viability of the strains was not equal, the germination percentage was measured to correct the initial proportion between the resistant and wild type strains. The initial frequency was determined in generation one, and the changes in frequency in the following generations were determined by RT-PCR using a 7500 Fast Real-Time PCR (Applied Biosystems)

and Sanger technology (Figure 10). The assay followed a completely randomized design, with four biological replicates. For each generation, ten Petri dishes with six leaves each were done for each replicate. Statistical analyses were performed using R version 3.5.1.

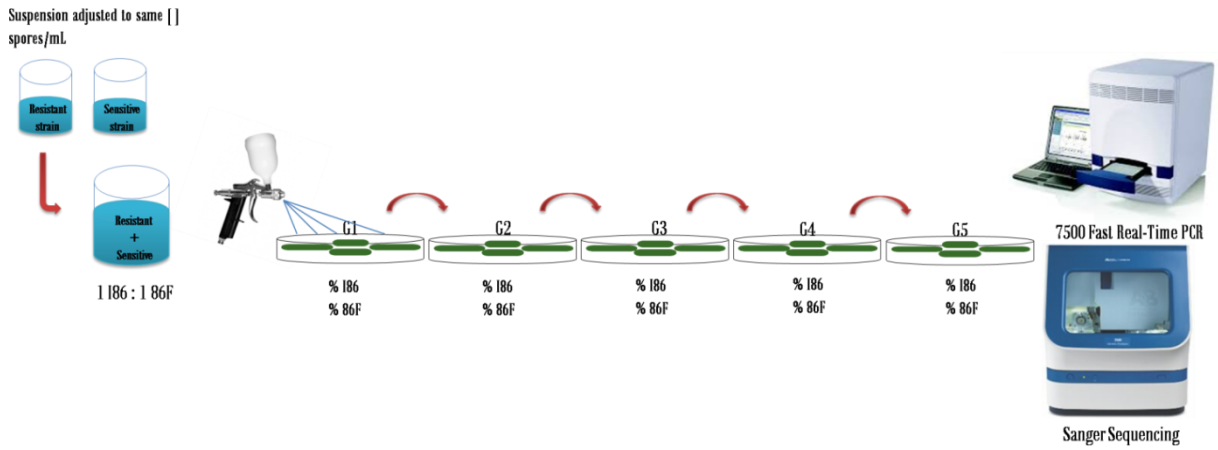

Figure 10. Competition assay between sensitive (100% I86- S) and mutated isolates (100% 86F- R) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves. Equal proportion of resistant (R) and sensitive (S) strains were mixed and propagated in non-treated leaves. At each generation, a suspension was prepared and inoculated in new soybean leaves. The frequency of mutant allele was measured at the end of five generations using a 7500 Fast Real-Time PCR (Applied Biosystems) and Sanger techonology systems.

4.8. Stability of mutation

The I86F substitution stability of the resistant isolates was measured by propagating the urediniospores for five successive generations with no selection pressure. At each generation, spores were collected for DNA extraction and 86F frequency quantification by RT-PCR using a 7500 Fast Real-Time PCR (Applied Biosystems) and Sanger technology. Three biological replicates composed the experiment. The assay followed a completely randomized design, with three biological replicates. For each generation, ten Petri dishes with six leaves each were done for each replicate. Statistical analyses were performed using R version 3.5.1

4.9. Quantitative genetic analysis of I86F using allele specific qPCR

An allele-specific real-time PCR methodology was developed for the quantitative detection of the I86F substitution. The frequency of the mutant allele was calculated using a standard curve experiment. A sample with 100% of 86F was serially diluted to create a standard curve, and the frequency of mutation of the experimental samples determined based on the standard curve. A primer was designed specifically for the mutant allele (Table 6), and

the specificity of the reaction was measured using a second standard curve. The second standard curve was built using a mix of mutant and wild type alleles, in a rate corresponding with the first serial dilution. The capacity to discriminate between wild type and mutant allele, and the non-specific amplification, was verified by comparing both standards curves (Figure 11). The primers and probe used for amplification were adapted from Simões et al. (2017) and are listed at Table 6. Taqman Universal PCR Master Mix was used for amplification, and the reactions were performed on 7500 Fast Real-Time PCR System (Applied Biosystems). The following conditions were applied: 2 min at 50°C, 10 min at 95°C and 40 cycles at 95°C for 15 s and 60 °C for 1 min.

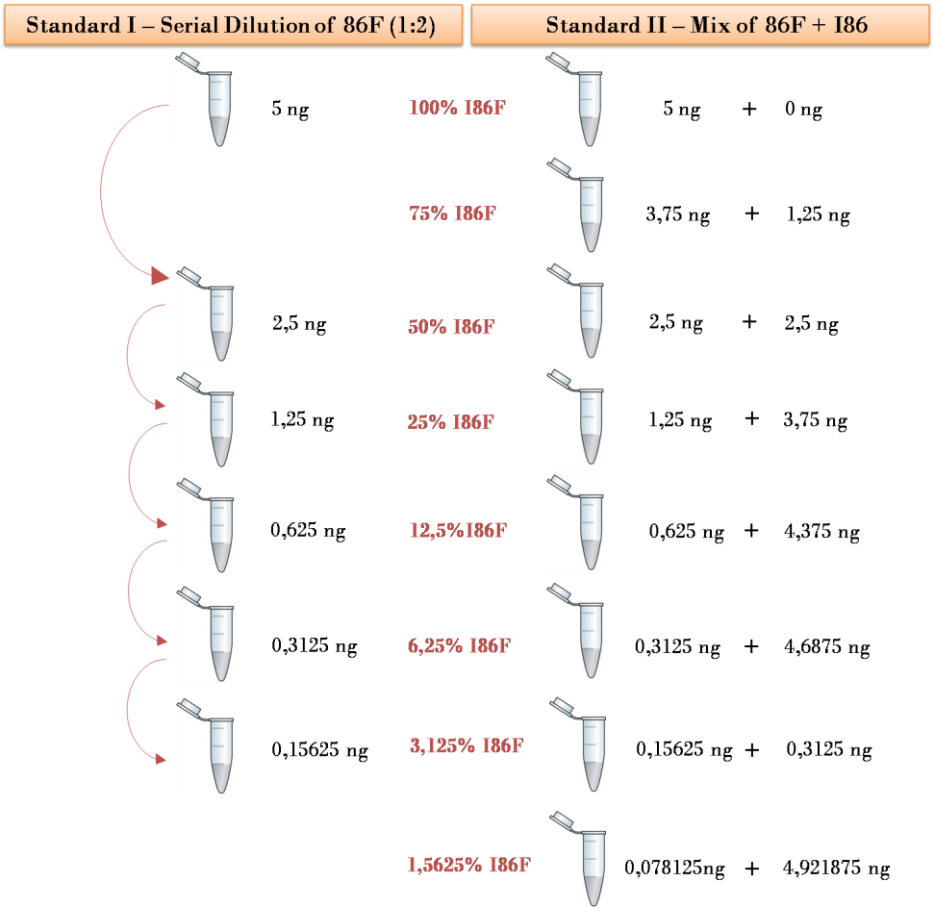

Figure 11. Allele discrimination using an allele specific qPCR. A sample with 100% of 86F was serial diluted to create a first standard curve. To assure the specificity of the primer to discriminate between the mutant and wild type allele in the pool of gDNA, a second standard curve was prepared. The second standard curve was built using a mix of mutant and wild type alleles, in a rate that corresponds with the first serial dilution. The reaction efficiency was measured by comparing both standards curves CTs.

Table 6. Primers and probe sequence for quantitative analysis of 86F frequency using quantitative PCR. In red the base corresponding to the 86F allele. The reverse primer is a common primer.

	Primers sequence	Amplicon size (bp)
Foward	AACAGCTAACTTGGTATTCCAGC**T**	
Reverse	AAACCACCGTAGATCACTGATGT	103
Probe	FAM-AACGGGTTGCGCTCTTACAGGAGGTATTC-BHQ1	

4.9.1. DNA yield quantification

Total gDNA was quantified using a fluorometric quantitation with dsDNA binding dye reagents (Quantus™ Fluorometer, Promega) according to the manufacturer's instructions. The gDNA concentration of all samples was adjusted to 2,5 ng/μL.

5. RESULTS

5.1. Single PCR product of sdh c gene

PCR products amplified with the primer set FW 5224 and RV 5223 for sdh c gene showed a clean single band in all samples with the expected PCR product size of 384bp (Figure 12).

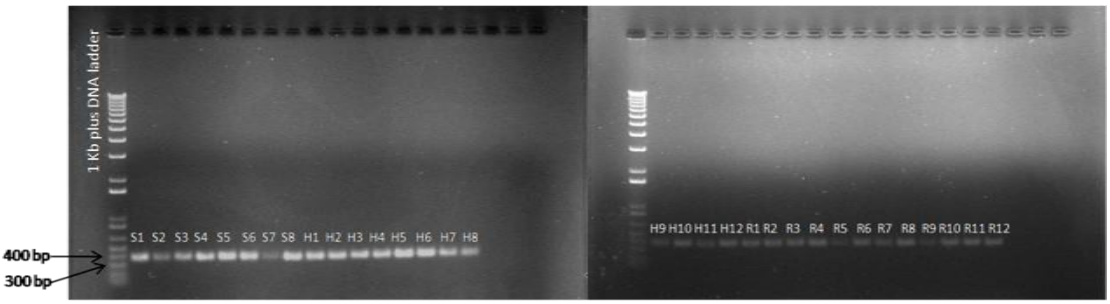

Figure 12. Electrophoresis gel of PCR products from *P. pachyrhizi* strains. PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega®), with a recovered yield of 30-40ng/µL after purification for a 25µL PCR reaction. S-Sensitive strains, H-Heterokaryon strains, R-Resistant strains. Agarose concentration on gel: 0,8%. TAE buffer 1X. Running time 40 min in 90V.

5.2. Selection of strains of each population in the analysis

Three strains of each population (S-Sensitive; H-Heterokaryon, R-Resistant) were selected based on the analysis of Sanger sequencing chromatograms. Strains with double-peaks at the SNP position were excluded for S and R strains. It was observed evenly-spaced peaks in the DNA sequences, with minimal noise (Figure 13). Sensitive (S) and 100% 86F strains (R) showed a single peak, while the heterozygous strains (H) showed a double peak. The base caller program designated "W" at base position 86 for the H strains, which means that one allele is T, while the other is an A (Figure 13).

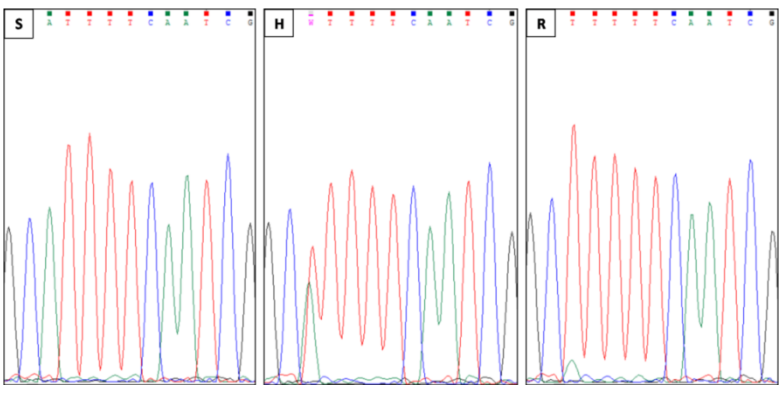

Figure 13. Sanger sequencing chromatograms of isolates with different SDHI sensitivity. S-Sensitive strain. H-Heterokaryon strain. R-Resistant strain. S and R are homozygous to I86 and 86F, respectively.

5.3. The different strains have different sensibility to SDHI fungicides

Detached leaf tests with the three strains indicated that higher 86F frequencies were associated with higher SDHI fungicide concentrations, with the wild type strain effectively controlled at lower doses compared with the heterokaryon and resistant strains. The differences in sensitivity among the three isolates were demonstrated by the Effective Concentration that kills 50% and 95% of the population, EC_{50} and EC_{95}, respectively (Table 6). The EC value was calculated based on the pathogen control using the JMP® Software and logistic regression model (Wang et al., 2017). Both carboxamides were phytotoxic (Benzovindiflupyr and Fluxapyroxad) at the highest dose of 100ppm (Figure 14).

Figure 14. Phytotoxicity symptoms at highest dose for the Benzovindiflupyr and Fluxapyroxad, as brown scorch lesions.

The EC_{50} values (ppm) for the sensitive population (S) is lower compared with the mutant strains (H and R) for Benzovindiflupyr and Fluxapyroxad. The Fluxapyroxad EC values showed differences among the three strains lower than that observed for Benzovindiflupyr. There are also differences between the mutant strains (H and R), with higher EC values for resistant strain (R) (Table 7). The homozygous 86F strain (R9) showed a phenotype with the highest resistance for both SDHI fungicides tested (Table 7). Benzovindiflupyr required 30X more ppm and Fluxapyroxad 5,3X more to kill half of the population when compared with the heterokaryotic strain. However, the maximum frequency of I86F reported in field population so far is around 50%.

Benzovindiflupyr was more effective than Fluxapyroxad for the sensitive strain, while the second was better against the mutant strains (Figure 15). An inversion in Benzovindiflupyr and Fluxapyroxad curves indicated a higher percentage of control at lower doses for Fluxapyroxad, in both H and R strains, compared with Benzovindiflupyr. Therefore, higher doses of Benzovindiflupyr were needed to achieve the same pathogen control. The performance of each molecule is essential in order to manage critical areas where the mutant frequency within a population is high. The disease severity assessment of dose-response curves for both SDHI fungicides is shown in Figures 16, 17 and 18, which represent S, H and R, respectively.

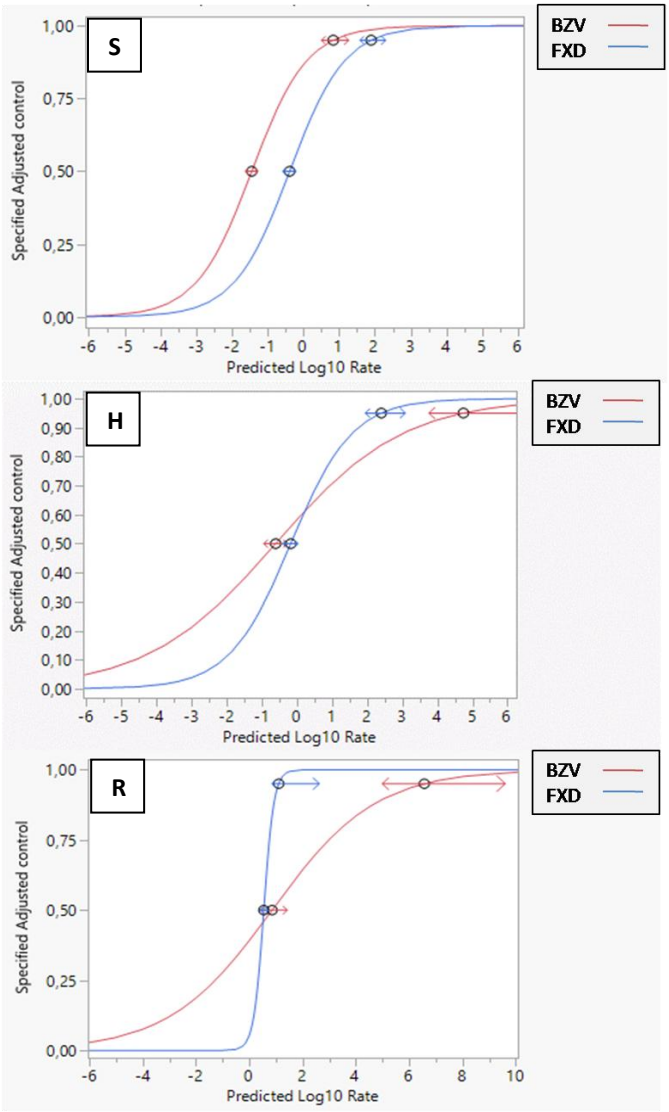

Figure 15. Sensitive (S), Heterokaryon (H) and Resistant strain (R) dose-response curves for Benzovindiflupyr (in red) and Fluxapyroxad (in blue) fungicides.

Table 7. Predicted EC 50% and 95% for the S-Sensitive, H-Heterokaryon and R-Resistant strains for the two carboxamides tested - Benzovindiflupyr and Fluxapyroxad.

Strain	Fungicide	EC	Predicted	Lower 95%	Upper 95%
S8	BENZO	**50**	**0,036**	0,024	0,054
		95	**6,821**	3,198	18,519
	FLUXA	**50**	**0,413**	0,263	0,626
		95	**78,283**	38,272	199,861
H10	BENZO	**50**	**0,248**	0,113	0,531
		95	**55380,821**	5743,980	1787521,164
	FLUXA	**50**	**0,670**	0,394	1,083
		95	**253,393**	88,332	1226,625
R9	BENZO	**50**	**7,205**	2,643	26,838
		95	**3722614,299**	97850,838	4067184144,500
	FLUXA	**50**	**3,531**	2,528	5,319
		95	**12,837**	7,116	430,700

The Resistance Factor (RF) was determined as the ratio of EC_{50}mutant/EC_{50}WT. The RF for the mutant 86F (R) strain is higher compared to the Heterokaryon strain, but the increase is more considerable for Benzovindiflupyr fungicide (Table 8). This difference suggests that the mutation affects the binding of the Benzovindiflupyr molecule more effectively than that of the Fluxapyroxad.

Table 8. Resistance Factor (RF) based on EC_{50} assessment for the 86F mutant strains. The Resistance Factor (RF) was determined as the ratio of EC_{50}mutant/EC_{50}WT

Strain	Resistance Factor (RF)	
	Benzo	Fluxa
S	1	1
H	6,9	1,6
R	200,1	8,5

5.4. Cross-resistance to SDHI fungicides

Cross-resistance occurs when resistance arises to one fungicide that also results in resistance to another fungicide. Usually, fungicides acting on the same target are considered cross-resistant to each other. The results showed a high correlation between the two carboxamides (R2=0,998) on the control of the three strains, with loss of sensitivity conferred by 86F mutation for both fungicides (Figure 19).

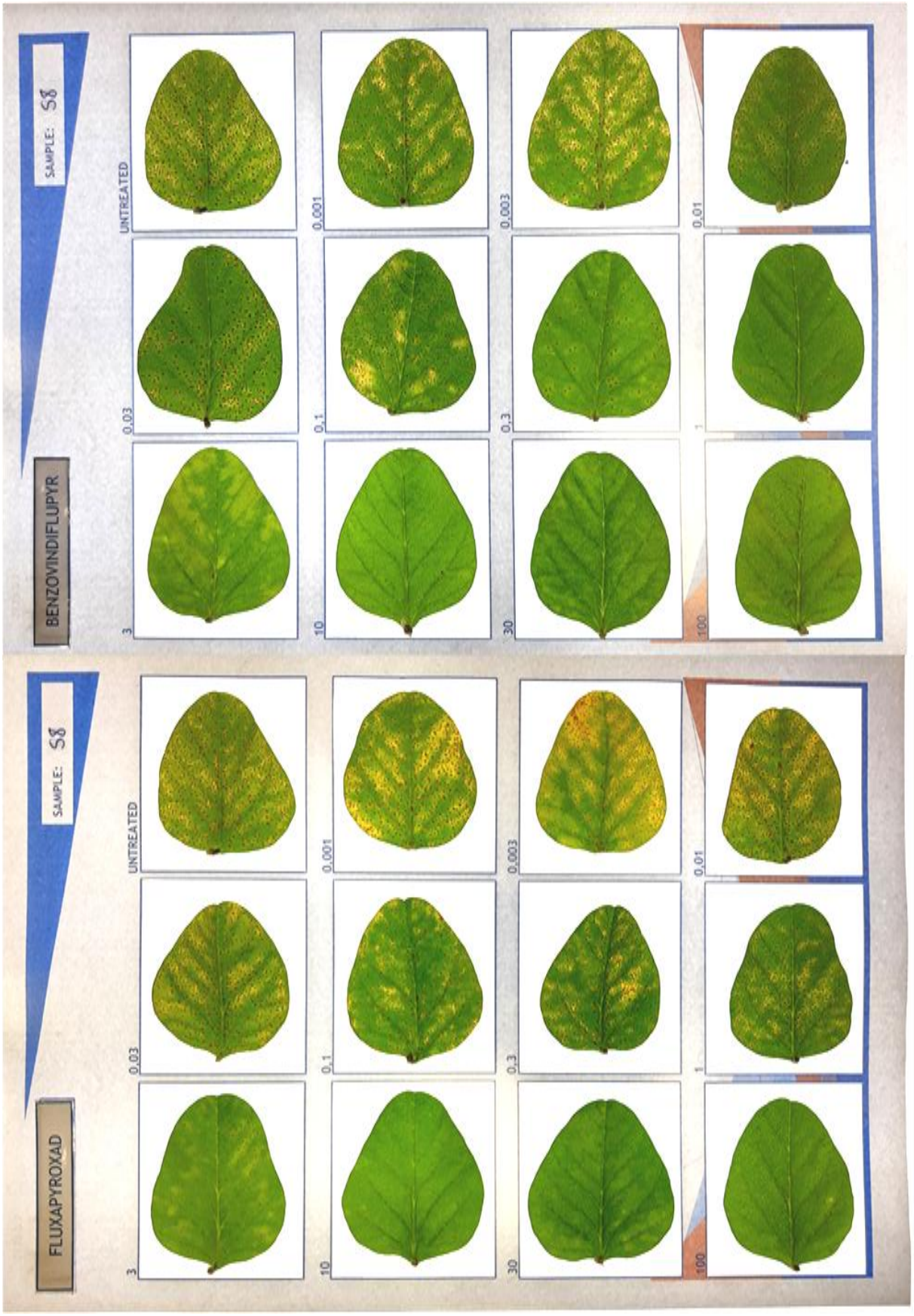

Figure 16. Benzovindiflupyr and Fluxapyroxad dose-response curve for the SDHI fungicide sensitive isolate (S). Doses ranging from 0,001 to 100 ppm of single formulated product.

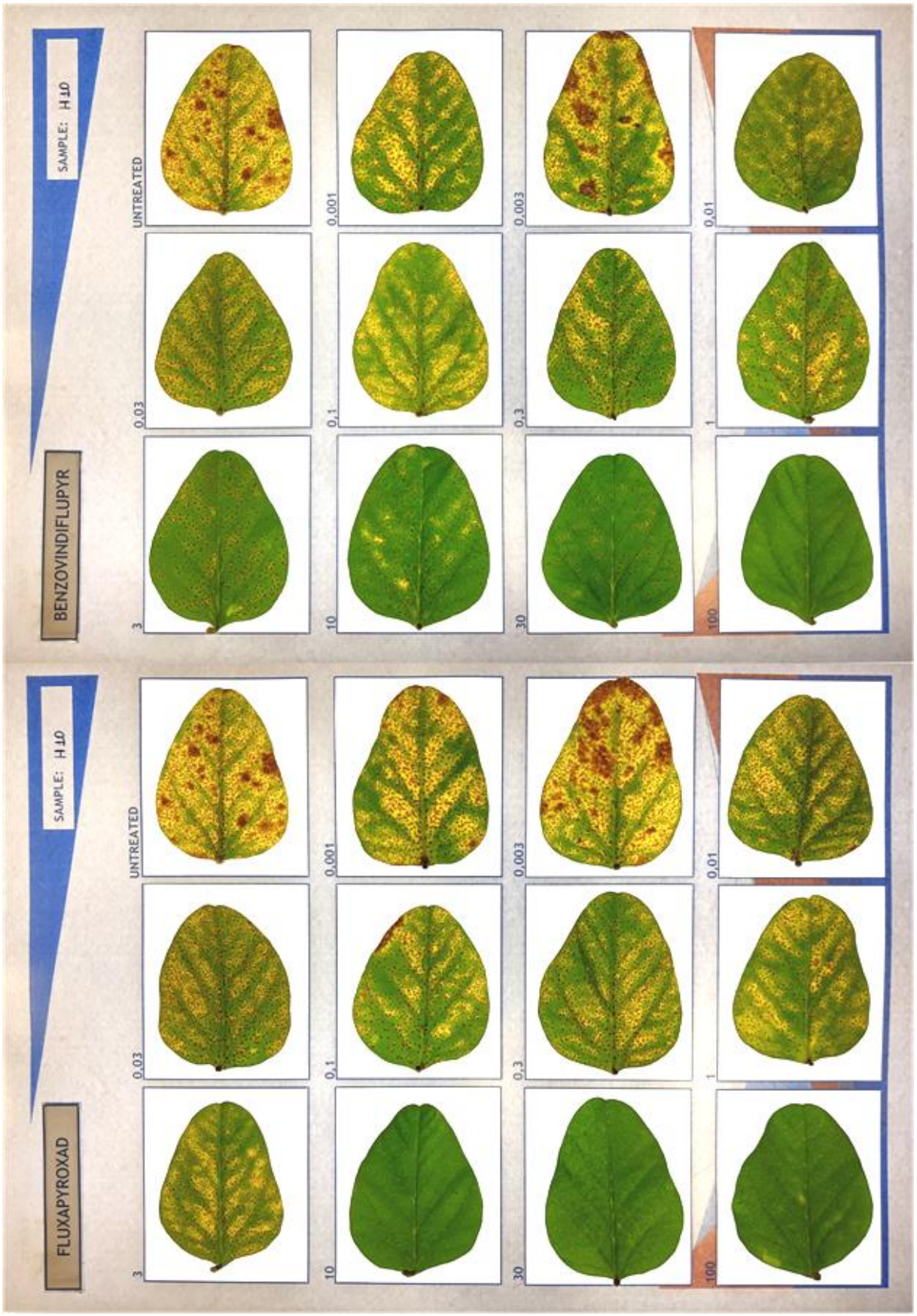

Figure 17. Benzovindiflupyr and Fluxapyroxad dose-response curve for the heterokaryon strain (H). Doses ranging from 0,001 to 100 ppm of single formulated product.

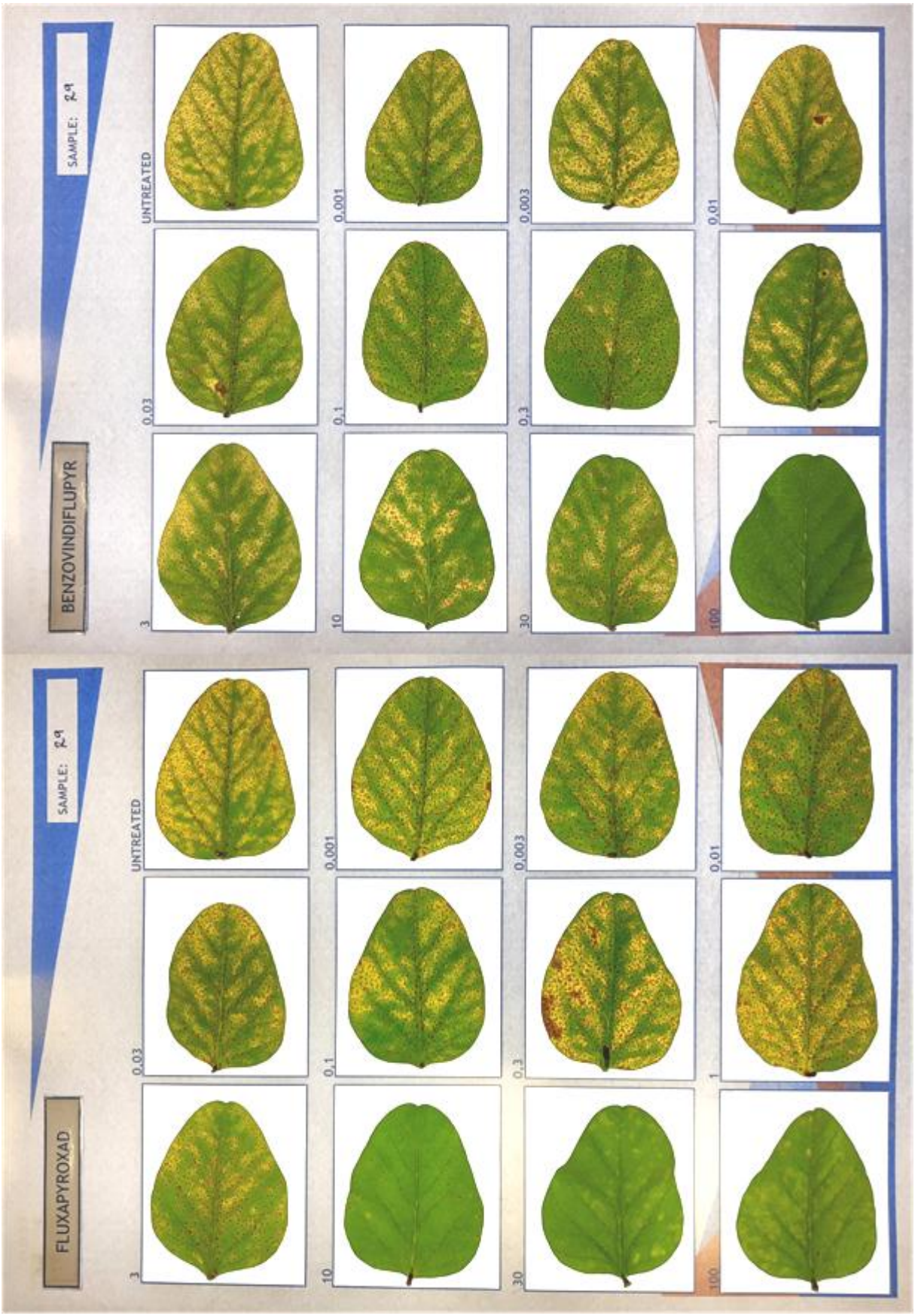

Figure 18. Benzovindiflupyr and Fluxapyroxad dose-response curve for the resistant strain (R). Doses ranging from 0,001 to 100 ppm of single formulated product.

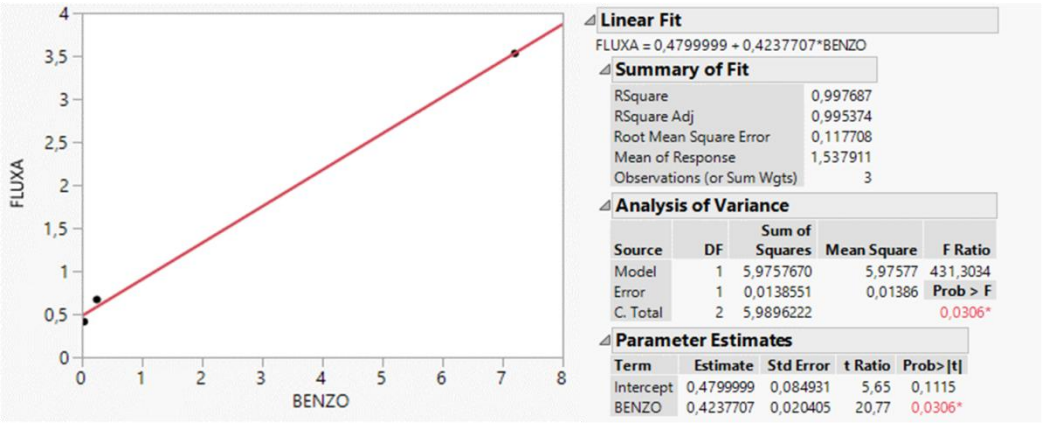

Figure 19. Bivariate fit analysis of correlation of the EC_{50} values for Benzovindiflupyr and Fluxapyroxad for the three strains of study. A Correlation coefficient of 0,998 was observed (R^2=0,998).

5.5. Biological characterization shows differences among the three populations

5.5.1. Appressorium development

The infection process among the strains when quantified by urediniospores germination and appressorium development was different. The Heterokaryotic and Resistance strains showed a higher percentage (statistically significant) of appressorium development when compared with the Sensitive strain (Figure 20 and Table 9).

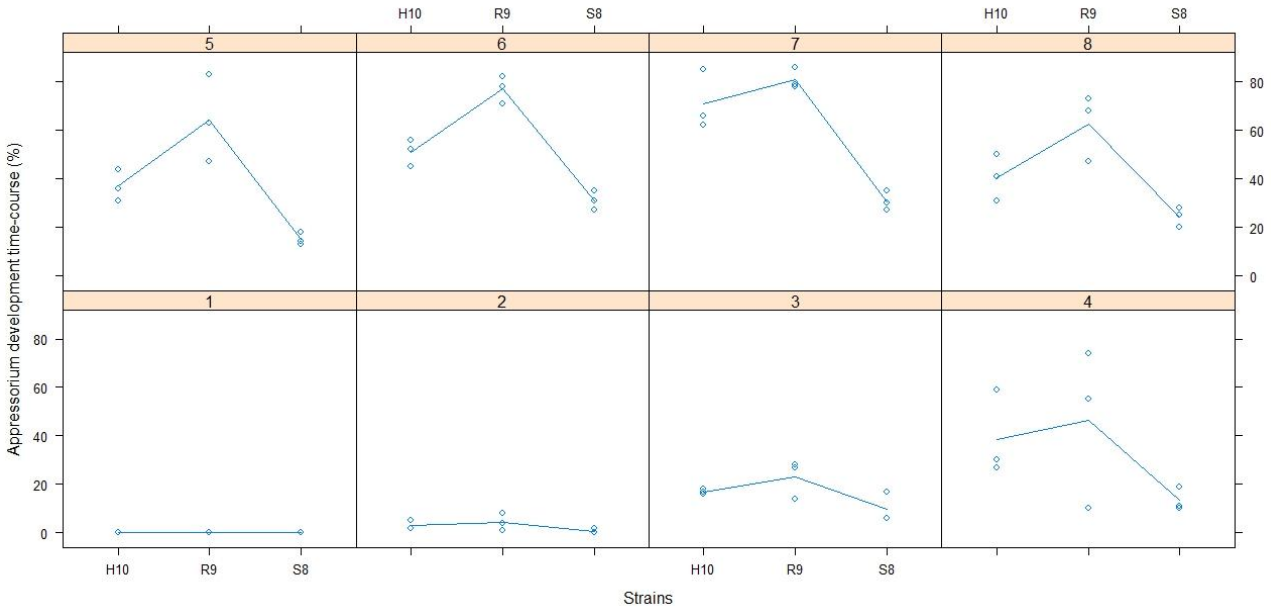

Figure 20. Appressorium development time-course for S-Sensitive, H-Heterokaryon and R-Resistant strains of *P. pachyrhizi* assessed during 1-8 hours.

Table 9. Analysis of Variance Table for appressorium development time-course of three SDHI fungicide sensitive strains.

Analysis of Variance Table					
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (> F)**
Strain	2	10336.2	5168.1	31.965	2.885e-10 ***
h.a.i	7	31922	4560.3	28.205	< 2.2e-16 ***
Residuals	62	10024	161.7	93.714	

Differences in multiple comparisons between S-H, S-R, and H-R also were observed (Table 10 and 11). The homozygous to 86F strain (R) showed a faster appressorium development compared with the Heterokaryotic (H) strain (Table 11).

Table 10. Contrasting the strains by EMMs comparison.

contrast	**estimate**	**SE**	**df**	**t.ratio**	**p.value***
H10-R9	-12.54167	3.67062	62	-3.417	0.0032
H10-S8	16.70833	3.67062	62	4.552	0.0001
R9-S8	2.925.000	3.67062	62	7.969	<.0001

*Pairwise comparisons with Tukey adjustment. Significance level : 0.05.

Table 11. Difference in appressorium development time course among S-sensitive, H-Heterokaryotic and R-resistant strains during 8 hours evaluation time. Means with the same letter do not differ by Tukey test considering α=5%

Strain	**emmean**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group***
S8	15.58333	2.595521	62	9.21405	21.95262	**a**
H10	32.29167	2.595521	62	25.92238	38.66095	**b**
R9	44.83333	2.595521	62	38.46405	51.20262	**c**

* P value adjustment: tukey method. significance level used: alpha = 0.05.
Means with the same letter do not differ by Tukey test considering α=5%

5.5.1. Latent period *in planta*

The latent period of the three strains, which is the time between infection and the onset of sporulation, was evaluated *in planta*. Plants were inspected every day for the onset of symptoms and spores releasing (Figure 21).

Figure 21. Relative duration of latent period *in planta*. The production of spores was followed every day until all the uredinia were releasing spores. The red arrows show uredinia closed, at the onset of spore release; blue arrows show uredinia releasing spores.

In general, the initiation of spores release occurred nine days after inoculation (d.a.i.), and by the 12th day, all treatments were releasing spores. The incubation at 25°C resulted in a shorter cycle with all strains releasing spores ten d.a.i. (Table 12).

Table 12. Number of plants with uredinia releasing urediniospores from 8 to 12 days after inoculation *in planta* in two incubation temperatures (20°C and 25°C) within growth chamber. S-sensitive strains (homozygous to I86), H-heterokayotic strain, R-resistant strain (homozygous to 86F).

T °C	Strain	d.a.i				
		8	9	10	11	12
20	S	0	0	0	8	12
	H	0	1	10	12	12
	R	0	0	1	10	12
25	S	0	0	12	12	12
	H	0	9	12	12	12
	R	0	5	12	12	12

The sensitive strain had a delayed sporulation time when compared to strains carrying the I86F mutation. At a temperature of 20°C, the heterokaryotic strain showed some advantage over sensitive and resistant strains, initiating the sporulation process earlier. Eleven d.a.i all lesions were sporulating for heterokaryotic strains, while resistant and sensitive strains delayed until the 12th day for complete uredinia opening. At 25°C, the latent period was shorter, with all strains sporulating only ten d.a.i. The resistant and heterokaryotic strains showed a shorter latent period than the wild type. However, the heterokaryotic strain seems to have a faster initial development than the 86F homozygous strain (Figure 22). The temperature of 30°C was not conducive to pustule development.

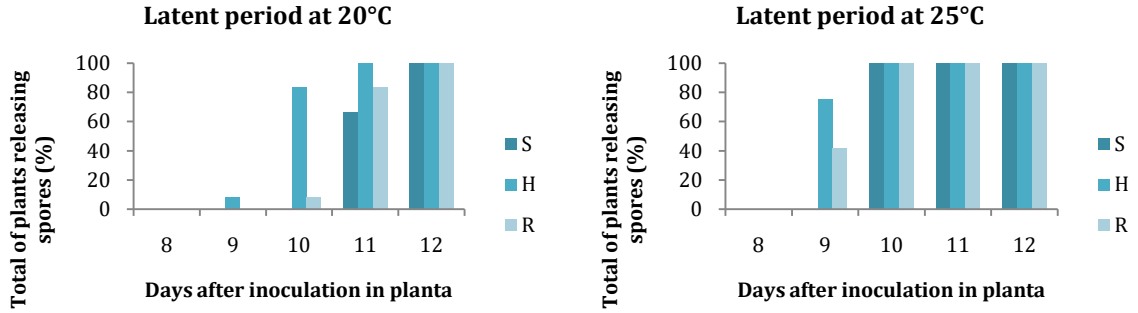

Figure 22. Latent period in two incubation temperatures (20°C and 25°C). Evaluations were done during twelve days and the total of plants releasing spores were counted. At day 12 all the uredinia were opened. At 25°C the latent period was shorter for all strains in comparison with 20°C latent period.

The block factor was added to control the wind variability among the growth chamber shelters. It can be notice at Table 13 that the effect of block was significant. The analysis of variance table is show at Table 13, and the effects of strains and the temperatures were not significant considering α=0,05. No interaction between the factors was observed.

Table 13. Analysis of Variance Table for Latent period of S-sensitive, H-heterokaryotic and R-resistant strains *in planta*. Model: Negative Binomial (link=log).

Analysis of Variance Table					
	Df	**Deviance Resid.**	**Df Resid.**	**Dev**	**Pr (> F)**
NULL			71	15.3789	
Block	2	19.3914	69	-4.0125	6.155e-05 ***
Strain	2	0.000	67	2.7601	1.0000
Temperature	1	1.811	66	0.9190	0.1748

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

5.5.2. Virulence *in planta* under different incubation temperatures (20°C, 25°C and 30°C)

The virulence measured under different incubation temperatures was performed in order to explore the impact of I86F substitution at the protein sequence of SDHC on pathogen fitness *in planta*. No interaction between the factors was observed. There were no differences in disease severity when the pathogen infection occurred at 20°C and 25°C of the incubation period (Table 14). At the temperature of 30°C, the pathogen did not induce disease symptoms. The effects of strain and d.a.i were significant (Table 14). Disease severity was higher when the Heterokaryotic strain infected the leaves than they were infected with either the sensitive (S) or resistant (R) strains (Table 15; Figure 23).

Table 14. Analysis of Variance Table for virulence under different incubation temperatures. Significant difference was observed among the strains for virulence *in planta.*

Analysis of Variance Table					
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (> F)**
Block	2	1640	819.9	4.4387	0.01295 *
Strain	2	15155	7577.5	41.0200	9.616e-16 ***
Temperature	1	655	664.7	3.5980	0.05923 .
d.a.i	2	11405	5702.5	30.8700	1.821 e-12***
Residuals	208	38423	184.7		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 15. Difference in disease severity (%) among S-sensitive, H-Heterokaryotic and R-resistant strains under different incubation temperatures. Means with the same letter do not differ by Tukey test considering α=5%

Strain	**emmean**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group***
S	20.34583	1.60177	208	16.49018	24.20148	**a**
R	25.5111	1.60177	208	21.6556	29.36676	**a**
H	40.12500	1.60177	208	36.26935	43.98065	**b**

* P value adjustment: tukey method. significance level used: alpha = 0.05.
Means with the same letter do not differ by Tukey test considering α=5%

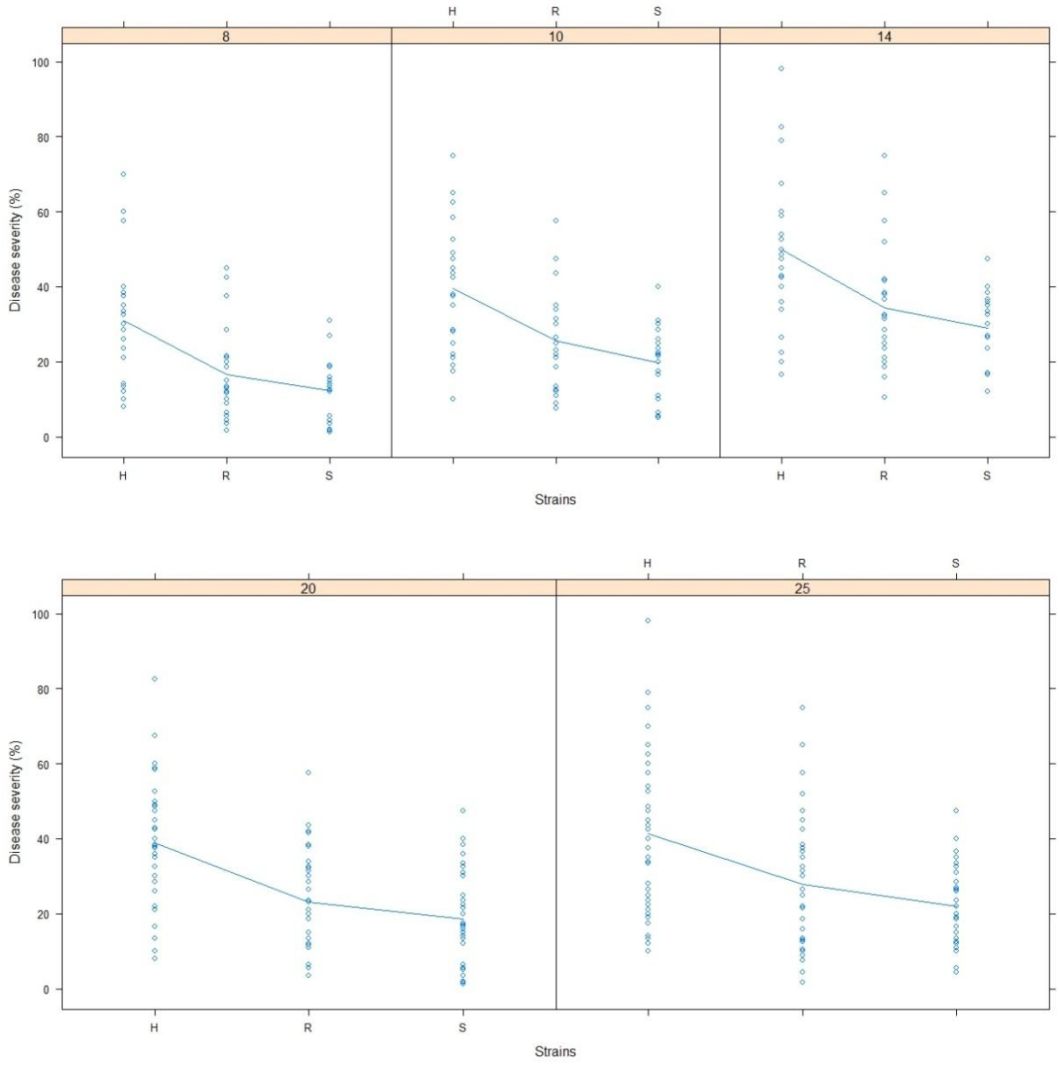

Figure 23. A) Comparison of in planta necrosis symptoms (Disease severity %) displayed by the mutants (R-resistant strain and H-heterokaryotic strain) in contrast with wild type strain (S-sensitive) at 8, 10 and 14 days after inoculation. B) Disease severity (%) at 20°C and 25°C for H, R and S strains. The assessment of soybean rust severity was done by using a diagrammatic scale.

The disease severity (%) was higher for the H-heterokayotic strain, independent of the temperature and in each d.a.i. analyzed, considering α=0.5 and Tukey method adjustment for multiple comparisons. No significant difference was observed between S-sensitive strains (homozygous to I86) and R-resistant strain (homozygous to 86F).

5.6. Quantitative genetic analysis of 86F using allele specific qPCR

Two standard curves validated the qPCR method (Figure 24). The first standard was a serial dilution of 100% 86F sample, and the second was a mix of pure 86F and I86 samples

(Figure 11). The serial dilution comprised 6 points, each with three replicates. The start gDNA quantity was 5 ng, and the dilution factor was 1:2.

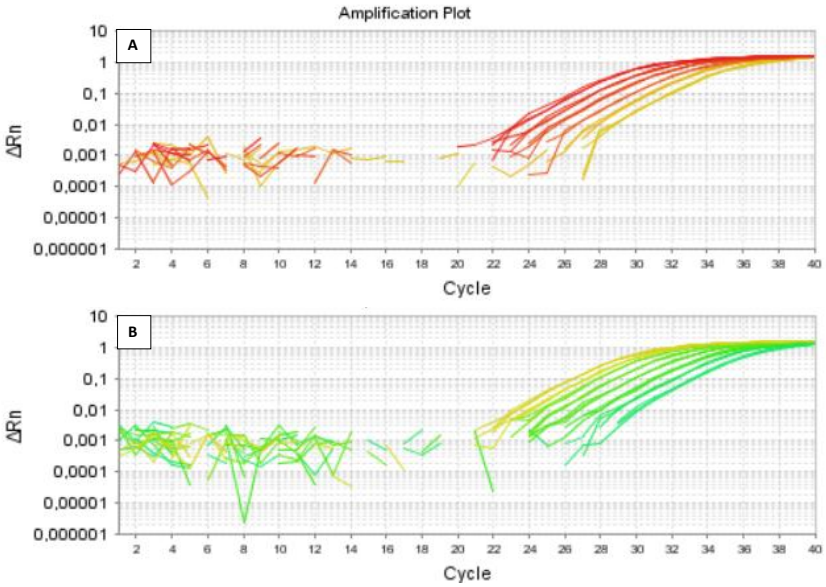

Figure 24. Amplification plot of standard curves. A) Standard curve I: serial dilution of 100% 86F isolate. B) Standard II: mix of pure 86F and I86 isolates. A spacing of 1 cycle between amplification curves for a 1:2 dilution is shown.

A negative control, which corresponded to a wild type sample with no mutant templates, was included alongside test samples to validate the assay efficiency in amplifying the mutant target only. The Ct of 36,8 for the negative control indicated the non-significant frequency of the mutant template. The Ct that corresponds to 1.56% 86F frequency was 33.4. A negative control of the reaction (blank), with no DNA template, did not show amplification (Figure 25).

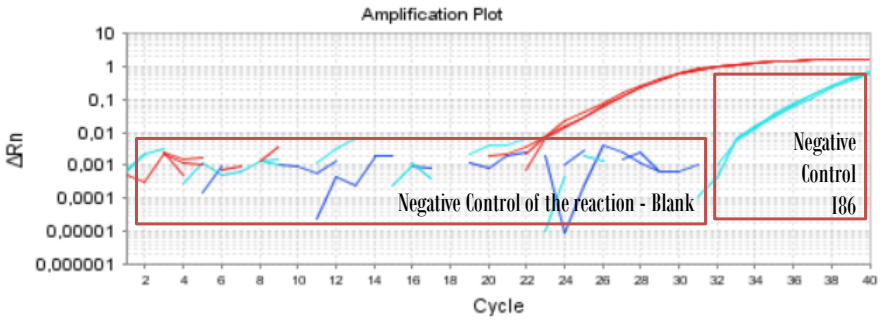

Figure 25. Amplification controls of PCR reaction assay. Negative control of the reaction or no template control (Blank). Negative control using a known wild type sample (I86).

The Standard curve (Figure 26) showed that the R^2 value reflected the linearity of the standard curve. Slope of -3,41 and a R^2:0,999 was observed for the assay.

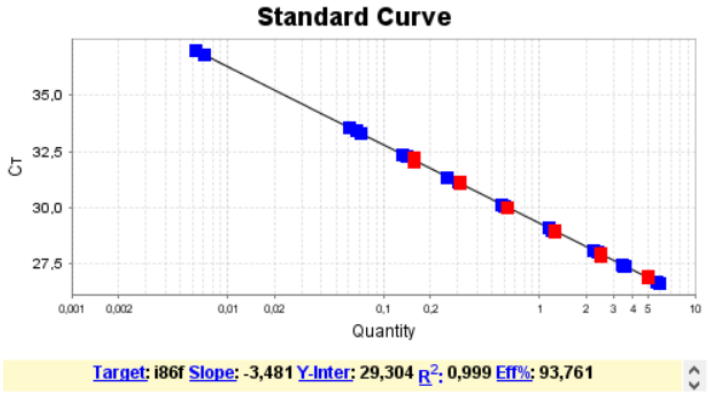

Figure 26. Standard curve and its reaction performance. Reaction efficiency of 93,761% and Correlation coefficient (R^2) of 0,999.

The correlation coefficient of the standard curve I (estimate the 86F frequency) and standard curve II (mixing the homozygous 86F and I86 samples) was R2=0.981 (Figure 27). The negative control showed no amplification.

Table 16. Potential to predict 86F mutation using quantitative PCR and standard curve method.

Standard I	Standard II		qPCR	
	I86F	I86	Quantity Mean	Quantity SD
	% I86F			
100,00	100,00	0,00	115,48	3,02
-	75,00	25,00	70,23	1,39
50,00	50,00	50,00	45,65	1,60
25,00	25,00	75,00	24,11	0,71
12,50	12,50	87,50	11,87	0,21
6,25	6,25	93,75	5,46	0,52
3,13	3,13	96,88	2,72	0,10
-	1,56	98,44	1,33	0,11
Negative control	0	100	0,13	0,01

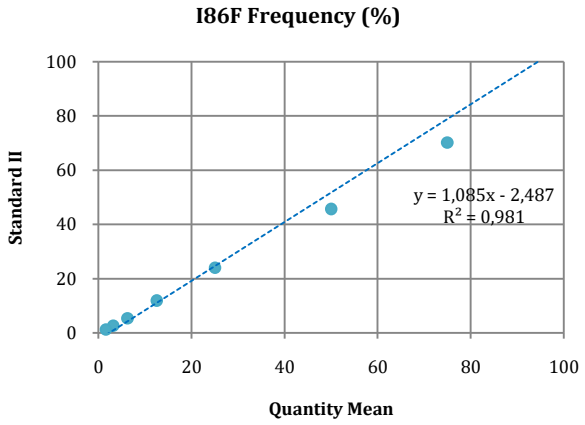

Figure 27. Correlation coefficient (R^2) between the two standard curves. Standard curve I: serial dilution of 100% 86F isolate. Standard II: mix of homozygous 86F and I86 isolates.

5.7. 86F frequency over five consecutive cycles under no fungicide pressure in competition assay with wild type strain and mutation stability assay

5.7.1. The allele frequency of 86F decreased in competition assays

Urediniospores suspensions prepared every 14 to 20 days by washing soybean leaves were inoculated in new detached leaves. No fungicide was applied. Each multiplication corresponded to one disease cycle, and five cycles were completed. The results showed a decrease (statistically significant) of 86F allele frequency throughout disease cycles under no selection pressure (Figure 28; Table 18). Three technical replicates were done in order to reduce the dilution and pipetting errors.

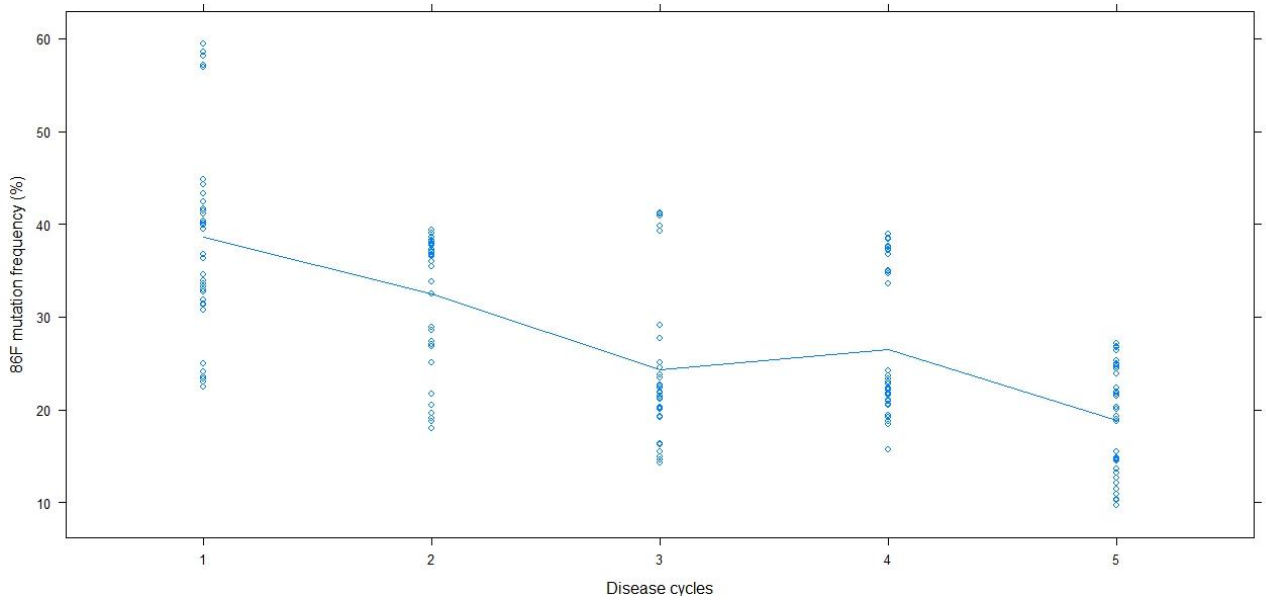

Figure 28. Frequency of I86F substitution in succinate dehydrogenase subunit c (sdh c) gene in competition assay between sensitive (S – 100% I86) and mutated isolates (R- 100% 86F) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using quantitative PCR.

Table 17. Analysis of Variance Table for I86F substitution frequency in competition assay between sensitive (S- 100% I86) and mutated isolates (R- 100% 86F) of *Phakopsora pachyrhizi* during five generations using quantitative PCR.

Analysis of Variance Table					
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (> F)**
Generations	4	8360.9	2090.22	51.882	<2.2e-16 ***
Sample	3	1393.7	464.58	11.531	6.726e-07 ***
Sample:qPCR Plate	8	3366.6	420.83	10.445	8.823e-12***
Residuals	164	6607.3	40.29		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

All biological replicates showed a decrease of 86F allele frequency throughout disease cycles under no selection pressure. No difference was observed from generation three to four,

followed by a decrease in generation five. An average reduction of 20% in 86F frequency was observed in five disease cycles (Table 18; Figure 29).

Table 18. Difference in 86F frequency (%) in competition assay between sensitive (S = wild type) and mutated isolates (R= resistant strain) of *Phakopsora pachyrhizi* during five generations of multiplication on non-treated soybean leaves using quantitative PCR. Means with the same letter do not differ by Tukey test considering α=5%

Generations	emmean	SE	df	lower.CL	upper.CL	.group*
1	38.59929	1.094029	168	35.75687	41.44171	**a**
2	32.51934	1.094029	168	29.67692	35.36176	**b**
3	24.26607	1.094029	168	21.42365	27.10849	**c**
4	26.44580	1.094029	168	23.60338	29.28822	**c**
5	18.87754	1.094029	168	16.03512	21.71996	**d**

* P value adjustment: Tukey method. significance level used: alpha = 0.05.
Means with the same letter do not differ by Tukey test considering α=5%

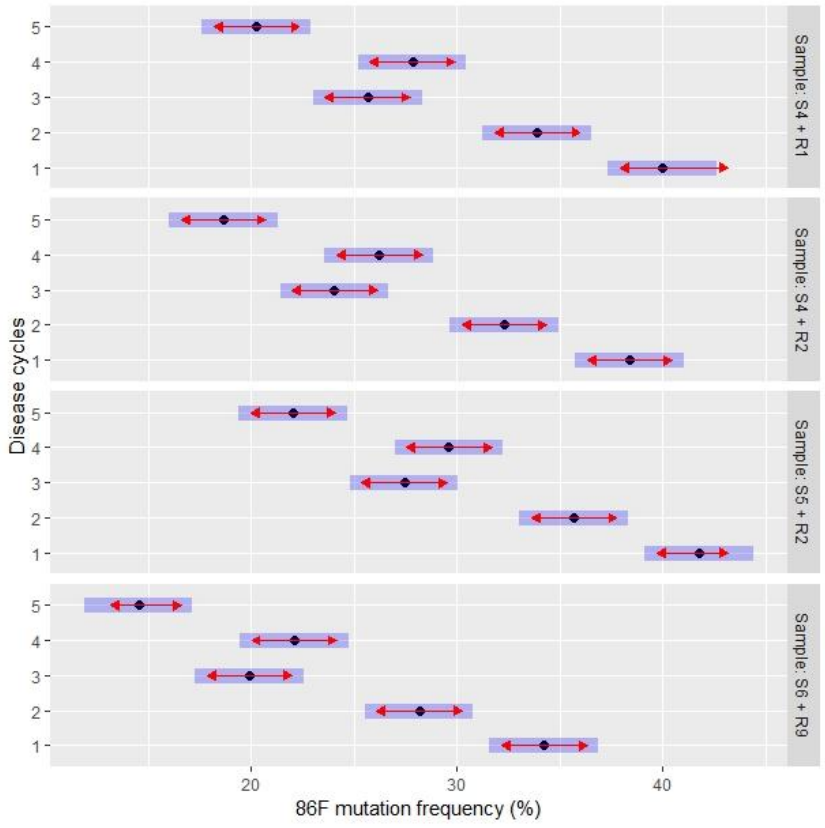

Figure 29. 86F mutation frequency (%) in succinate dehydrogenase subunit c (sdh c) gene in competition assay between sensitive (S = wild type) and mutated isolates (R= resistan strain) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using quantitative PCR. The blue bars are confidence intervals for EMMs, and the red arrows are for comparisons among factor levels. If an arrow from one mean overlaps an arrow from another group, the difference is not "significant," based on Tukey Method and α = 0.05.

The decrease of 86F allele frequency throughout five disease cycles under no selection was also corroborated using the Sanger sequencing of the samples. A little discrepancy was

observed probably due to the sensibility for SNP detection using Sanger, which is about 5% (Table 19).

Table 19. 86F frequency assessment of mutated (R= resistant strain) and sensitive (S = wild type) strains of *Phakopsora pachyrhizi* in competition during five disease cycles on non-treated soybean leaves using Minor Variant Finder (MVR) software to analyses Sanger sequencing results.

Strains	Generations	Forward percent	Reverse percent	86F frequency (%)
S4 + R2	1	66,3	53,4	59,85
	2	52,9	38,1	45,5
	3	31,7	19,3	25,5
	4	34,5	22,2	28,35
	5	28,2	15,6	21,9
S6 + R9	1	54,5	37,5	46
	2	42,6	28,7	35,65
	3	35	21,7	28,35
	4	32,6	21,5	27,05
	5	35,4	23,3	29,35
S5 + R2	1	63,8	51,9	57,85
	2	56,1	42,8	49,45
	3	32,7	20,6	26,65
	4	38,4	24,9	31,65
	5	40,7	28	34,35
S4 + R1	1	56.9	43.6	50.25
	2	52.6	39	45.8
	3	48.8	35.7	42.25
	4	51.5	37.5	44.5
	5	40.7	28	34.35

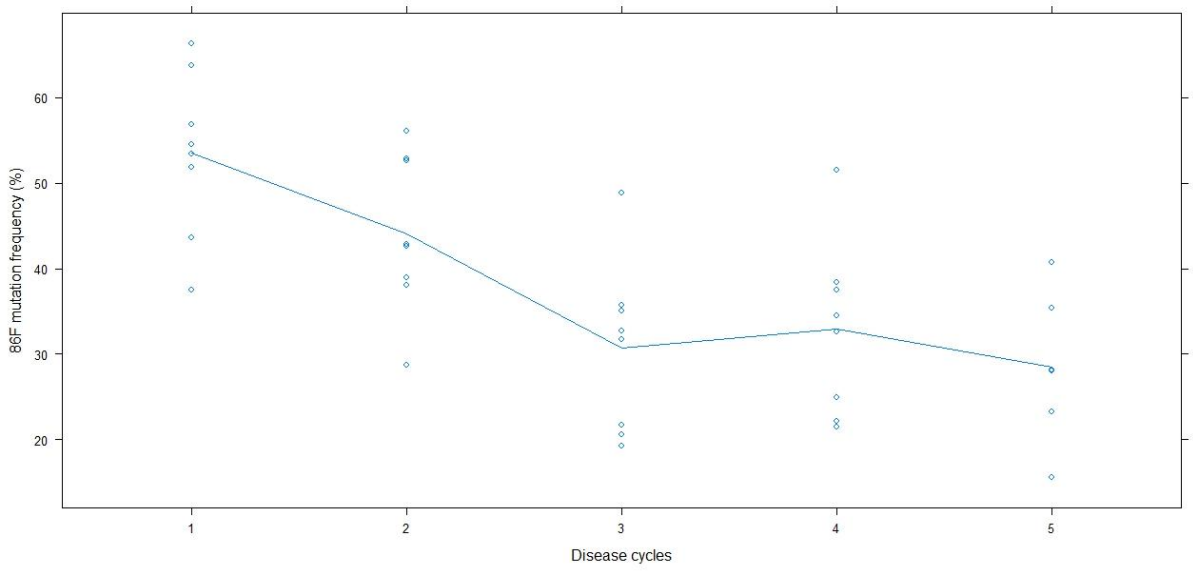

Figure 30. Frequency of I86F substitution in succinate dehydrogenase subunit c (sdh c) gene in competition assay between sensitive (S = wild type) and mutated isolates (R= resistan strain) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using MVF software to analyze Sanger sequencing results.

5.7.2. The frequency of the 86F allele is stable in pure cultures over disease cycles

The stability assay revealed that I86F mutation tends to be stable when resistant strains alone are cultured in the first three disease cycles with no selection pressure (Table 20). A little decrease in mutation frequency was observed in the fourth and fifth generations (Figure 31).

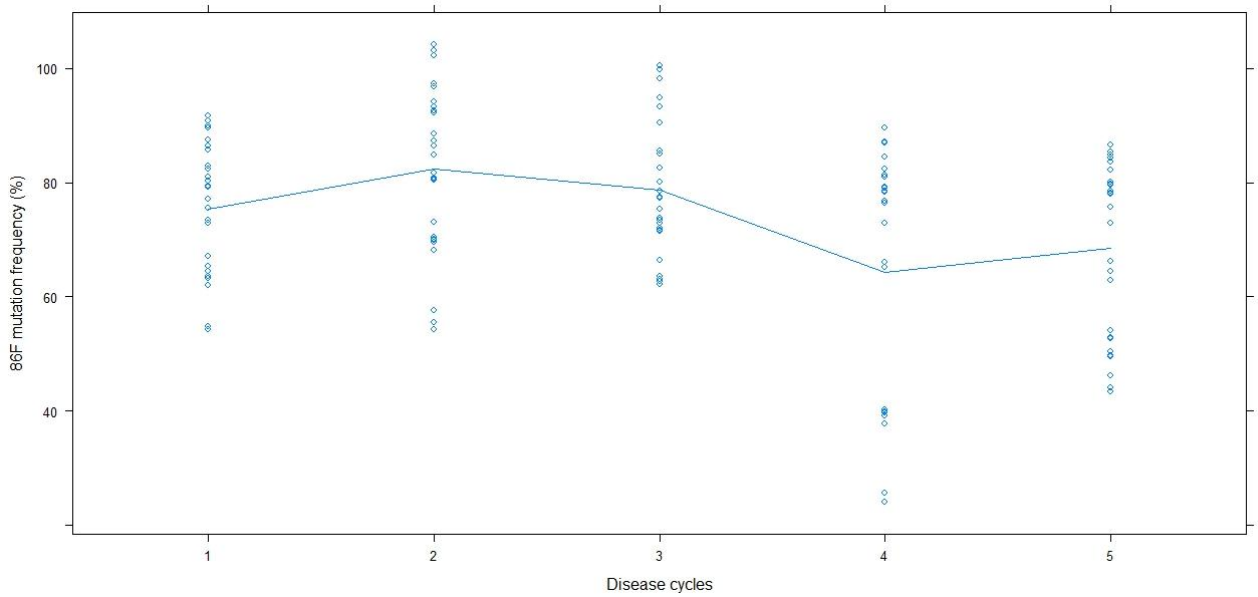

Figure 31. Frequency of I86F mutation in succinate dehydrogenase subunit c (sdh c) gene in mutation stability assay of mutated isolates (R2, R1 and R9) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using quantitative PCR.

Table 20. Analysis of Variance Table for 86F mutation stability of mutated isolates (R= resistant strain) of *Phakopsora pachyrhizi* during five generations using quantitative PCR.

Analysis of Variance Table					
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (> F)**
Generations	4	5843.7	1460.9	20.2485	8.228e-13 ***
Sample	2	20351.1	10175.5	141.0346	<2.2e-16 ***
Sample:qPCR Plate	6	2385.0	397.5	5.5094	4.445e-05 ***
Residuals	122	8802.2	72.1		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

A statistically significant difference was observed in the fourth and fifth generation of disease cycle by quantitative PCR, with a decrease of 86F allele frequency (%) when cultured alone in non-treated leaves. An increase of 86F frequency was observed from generation 1 to generation 2 (Table 21). All biological samples followed the same pattern of 86F frequency fluctuation through disease cycles (Figure 32).

Table 21. Difference in 86F frequency (%) for mutated isolates (R= resistant strain) of *Phakopsora pachyrhizi* during five generations of multiplication on non-treated soybean leaves using quantitative PCR. Means with the same letter do not differ by Tukey test considering α=5%

Generations	emmean	SE	df	lower.CL	upper.CL	.group*
2	82.36746	1.634685	122	78.10199	86.63293	**a**
3	78.69162	1.634685	122	71.42614	79.60674	**ab**
1	75.34127	1.634685	122	71.07580	79.60674	**b**
4	64.35479	1.634685	122	60.08932	68.62027	**c**
5	68.55274	1.634685	122	64.28726	72.81821	**c**

* P value adjustment: Tukey method. significance level used: alpha = 0.05.
Means with the same letter do not differ by Tukey test considering α=5%

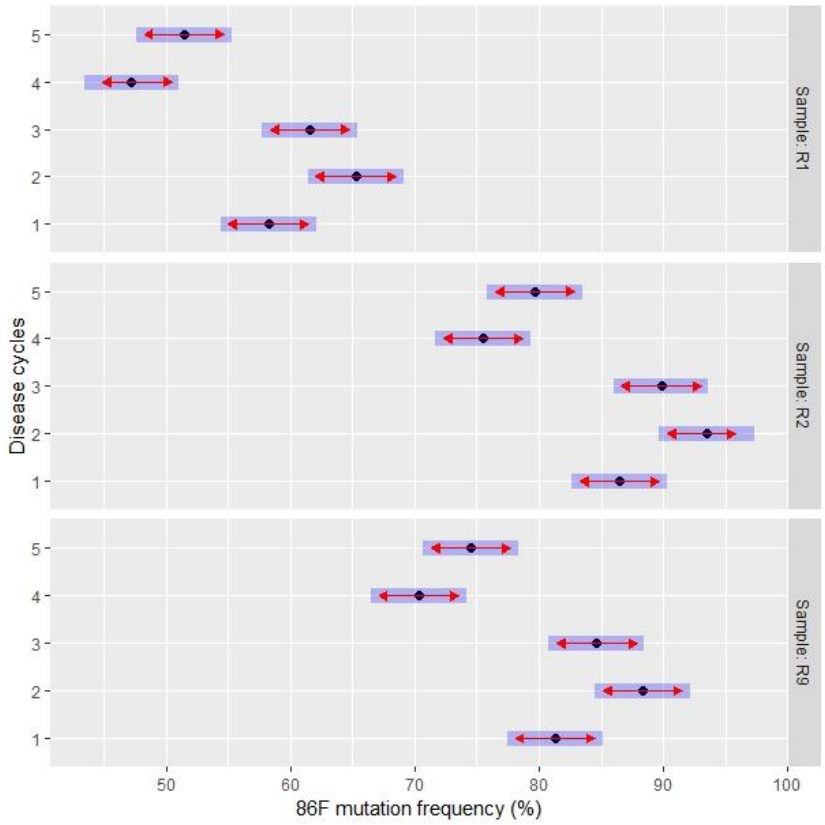

Figure 32. 86F mutation frequency (%) in succinate dehydrogenase subunit c (sdh c) gene in mutation stability assay of mutated isolates (R= resistan strain) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using quantitative PCR. The blue bars are confidence intervals for EMMs, and the red arrows are for comparisons among factor levels. If an arrow from one mean overlaps an arrow from another group, the difference is not "significant," based on Tukey Method and α = 0.05.

The stability of the allele 86F frequency in pure cultures over five generation cycles with no selection was maintained using the Sanger sequencing of the samples (Figure 33). No significant change in 86F frequency (%) was observed over five consecutive disease cycles in pure cultures using Sanger sequencing for SNP detection (Table 22). A little discrepancy was observed probably due to the qPCR method sensibility.

Table 22. 86F frequency assessment of mutation stability over five generations in untreated soybean leaves using Minor Variant Finder (MVR) software to analyses Sanger sequencing results.

Strains	Generations	Forward percent (%)	Reverse percent (%)	Mean(%)
	1	90,5	86,4	**88,5**
	2	91,9	86,9	**89,4**
R1	**3**	89,3	85,7	**87,5**
	4	89	83,7	**86,4**
	5	88,9	82,8	**85,9**
	1	93,2	90,7	**92,0**
	2	94,3	91	**92,7**
R2	**3**	95,7	92,2	**94,0**
	4	96	92,8	**94,4**
	5	93,7	92,1	**92,9**
	1	90,9	86,3	**88,6**
	2	92,2	88,3	**90,3**
R9	**3**	91,7	88,7	**90,2**
	4	92,2	88,7	**90,5**
	5	92,9	88,9	**90,9**

At Figure 33 it can be noticed that 86F allele seems to be maintained over the generations tested even if it is not in complete homozygosis when Sanger sequencing results were analyzed using MVF software.

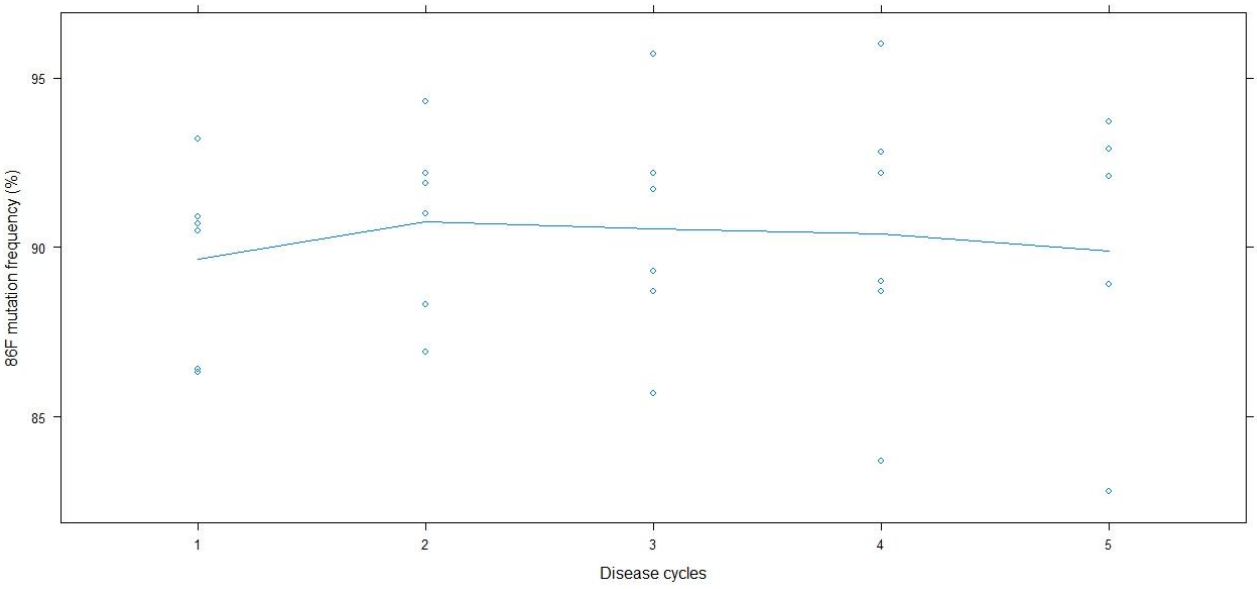

Figure 33. Frequency of I86F mutation in succinate dehydrogenase subunit c (sdh c) gene in mutation stability assay of mutated isolates (R2, R1 and R9) of *Phakopsora pachyrhizi* during five disease cycles on non-treated soybean leaves using MVF software to analyze Sanger sequencing results.

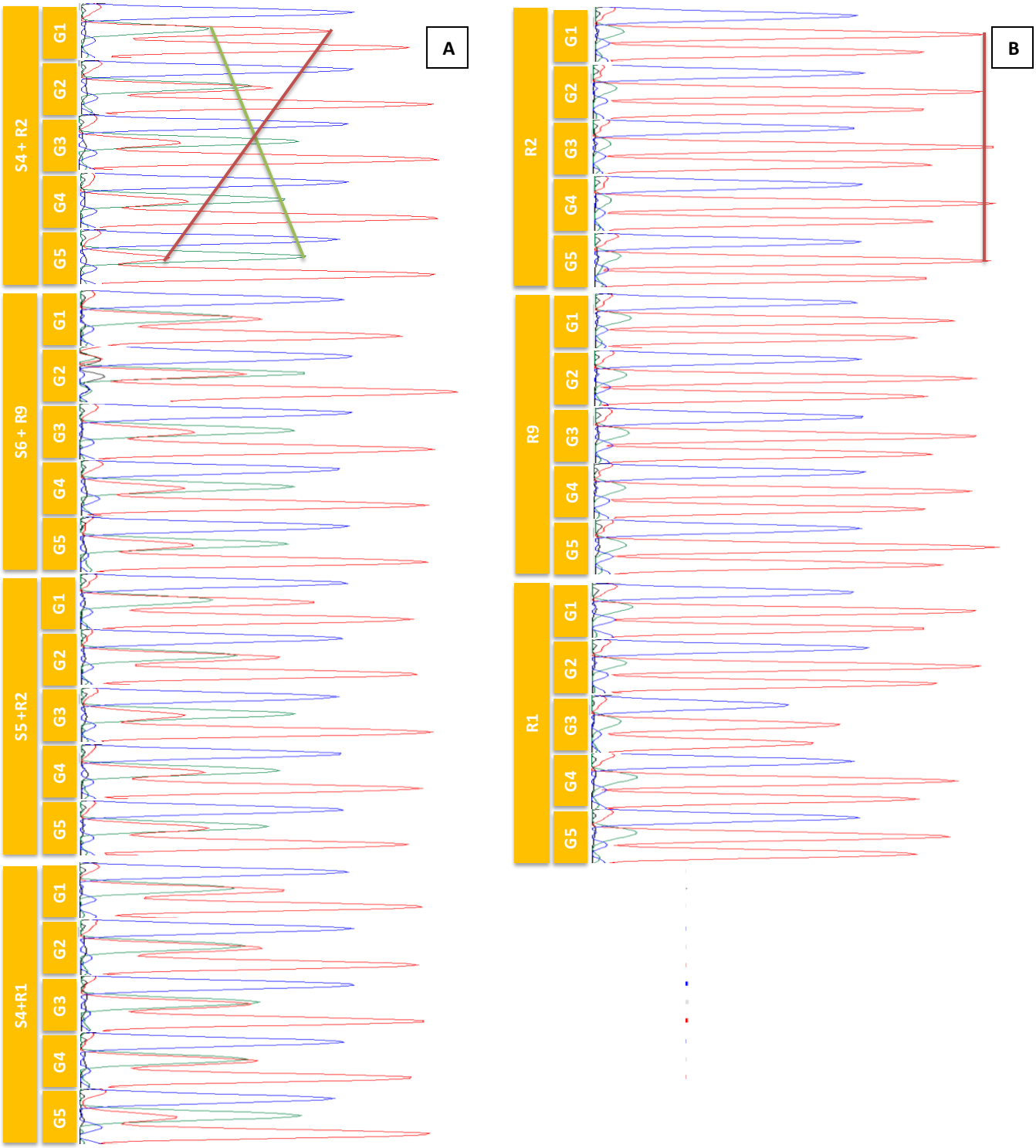

Figure 34. Analysis of Sanger sequencing chromatograms for competition and mutation stability tests after five cycles. A) In the competition assay, the frequency of I86 allele increased as generation proceeds, what imply some advantage of allele I86 over allele 86F, in all the four biological replicates (S4+R2, S6+R9, S5+R2, S4+R1). B) It the mutation stability assay, the frequency of 86F was maintained over five generations (R2, R9, R1).

6. DISCUSSION

This work presented for the first time the obtaining, biological characterization, and genotyping of populations of the soybean rust pathogen *P. pachyrhizi* susceptible and resistant to SDHI fungicides. Susceptible strains were collected from the fields and maintained in growth chambers. Resistant strains were obtained in the lab under the selection pressure of the fungicide. Single uredinium lesion was isolated from leaves sprayed with 45 ppm of fungicide and multiplied under a maintenance dose to reach homozygosis for the resistant allele 86F. Three strains of three populations screened for the allele frequencies 100% I86 susceptible; 50% I86/50%86F resistant; 100% 86F resistant were selected for characterization. Up to date field populations carrying 100% of the mutated 86F allele were not identified (Simões et al., 2017). The strains obtained in this study are a valuable resource to understand the behavior of such mutations over several disease cycles.

Both sdh c resistant strains, the heterokaryotic (H) and the homokaryotic (R), (I86:86F and 86F:86F, respectively), showed higher EC_{50} values compared to those of the wild type when submitted to either Benzovindiflupyr or Fluxapyroxad treatments. However, EC_{50} values were higher for strains 86F (R) when compared to strains I86F (H). Similar results were reported by Simões et al. (2017) when analyzing populations of *P. pachyrhizi*. The higher the frequency of 86F, the higher the EC_{50} value. Also, the EC_{50} value increase for Fluxapyroxad was lower than that for Benzovindiflupyr.

The level of fungicide resistance is usually determined relating the concentration needed to reduce life parameters, such as mycelium growth and spore germination by 50%. A mutant can be designated resistant to a fungicide if the EC_{50} value is at least the double of that applied for the wild type sensitive strain (Delp & Dekker, 1985). The populations studied here presented six times over the EC_{50} value for Benzovindiflupyr, but only 1.6 fold for Fluxapyroxad. The nature of the resistance may vary according to the pathogen genetic background and the active fungicide component, but studies at population levels always consider cross-resistance between different molecules of the same class. Although all SDHI fungicides belong to the same cross-resistance group, structural variations may suggest differences in binding properties to the target enzyme. Fluxapyroxad binding site seems to be less affected by isoleucine to phenylalanine substitution than Benzovindiflupyr. SDHIs are structurally diverse but share an essential characteristic feature, which is the amide bond (Sierotzki, & Scalliet, 2013). The core, which is essential for binding and in vivo potency, varies among chemical groups, and the most described types in terms of marketed molecules are the pyrazole carboxamides. The core of these molecules enters deeply into the active site of the SDH enzyme. The SDHI molecules have two main components, the

linker and the hydrophobic rest. The most frequent linker is a phenyl group critical for its activity. The hydrophobic rest is likely to be partially exposed at the surface of the enzyme but also considered relevant for biological spectrum and potency (Sierotzki & Scalliet, 2013). Mutation at the same amino acid position can have a completely different sensitivity pattern toward SDHI fungicides. It is crucial to determine the genetic background in order to evaluate the cross-resistance behaviors toward these fungicides. Also, relative variation in sensitivity among isolates of the same species carrying the same mutation indicates that factors other than target sequences involve the sensitivity to SDHIs. Despite the improvement of molecular methods to detect and to monitor resistance in the field, when or where resistance will occur is still challenging to predict. By creating mutants in the lab and using fitness measurements, we expect to enhance the ability to predict agricultural problems.

Both resistant strains, the heterokaryotic (H) and the homokaryotic (R), showed a faster initial development considering the percentage of appressorium formation compared to the wild type strain (S). A significant difference, however, was also found between resistant 86F (R) and I86F (H) strains. Latent periods ranged from 9.25 to 11.3 days, depending on the strain and the temperature treatments. The latency period of the strains was not affected by the temperature treatments 20°C and 25°C; however, at 25°C the latency was equally reduced for all strains. At 20°C the differences in latency among the strains were more evident, revealing a shorter latent period for strain I86F (H) when compared with both 86F (R) and I86 (S) strains.

Disease severity provoked by heterokaryon strains (H) in the absence of fungicide was higher when compared with both wild type (S) and resistant strains (R). However, temperatures of 20°C and 25°C did not affect the results in either case. Otherwise, the temperature of 30°C was not conducive to disease development. Interestingly, the virulence test performed with the mutant strains showed that I86F substitution could lead to increased symptoms in the absence of the carboxamide treatment. The reason for this remains unclear and will require further investigation. Scalliet (2012) reported a similar response in studies with SDH mutants of *Mycosphaerella graminicola*. Even though the reasons for this increased symptomology remain elusive, the data indicated that sdh c mutation could influence the biology of the *P. pachyrhizi*, leading to the faster initial development and a shorter life cycle.

The faster initial development measured by appressorium development and latency suggested that resistant strains, both heterokaryotic (H) and homokaryotic (R), are more aggressive when compared with wild type strain (S) under no fungicide selection pressure. These types of strains with a shorter latent period are considered more aggressive epidemiologically since they can undergo more disease cycles within the same season.

The infection success of most pathogenic fungi depends on leaf wetness. According to Furtado et al. (2010), an interruption of wetness one hour after inoculation causes average reductions in the germination of *P. pachyrhizi* isolates from 56 to 75% and appressorium formation from 84 to 96%. A faster initial development would allow the infection process in less favorable environments, increasing ASR severity, and yield losses.

Resistant strains with shorter life cycles require an adjustment of the interval between fungicide spray. The timing of application in the absence of symptoms is crucial to disease management. The disease control based on preventive applications proved to be more efficient than the curative applications (Juliatti et al., 2016). However, the increase in fungicide applications is not that simple to consider due to consequent increases in production costs and the impact on pathogen evolution. The treatment program of the most common SDHI fungicides recommends a maximum interval of 14 days between applications and two applications of the same fungicide per crop growing season. The selective pressure exerted by a fungicide application program on the pathogen population must also consider the mode of action (MOA) rotation. The reduced number of MOA fungicides requires a careful evaluation of the pathogen potential to evolve resistance. Thus, it is essential to maximize the fungicide viability of currently available molecules.

The allelic frequencies of up to 100% I86F were not described in field samples during monitoring programs for the 2016/2017 crop season (Simões et al., 2017). In the FMC monitoring program of season 2018/2019, the highest 86F frequency found in the field was 54% (data not shown). As suggested by Simões et al. (2017), and observed in sensitivity studies of field populations, the highest I86F frequency of about 50% suggests that heterozygous strains (H) are fitter than those 86F homozygous (R).

It seems like the fungicide exerts a selective pressure, under which resistant genotypes are fitter than sensitive genotypes. However, this selective advantage may be offset by fitness costs. Fitness penalties may reduce the overall selective advantage of resistance under no fungicide treatment, but more significantly, they may lead to reversals of resistance given sufficient gaps in treatment (Hawkins & Fraaije, 2018). Variation in fitness between individuals may arise from differences in performance at any stage of the life cycle, such as viability, mating success, and fecundity (Antonovics & Alexander, 1989).

The fitness can be estimated using the rate of change in allelic frequencies in controlled conditions in the presence and absence of the selection pressure. The results here showed that the frequency of 86F allele decreased in mixed populations over five disease cycles under no fungicide pressure in both experiments evaluated (the qPCR and the Sanger sequencing

methods). The allele I86 (S) had a competitive advantage over the 86F (R) in this study, despite the faster initial development and virulence of the 86F resistant strain. This type of prediction involving competitive assays is strategic for resistance management programs. Reducing the selection pressure of SDHI fungicides could lead to a decrease in the frequency of the mutant allele in field populations. However, the competition ability between heterokaryotic (H) and sensitive strain (S) requires also investigation.

Similar studies were developed by Klosowski et al. (2016) for isolates carrying the Y131F+I475T and Y131F+K12R mutations in cyp 51 gene. However, in this case, QoI resistant strains with F129L mutation was not different from the initial frequency after cultured with sensitive isolate during four disease cycles (Klosowski et al., 2016). Also, the Mancozeb, the multisite fungicide evaluated, did not affect the competitive dynamics of the alleles frequency in both cyt b and cyp 51 mutants when they were co-cultivated with the wild type. The authors observed that the multisite fungicide equally inhibited both mutant and sensitive strains. According to the FRAC QoI working group, the analysis of historic samples showed that the F129L mutation was present at significant levels from at least 2012/13 season, and a high frequency of F129L mutation observed in the majority of samples since season 2014/15 in Brazil. Bolivia and Paraguay, in both cultivated and volunteer soybean plants, reported the high F129L mutation frequency. The advantage over sensitive isolate might explain why the F129L substitution in cyt b is considered stable.

The 86F mutation frequency in this study did not seem to decrease during five consecutive cycles of multiplication, suggesting that it has some stability if not in competition with the wild type strain. The results of the qPCR analysis regarding the 86F allele frequency in pure culture showed no variation for at least the first three disease cycles. Data strongly supported by the results obtained using the sequencing method. It seemed that fourth and fifth disease cycles also kept a stable allelic frequency. However, dilution errors may have contributed to a slight variation after the third cycle, effect not observed by the Sanger sequencing method.

Competition experiments between resistant 86F (R) and I86 wild type (S) strain showed that I86 allele promoted an advantage over the 86F, suggesting a fitness cost associated with the isoleucine to phenylalanine substitution at SDHC protein sequence. The fitness cost is probably the cause of the absence of homozygous resistant strains in field conditions. Conversely, field strains maintained F129L mutations in homozygosis. According to the FRAC SDHI working group (2018), SDHI-sensitivity in monitoring programs from the season 2017/18 was comparable to the previous season, I86F mutation did not spread in the field. Even with phenotypes with a decreased sensitivity to SDHI fungicides, field efficacy of SDHI-containing

fungicides remained generally good (FRAC). Recently, we identified an F129L mutation in homozygosis in the 86F resistant strain. This mutation is the first case of strains in homozygosis detected for both I86F and F129L substitutions, at sdh c and cyt b, respectively, and they will be further investigated using the same approach developed here.

The competition and mutation stability assays developed in this study comprised approximately 80 days (5 disease cycles), which is related to the soybean free-period comprised from 60 to 90 days in general. The individuals that survive the soybean free-period, after three to four fungicide applications during the season, will establish a new population in the next regular crop season, and the penalty associated with I86F substitution will reduce the mutated allele frequency and delay or avoid the fixation of resistance. *P. pachyrhizi* urediniospores travel long distances with the wind, enabling high gene flow among populations. Consequently, they are likely to disseminate mutant and wild type alleles across a large geographical area. When in the absence of carboxamide treatment, the sensitive foreign individuals will compete with 86F resistant isolates, potentially leading to reversals of resistance if given sufficient gaps between treatments.

ASR disease management must consider *P. pachyrhiz* biology cycle of field populations, accounting for the reduction in latency for resistant strains and the residual activity of protective molecules in field conditions. Also, there is a need for new MOA molecules as the availability of different MOA fungicides is restricted. Of equal importance is a detailed understanding of their mode of action and the consequences of target modifications.

6.1. Considerations and conclusions

This study contributed to describing the increased resistance of strains R (100% 86F) compared to H (50% I86:86F 50%) toward SDHI fungicides. Besides, we identified a fitness cost associated with resistance (allele in homozygosis) when no fungicide is applied. Resistant strains of *P. pachyrhizi* were more aggressive than wild type and have a selective advantage under fungicide pressure in the field. However, when in competition with wild type strains under no fungicide pressure, they were less adapted.

Resistant strains of *P. pachyrhizi* were more aggressive than wild type strains and have a selective advantage under fungicide pressure in the field. However, when in competition with wild type strains and under no fungicide pressure, they were less adapted. With the sensitivity loss towards all the available mode of action fungicides, it is important to understand if a fitness cost

would reduce the mutation frequency in field populations (and the time it would take), for disease management in fungicide programs.

The incubation and latent periods for strains carrying the 86F mutation reported here were shorter compared with those for wild type strains. Although these data allowed significant premises about the biology of *P. pachyrhizi*, other measures, such as the number of urediniospores produced per lesion and the spore viability may provide a more accurate analysis. Also, in this study, although the experiments employed non-treated soybean plants, the propagation of resistant strains in the lab was dependent on a maintenance dose of Elatus®, and therefore in continuous fungicide stress. At this point, we can not exclude the possibility that the selection of mutations other than directly related to I86F may be responsible for the fast initial growth of resistant strains. Additional investigations using a more significant number of field isolates and more technical replicates may verify the data presented here. Also, a competitive assay considering sensitive strains and resistant heterokaryons should improve the conclusions.

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