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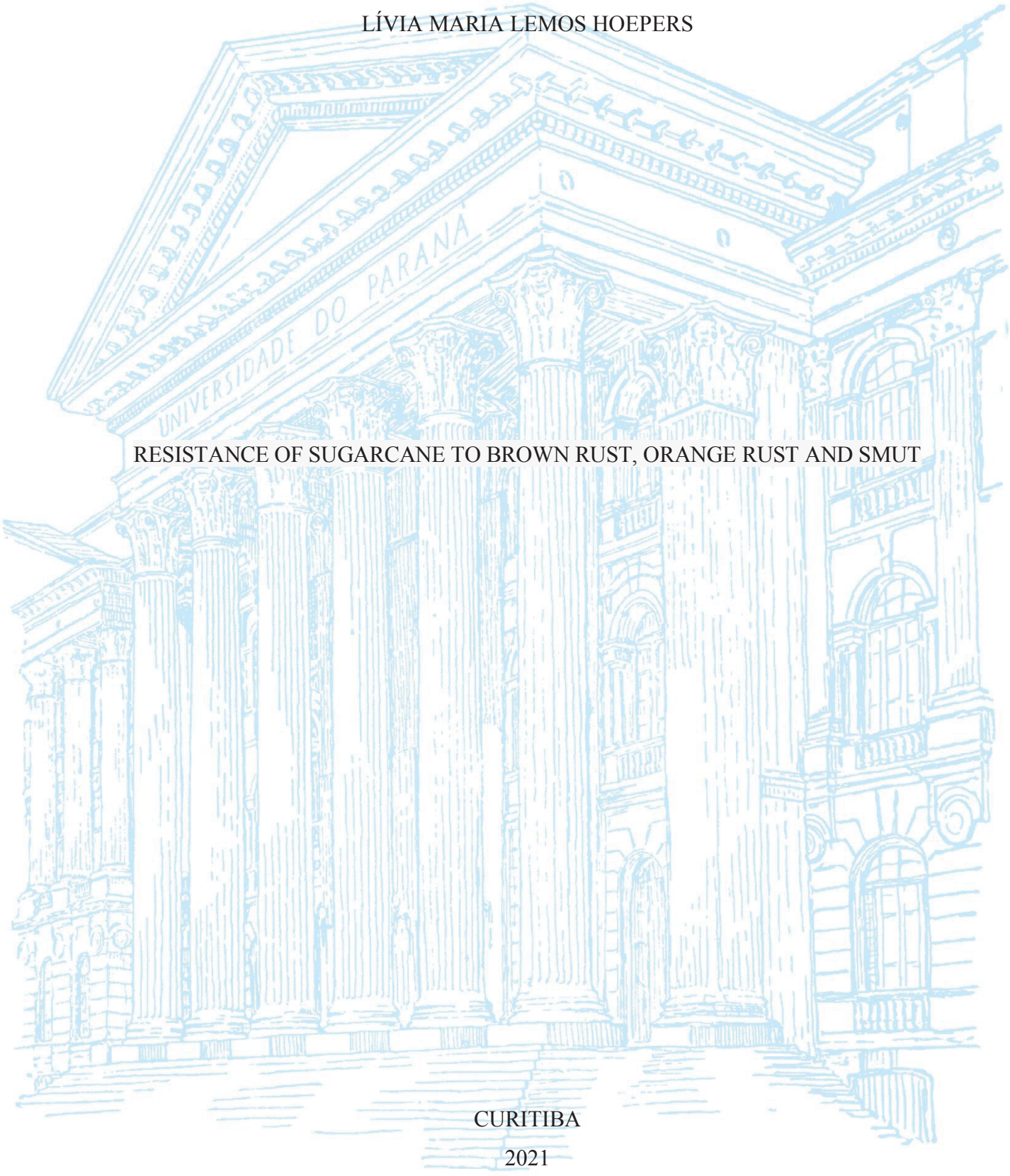

RESISTANCE OF SUGARCANE TO BROWN RUST, ORANGE RUST AND SMUT

CURITIBA

2021

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RESISTANCE OF SUGARCANE TO BROWN RUST, ORANGE RUST AND SMUT

Tese apresentada ao curso de Pós-Graduação em Agronomia – Produção Vegetal, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências.

Orientador: Dr. Henrique da Silva Silveira Duarte

Co-orientadores: Drª Lucimeris Ruaro, Dr. Ricardo Augusto de Oliveira, Drª Renata Faier Calegario, Dr. João Carlos Bespalhok Filho

CURITIBA

2021

Hoepers, Lívia Maria Lemos
Resistance of sugarcane to brown rust, orange rust and smut. / Lívia Maria Lemos Hoepers. - Curitiba, 2021.

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Agronomia - Produção Vegetal.
Orientador: Henrique da Silva Silveira Duarte.
Coorientadores: Lucimeris Ruaro, Ricardo Augusto de Oliveira, Renata Faier Calegario, João Carlos Bespalhok Filho.

1. Cana-de-açúcar - Fungos. 2. Cana-de-açúcar - Resistência a doenças e pragas. 3. Fungos-da-ferrugem - Brasil. I. Duarte, Henrique da Silva Silveira. II. Ruaro, Lucimeris. III. Oliveira, Ricardo Augusto de. IV. Calegario, Renata Faier. V. Bespalhok Filho, João Carlos. VI. Título. VII. Universidade Federal do Paraná.

Sistema de Bibliotecas/UFPR
Guilherme Luiz Cintra Neves - CRB9/1572

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIENCIAS AGRARIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO AGRONOMIA
(PRODUÇÃO VEGETAL) - 40001016031P6

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CURITIBA, 30 de Março de 2021.

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AGRADECIMENTOS

À minha família, em especial meus pais, José e Ivete, pelo apoio e exemplo de caráter, honestidade e perseverança, além de me guiarem e apoiarem que eu seguisse o caminho do conhecimento, e ao meu sobrinho Daniel, por ser uma luz na minha vida.

À minha querida tia Marinalva e minhas primas/irmãs, Cris e Carol, por terem me acolhido de maneiras que eu nem sabia que podia ser acolhida, eu ter ganhado vocês foi uma das melhores coisas que me aconteceu.

Às irmãs que a vida me deu, Danielle Stefanello Mathias, Raisa Fantin, Danielle Dutra Martinha, Mariana Franca e Juliane Borella por me apoiarem diante das piores adversidades que enfrentei, pra sempre vou levá-las comigo aonde quer que eu vá.

Ao meu orientador Dr. Henrique da Silva Silveira Duarte, por ter aceitado me guiar nessa empreitada, por não ter desistido mesmo diante dos percalços no caminho e por ter confiado na minha capacidade desde o início.

Aos meus co-orientadores Dra. Lucimeris Ruaro, Dr. Ricardo Augusto de Oliveira, Dr. João Carlos Bespalhok Filho e Dra. Renata Faier Calegario por todo suporte na execução desse trabalho e por sempre estarem dispostos a me auxiliar.

À Dra. Ana Cristina Fiori-Tutida e ao Dr. Roberto Giacomino Chapola, por aceitarem o convite e pelas contribuições em minha banca de defesa.

Aos técnicos do PMGCA/UFPR em Paranavaí, Fabinho, Guilherme, Alessandro, Mineiro, Ailton, Carlos e Alexandro, bem como à equipe de campo, em especial ao Alexandre (em memória), pela ajuda e colaboração para que os ensaios fossem realizados, e à Vera, pela amizade e dedicação em todos os momentos que estivemos juntas.

Aos técnicos do LAEM e LEMID, Izabel, Tiago e Simone, por toda a ajuda nesses 4 anos de trabalho.

À minha estagiária Mayara, por ter sido dedicada e responsável na condução dos experimentos.

Aos meus grandes amigos Anderson, Eloísa e Jeferson, por me mostrarem o quanto a fitopatologia é incrível e me apoiarem, dando todo o suporte que precisei na mudança de linha de pesquisa, devo isso a vocês.

Aos meus amigos Marlon, Xadu, Leo e Fabio por todo apoio, momentos de descontração e conversas aleatórias cheias de sentido que por muitas vezes me ajudaram a ter pontos de vista diferentes sobre os problemas da vida e assim chegar a uma solução.

Aos meus colegas e amigos do LEMID, que conheci por ocasião do doutorado, mas que permanecerão por toda a vida. Obrigada por estarem ao meu lado nessa trajetória, por poder compartilhar minhas alegrias e sempre ser amparada nos momentos de dificuldade, sem vocês tudo seria muito mais difícil.

À secretária do Programa de Pós-Graduação da UFPR, Lucimara Antunes, sempre muito solícita em ajudar todos os alunos com a parte burocrática da pós-graduação.

A todos os professores do Programa de Pós-Graduação em Produção Vegetal da Universidade Federal do Paraná.

À Universidade Federal do Paraná e ao Curso de Pós-Graduação em Produção Vegetal, pela oportunidade de aprendizado e realização do doutorado.

À RIDESA e ao PMGCA/UFPR, pelo suporte para que meus experimentos pudessem ser concluídos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

A todos, que porventura não tenha citado, mas que de alguma maneira contribuíram para que tudo isso se tornasse realidade.

“The scientist is not the man who provides the true answers; is the one who asks the real questions”. (Claude Lévi-Strauss)

RESUMO

O Brasil é o maior produtor mundial de cana-de-açúcar, com aproximadamente 40% do total da produção mundial da cultura. Dentre os fatores que causam reduções na produtividade, destacam-se as doenças, em especial a ferrugem marrom (*Puccinia melanocephala*), ferrugem alaranjada (*Puccinia kuehnii*) e o carvão (*Sporisorium scitamineum*). As ferrugens são causadoras de pústulas salientes na face abaxial das folhas, gerando redução dos níveis de fotossíntese e, consequentemente, da produção. O carvão pode gerar redução da quantidade e da qualidade do açúcar produzido, além da morte dos colmos e redução da longevidade do canavial, por meio da produção de estruturas denominadas chicotes. Em cana-de-açúcar, o principal método de controle de doenças é a resistência genética, para tanto, os programas de melhoramento da cultura buscam genótipos que aliem resistência às principais doenças a altas produtividades. Contudo, os métodos para avaliação do comportamento dos genótipos em relação à resistência às ferrugens e ao carvão não são padronizados, sendo que novas técnicas como a utilização de marcadores moleculares podem ser utilizadas em prol da maior eficiência no desenvolvimento de cultivares de cana-de-açúcar. Dessa maneira, o presente estudo teve por objetivo: i) verificar a resposta de genótipos de cana-de-açúcar às ferrugens alaranjada e ferrugem marrom e obter a classificação destes quanto a sua resistência; ii) validar o marcador molecular G1 na detecção de resistência à ferrugem alaranjada em genótipos brasileiros de cana-de-açúcar, iii) propor uma metodologia padronizada para classificação de genótipos de cana-de-açúcar quanto à sua resistência ao carvão (*S. scitamineum*) e apresentar a reação de 35 genótipos brasileiros de cana-de-açúcar à doença. Os resultados encontrados nessa pesquisa mostraram que: i) Dos genótipos avaliados para ferrugem marrom, 4 foram classificados como suscetíveis, 6 como moderadamente suscetíveis e 1 apresentou resistência moderada. Para a ferrugem alaranjada, 3 genótipos foram classificados como suscetíveis, 7 como moderadamente suscetíveis e 1 como moderadamente resistente. A avaliação e classificação de genótipos de cana-de-açúcar é uma ferramenta que auxilia em ensaios preliminares e escolha de genótipos promissores para fases mais avançadas dos programas de melhoramento e fornece informações aos produtores na escolha das cultivares que serão plantadas; ii) Dos 63 genótipos suscetíveis, 27 apresentaram presença de banda relativa ao marcador molecular G1. Dos 17 genótipos classificados como resistentes por meio do teste a campo, 10 não apresentaram a respectiva banda. Foi calculado o coeficiente Phi (ϕ) de correlação, por meio do qual observou-se que não existe correlação estatisticamente significativa entre resistência fenotípica e presença do marcador. De acordo com o observado, G1 não apresenta potencial de uso em seleção assistida para resistência de cana-de-açúcar à ferrugem alaranjada nas condições testadas; e iii) A maior parte dos genótipos testados para carvão (~78%) apresentou algum nível de resistência, dessa maneira, o método para a obtenção de resultados mais reproduzível quanto a classificação de resistência de genótipos de cana-de-açúcar ao carvão é recomendar para novos ensaios utilizar o genótipo padrão de suscetibilidade RB935621 e pelo menos um dos padrões de resistência (RB985476, CV6945 e RB956911), avaliar a incidência de chicotes em intervalos aproximados de 30 dias, partindo do surgimento dos primeiros chicotes, até por volta dos 250 dias da cultura, quando o número de perfilhos tende a se estabilizar, obter a AACPD e consequentemente a percentagem relativa da doença e no final enquadrar os genótipos quanto ao seu nível de resistência de acordo com os intervalos de cada nível de resistência ao carvão da cana-de-açúcar.

Palavras-chave: *Puccinia melanocephala*. *Puccinia kuehnii*. *Sporisorium scitamineum*. Resistência genética. Screening.

ABSTRACT

Brazil is the world's largest sugarcane producer with approximately 40% of the world's total crop production. Among the factors that reduce productivity, diseases stand out, particularly brown rust (*Puccinia melanocephala*), orange rust (*Puccinia kuehnii*) and sugarcane smut (*Sporisorium scitamineum*). The rusts originate salient pustules in the abaxial face of the leaves reducing photosynthesis levels, therefore, production. The smut can reduce quality and quantity of sugar produced, besides the death of the culms and reduction of the longevity of the cane field through production of structures called "whips". In sugarcane the main method of disease control is genetic resistance, therefore, the culture improvement programs look for genotypes that ally resistance to the main diseases and high productivity. However, the evaluation methods of genotype behavior regarding their resistance to rusts and sugarcane smut are not standardized, in view of new techniques such as the usage of molecular markers can be used for higher efficiency in sugarcane cultivars development. As such, the present study aimed: i) verify the reaction of sugarcane genotypes to brown and orange rust in terms of their resistance using leaf whorl inoculation technique; ii) validate the molecular marker G1 in the detection of resistance to orange rust in Brazilian sugarcane genotypes, iii) propose a standardized methodology for sugarcane genotypes classification regarding their resistance to smut (*S. scitamineum*) and present the reaction of 35 Brazilian sugarcane genotypes to the disease. The results found in this research show that: i) of the genotypes evaluated for brown rust, 4 were classified as susceptible, 6 as moderately susceptible and 1 showed moderate resistance. For orange rust, 3 genotypes were classified as susceptible, 7 as moderately susceptible and 1 as moderately resistant. The evaluation and classification of sugarcane genotypes is a tool that aids preliminary tests and selection of promising genotypes for more advanced steps in the improvement programs and provides producers information in the choice of cultivars to be planted; ii) Of the 63 susceptible genotypes, 27 exhibited presence of relative band to the molecular marker G1. Of the 17 genotypes classified as resistant through field test, 10 did not presented such band. The correlation Phi (ϕ) coefficient wherewith was observed that there is no statistically significant correlation between phenotypic resistance and the presence of the marker. According to the observed, G1 does not presents usage potential for assisted selection of sugarcane resistance to orange rust in the tested conditions; and iii) Most of the genotypes tested for smut (~78%) presented some level of resistance, in this way, the most reproducible method of obtaining results regarding resistance screening of sugarcane genotypes to smut is to recommend for new tests to use the standard susceptible genotype RB935621 and at least one of the resistance standards (RB985476, CV6945 e RB956911), evaluate the whips incidence at approximately 30-day intervals, starting from the emergence of the whips, until around 250 days of culture, when the number of tillers tends to stabilize, obtain the AUDPC and consequently the relative percentage of the disease and in the end fit the genotypes regarding their resistance level according to the intervals on each level of resistance to sugarcane smut.

Keywords: *Puccinia melanocephala*. *Puccinia kuehnii*. *Sporisorium scitamineum*. Genetic resistance. Screening.

CONTENTS

1. GENERAL INTRODUCTION

Sugarcane is one of the most important crops in Brazil, being the world's largest producer currently (FAOSTAT, 2021), with 760 million t estimated production in 2019/20 harvest and 77,3 t ha^{-1} average productivity (CONAB, 2020) corresponding to approximately 40% of the worldwide production (FAOSTAT, 2021). In the state of Paraná, the sugarcane plantation occupied a total area of 516,6 thousand hectare in the 2019/20 harvest, being among the largest producing states of the country (CONAB, 2020).

Since the introduction of the culture in Brazil, there were significant changes in the cultivars genetic improvements, in addition to the increase of the production areas, thereby, over the years, greater socio-environmental restrictions have been imposed around the handling of the cane field, like the pre-harvest burning. These factors contributed to the occurrence of conditions that favor the development of diseases, which made them become the second most cause for reduced productivity and quality of the raw material produced (SIMÕES NETO et al., 2016). Among the fungal diseases, three stand out: orange rust, brown rust and sugarcane smut.

The causal agent of brown rust is the fungus *Puccinia melanocephala* (H. and P. Syd), mandatory parasite of the leaf tissues and is present in almost all producing regions of the world. This disease had great importance in Mexican crops in the early 1980s, with losses of up to 50% in the culms production being recorded (PURDY et al., 1983). In Brazil, the most expressive epidemic occurred in the year of 1992, when nearly US$107 mi in losses were recorded, corresponding to 8,74% of the production because the main cultivars planted were susceptible to the pathogen (MOURA et al., 1999).

The orange rust, caused by the fungus *Puccinia kuehnii* (W. Krüger) E. J. Butler, had great importance in the Australian cultivars in the 2000s, where it caused a significant reduction in production (24%) of the culture in Australia, in need of replacement of the most planted cultivar of the country that was very susceptible to the disease (about 45% of the cultivated area). The losses caused that year were estimated in 210 million dollars, and there are reports of up to 50% in reduced yield in the sugarcane fields affected by the disease (MAGAREY et al., 2001). In Brazil, the orange rust was first detected in the 2009/10 crop in the city of Araraquara, SP, leading to replacement of four important cultivars in Brazil, SP89-1115, SP84-2025, RB72454 (BARBASSO et al., 2010) and, few years later, SP81-3250 because they showed susceptibility to the disease. It is present in all Brazilian producing regions and is considered one of the main diseases that affects the cane fields because their spores are easily

spread by the wind (ZHAO et al., 2011). When infected by the rusts, the sugarcane leaves exhibit salient pustules with yellowish to brownish color, with rupture of the leaf blade epidermis, generating reduction in productivity due to reduction of chlorophyll, photosynthetic efficiency, transpiration rates and leaf photosynthesis (ZHAO et al., 2011). The pustules can be distributed throughout the leaf blade, being that the brown rust exhibit elongated lesions, focusing in the middle portion towards the tips of the leaf blade (MINCHIO et al., 2011), and the orange rust shows smaller lesions, focused in the base towards the middle of the leaves (MAGAREY et al., 2001; KLOSOWSKI et al., 2013).

The sugarcane smut is caused by the fungus *Sporisorium scitamineum* (Syd.) and was one of the first diseases reported in the culture, in 1877, in South Africa (RICAUD et al., 1989). Since then, the disease has spread to other sugarcane producers in the world, arriving in Brazil in the year of 1946, infecting primarily sugar cane fields in São Paulo state and spreading to all sugarcane producing regions of Brazil (BERGAMIN FILHO et al., 1987). The damages caused by the disease to the crops occurs due to the reduction of the quality and amount of juice, death of the culms and the need of early renewal of the cane field, or yet making unfeasible the use of more productive cultivars since the resistance of the planted genotypes is one of the determining factors in the incidence of the disease, besides the presence of the initial inoculum and occurrence of favorable environmental conditions for the development of the pathogen (SANTOS et al., 2004; MANSOOR et al., 2016; HUANG et al., 2018).

The recognition of the disease is made by noting the emergence of a typical filiform structure, called “whip”, this structure being the result of a modification of the plant meristem, induced by the causal agent. The whips vary in size and can reach more than one meter in length and are composed of a central column consisting parenchymal elements and vascular bundles, covered by the fungus teliospores, which are initially protected by a silvery membrane that breaks, exposing the teliospores, which are easily carried by the wind and rainwater (RICAUD et al., 1989). In very susceptible cultivar, the disease symptoms can be observed before the appearance of the whip, through changes in the plant growth habit, which could present clump overgrowth, thinning of the culms, more upright tillers with leaf and internode shortening and discoloring of the internal culm tissues (BERGAMIN FILHO et al., 1987, RICAUD et al., 1989, COMSTOCK, 2000).

The usage of genetic resistance in sugarcane is considered one of the best disease control methods (TOKESHI and RAGO, 2005). The development of resistant cultivars happens by transferring resistance alleles from parents with known characteristics, however, new resistant

cultivars need ongoing development due to the plant-pathogen interaction, that leads to a co-evolution between pathogen and host (ALZATE-MARIN et al., 2005).

The breeding process is carried out over a period of 12-15 years of selection and field experimentation (MATSUOKA et al., 2009). The disease resistant genotype selection is commonly based on phenotypic evaluation of genotypes after the occurrence of natural infection and artificial inoculation can be used in the final stages (when the number of genotypes is reduced), or yet in the greenhouse (SOOD et al., 2009). This type of selection demands space, time, trained staff, favorable environmental conditions for the occurrence of the disease and rely on the inoculation success (SOOD et al., 2013).

The molecular markers can be quite useful in the alleles transfer process, because, if they are strongly linked to the resistance alleles, they can be used in the marker-assisted selection (MAS) process (ALZATE-MARIN et al., 2005). MAS is the usage of markes that are strictly linked to genes of interest, which facilitates target gene identification in the populations derived from countless crosses, basing, in this way, characterization of a given genotype in their genotypic characteristics (PATHANIA et al., 2017). The screening of genotypes using molecular techniques in sugarcane has been employed for some diseases, like root rot caused by *Pachymetra chaunorhiza* (MCINTYRE et al., 2005) and brown rust (*Puccinia melanocephala*) (BARRETO et al., 2017).

In sugarcane improvement programs, genotypes susceptible to major diseases are eliminated, not advancing to subsequent selection stages. However, those with intermediate resistance reaction must be studied if their productivity characteristics are interesting (IDO et al., 2006). Therefore, the efficiency of the evaluation process and classification of sugarcane genotypes as for resistance to major diseases is of utmost importance for improvement programs and also for technicians and producers, once knowing the reactions of genotypes assists in choosing and positioning of cultivars in adequate places and planting times.

In this way, the present study aimed to: i) verify the reaction of sugarcane genotypes to brown and orange rust in terms of their resistance using leaf whorl inoculation technique; ii) validate the G1 molecular marker in detecting the resistance to orange rust in Brazilian sugarcane genotypes; iii) propose a standardized methodology for sugarcane genotype classification as of their resistance to sugarcane smut (*S. scitamineum*) and present the reaction of 41 sugarcane genotypes to the disease.

2. CHAPTER I - REACTION OF SUGARCANE GENOTYPES TO BROWN AND TO ORANGE RUST BY LEAF WHORL INOCULATION[1]

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[1] This manuscript is published on Ciência Rural.

ABSTRACT

In this research eleven sugarcane genotypes were classified in relation to their resistance to brown rust, and eleven to their resistance to orange rust. Artificial inoculation was carried out in the leaf whorl of 165-day-old plants in the city of Paranavaí, Paraná State, Brazil, in 2017. The evaluation was performed 30 days after inoculation, using a rating scale. Among the genotypes tested for brown rust, four were classified as susceptible, six as moderately susceptible and one presented moderate resistance. For orange rust, three genotypes were classified as susceptible, seven as moderately susceptible and one as moderately resistant. The evaluation and classification of the reaction of sugarcane genotypes to the rusts is an important tool that assist in preliminary trials and selection of promising genotypes for more advanced stages of breeding programs and provides information to producers on the choice of cultivars to be planted.

Key words: *Saccharum* spp**,** *Puccinia melanocephala*, *Puccinia kuehnii*, classification, resistance.

RESUMO

Neste trabalho onze genótipos de cana-de-açúcar foram classificados em relação à sua resistência à ferrugem marrom, e onze quanto à resistência à ferrugem alaranjada. Foi realizada a inoculação no "cartucho foliar" de colmos com 165 dias, no município de Paranavaí, PR, em 2017. A avaliação foi feita aos 30 dias após a inoculação, utilizando uma escala de notas. Dos genótipos avaliados para ferrugem marrom, quatro foram classificados como suscetíveis, seis como moderadamente suscetíveis e um apresentou resistência moderada. Para a ferrugem alaranjada, três genótipos foram classificados como suscetíveis, sete como moderadamente

suscetíveis e um como moderadamente resistente. A avaliação e classificação da reação de genótipos de cana-de-açúcar às ferrugens é uma ferramenta importante que auxilia em ensaios preliminares, na escolha de genótipos promissores para fases mais avançadas dos programas de melhoramento além de fornecer informações aos produtores na seleção das cultivares que serão plantadas.

Palavras-chave: *Saccharum* spp.**,** *Puccinia melanocephala*, *Puccinia kuehnii*, classificação, resistência.

Sugarcane is an important crop in Brazil, with a production estimated in 747 million tons in the 2017/2018 harvest, which corresponds to about 39% of the world total, giving the country the title of largest world producer (FAOSTAT, 2020). Yield is influenced by factors such as crop genetic characteristics, climatic factors, soil physicochemical characteristics, nutrient availability, pests, weeds and diseases (GILBERT et al. 2006; LIMA et al. 2017).

Among the diseases, the rusts stand out. Brown rust, caused by the fungus *Puccinia melanocephala* Syd. & P. Syd., was first reported in Brazil in the municipality of Capivari (SP), in 1986, and spread rapidly throughout the other producing regions of the country (AMORIM et al. 1987). It promotes reduction of photosynthetic area mainly in plants aged 4 to 7 months causing delay in plant development in susceptible cultivars, being reported yield reductions of 10 to 50% (MAGAREY et al. 2001). Orange rust, caused by *Puccinia kuehnii* (W. Krüger) E. J. Butler, was first observed in Brazil in 2009, when it affected the production of three highly susceptible cultivars, SP89-1115, RB72454 and SP84-2025, corresponding to 10% of sugarcane plantations in the country at the time (BARBASSO et al. 2010). Like *P. melanocephala*, the fungus produces pustules on the leaf surface, reducing photosynthetic activity, production and quality of the final product (ZHAO et al. 2011). In Brazil, yield reduction have ranged 20 to 40% tons of cane per hectare in susceptible cultivars, caused by this disease (CRUZ et al. 2014).

Although, fungicide application is employed as an emergency technique, the main control method of sugarcane rusts is the use of resistant cultivars (ROTT et al. 2016). For such a control measure to be adopted efficiently, it is necessary to know the level of genotype resistance to diseases. Most of the studies on classification of Brazilian genotypes regarding their resistance to diseases are dedicated to orange rust (ARAÚJO et al. 2013; KLOSOWSKI et al. 2015; CHAPOLA et al. 2016; URASHIMA et al. 2018), with those focusing on brown rust being prior to the arrival of *Puccinia kuehnii* in the country (IDO et al. 2006). Thus, the

objective of this study was to verify the reaction of sugarcane genotypes to brown and orange rust in terms of their resistance using leaf whorl inoculation technique.

Two experiments (one experiment for each disease) were conducted in 2016/17 for evaluation of genotypes in relation to resistance to brown rust and to orange rust in an experimental area in the municipality of Paranavaí, Paraná, Brazil (23°05' S; 52°26' W, 470 m asl), where a meteorological station was also installed to obtain climate data. The design used in both experiments was that of casualized blocks, with eleven treatments and four repetitions. The experimental unit consisted of two plants per genotype, spaced by 0.5 m, and 2 m between treatments.

The genotypes evaluated for brown rust were: CTC-4, RB835486, RB966229, RB036065, RB036147, RB056388, RB106803, RB106811, RB106814, RB106819, RB106822; and for orange rust were: SP81-3250, RB72454, RB006629, RB036059, RB036145, RB036153, RB036163, RB056388, RB106803, RB106819, RB106822. Genotypes with known reaction to rust were included in the experiments. For brown rust, CTC-4 were used as susceptible standard; while RB106819 were used as resistance standards. For orange rust, susceptible standard were RB72454 and RB106819 as resistant standard. The other genotypes were chosen for their genetic potential of interest for the sugarcane improvement program.

The seedlings used came from individualized buds, which were planted in tubes containing commercial substrate and filter cake in a ratio of 1:1, on October 19, 2016, and kept in a greenhouse with irrigation by sprinkling until 60 days. After this period, the seedlings were manually transplanted to the field.

To obtain spore suspension for plant inoculation, urediniospores of *P. melanocephala* and *P. kuehnii* were collected in the experimental station of UFPR in Paranavaí, PR, using a vacuum pump and a glass collector at 15 days before inoculation by aspirating the surface of symptomatic leaves from susceptible cultivars, as described by SOOD et al. (2009) and stored in a freezer (± 2 °C). The preparation of suspensions of the two rusts occurred in the same way, adding the spores in distilled water and shaking, in order to homogenize each suspension. The concentration was adjusted to 10^4 viable spores mL^{-1} using a hemacytometer (Neubauer chamber, Optik Labor, Germany) (SOOD et al. 2009), adding 0.1% of Tween 20 to the final volume of each suspension.

Inoculation was performed 105 days after transplanting the seedlings to the field, using 0.5 mL of suspension (water + spores + Tween 20), which was placed separately inside the leaf whorls of six individual stalks per replicate using a repeater pipette, being inoculated the three

tallest stalks of each plant, which were identified for further evaluation by cutting 1/3 of the top of the tallest leaves.

After 30 days of incubation, the inoculated leaves were evaluated, after they emerged from the whorl, where the symptoms were demonstrated as a band of pustules (in susceptible varieties). The symptoms of rust were assessed on the 0-4 scale (SOOD et al. 2009), with 0 - no symptoms, 1 - chlorotic flecks, 2 - orange-brown lesions, without sporulation, 3 - one to five pustules with sporulation (production of urediniospores), and 4 - six or more coalescent pustules with sporulation resulting in leaf necrosis. Treatments were analyzed based on the averages obtained in the field and classified according to the modified SOOD et al. (2013) scale, in which the genotypes with 0 - 1 notes were considered resistant; 1.1 - 2, moderately resistant; 2.1 - 3, moderately susceptible and 3.1 - 4, susceptible.

The average temperature between the inoculation and plant evaluation (30 days) was 21.5 ºC, with a minimum of 15.5 ºC and a maximum of 25.7 ºC, and according to SANJEL et al. (2019) the ideal mean temperature for the development of both rusts is between 20 and 22.2 °C.. Average relative humidity of air during 30 days was 80%, with accumulated precipitation of 177.6 mm.

In the inoculated stalks, the symptoms were observed in bands of pustules well defined in the youngest leaf, the one that had contact with the urediniospores and its growth occurred between the inoculation and the evaluation. Based on the evaluation method proposed by SOOD et al. (2009), of the genotypes tested for brown rust, four were classified as susceptible, six as moderately susceptible and only one showed moderate resistance. Genotypes inoculated with *P. kuehnii* showed a similar pattern, three of them were classified as susceptible, seven as moderately susceptible and one as moderately resistant (Table 1).

In this study, it was observed that there are differences between the resistance levels of the genotypes for both brown and orange rust. SOOD et al. (2013) pointed out that the leaf whorl artificial inoculation of *P. melanocephala* and *P. kuehnii* can be performed even at seasons of the year when rust spores are not available in the field, because preserved urediniospores can be used. Because it is carried out in the field, leaf whorl inoculation may be a way to take advantage of routine trials of sugarcane breeding programs, in order to expose the genotypes to a condition favorable to the occurrence of the disease, assisting in preliminary trials and the selection of promising genotypes for more advanced phases of the programs (SOOD et al. 2009). In addition, the evaluation and classification of genotypes in relation to their resistance provides information for producers on the choice of cultivars to be planted.

Table 1. Brown and orange rust rating of sugarcane genotypes based on leaf whorl artificial inoculation

Brown rust			Orange rust		
Genotype	Score*	Rating	Genotype	Score*	Rating
RB966229	4.00	Susceptible	RB036145	4.00	Susceptible
RB056388	3.96	Susceptible	RB106803	3.96	Susceptible
RB835486	3.75	Susceptible	SP81-3250	3.88	Susceptible
RB036065	3.25	Susceptible	RB106822	3.04	Moderately susceptible
CTC-4	3.00	Moderately susceptible	RB056388	3.00	Moderately susceptible
RB106803	3.00	Moderately susceptible	RB72454	3.00	Moderately susceptible
RB106822	3.00	Moderately susceptible	RB036163	2.96	Moderately susceptible
RB106811	2.92	Moderately susceptible	RB036153	2.83	Moderately susceptible
RB106814	2.88	Moderately susceptible	RB106819	2.83	Moderately susceptible
RB036147	2.75	Moderately susceptible	RB036059	2.75	Moderately susceptible
RB106819	2.00	Moderately resistant	RB006629	2.00	Moderately resistant

* Mean severity score of 6 observations per treatment, according to the modified scale of SOOD et al. (2013) in which: scores of 0 - 1: resistant; 1.1 - 2: moderately resistant; 2.1 - 3: moderately susceptible and 3.1 - 4: susceptible.

Acknowledgements

This study was financed in part by the 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil' (CAPES) – Finance Code 001 and 'Rede Interuniversitária para o Desenvolvimento Sucroalcooleiro (RIDESA/PMGCA/UFPR)'. The seventh author received research fellowship from National Council for Scientific and Technological Development (CNPq)/Brazil.

Declaration of conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Authors' Contributions

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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3. CHAPTER II - VALIDATION OF THE G1 MOLECULAR MARKER ASSOCIATED WITH RESISTANCE TO ORANGE RUST IN BRAZILIAN SUGARCANE GENOTYPES[2]

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[2] This manuscript is published on Sugar Tech journal.

ABSTRACT

Orange rust, caused by the fungus *Puccinia kuehnii* is an important disease of sugarcane (*Saccharum* sp.). A molecular marker (G1) was developed to identify the resistance of sugarcane to orange rust. The aim of the present study was to validate the molecular marker G1 in the detection of resistance to orange rust in Brazilian sugarcane genotypes. 80 genotypes were analyzed by PCR, being classified according to the presence or absence of the marker. For field evaluation of orange rust resistance, a randomized block experiment was conducted, with the same 80 genotypes using four replications. Artificial inoculation was performed in the leaf whorl, with an evaluation made at 30 days after inoculation, using a scale from zero (without symptoms) to four (more than five pustules with sporulation), in which cultivars with an average score between zero and two were classified as resistant, and a score greater than two, susceptible. Of the 17 genotypes classified as resistant through the field test, 10 did not present the respective band. Of the 63 susceptible genotypes, 27 had the band relative to the molecular marker G1. The selection accuracy of G1 marker (the efficiency of predicting resistant phenotype) was 22.86%. The Phi correlation coefficient (ϕ) was calculated, through which it was observed that the variables are independent, with no statistically significant correlation between them. The results indicate that the G1 marker had a low efficiency to predict the resistance to orange rust in clones under selection in a Brazilian breeding program and Brazilian commercial cultivars of sugarcane.

Key words: *Saccharum* spp., *Puccinia kuehnii*, marker-assisted selection, MAS.

3.1 Introduction

Sugarcane is one of the most important crops in Brazil, and the country is currently the world's largest producer, with an estimated production of 747 million tons in the 2018 harvest, followed by India, China and Thailand (FAOSTAT, 2020). Brazilian production corresponds to approximately 40% of the world total, and the country stands out with the largest planted area (approximately 10 million hectares), and an average productivity of 75.78 ton ha^{-1} (CONAB, 2019).

The sugarcane production is influenced by genetic characteristics of cultivars, climate, availability of nutrients, attack by pests and incidence of diseases, among other factors (Lima et al. 2017). Among fungal diseases, orange rust, caused by *Puccinia kuehnii* (Krüger) Butler, is present in several sugarcane producing countries, being considered one of the main diseases affecting cane fields, as it produces pustules on the leaf surface, reducing photosynthetic activity and, consequently, the yield and quality of the final product (Zhao et al. 2011).

The use of genetic resistance in sugarcane is considered one of the best methods of disease control (Tokeshi and Rago 2005). The development of resistant cultivars usually occurs by transferring resistance alleles from parents with known characteristics. Thus, new resistant cultivars need to be constantly developed, due to the plant-pathogen interactions, which lead to a co-evolution between the pathogens and the host (Alzate-Marin et al. 2005).

The sugarcane breeding process is carried out over a period of 12 to 15 years of field selection and experimentation (Matsuoka et al. 2009). The selection of genotypes resistant to orange rust is based on the phenotypic evaluation of the genotypes after the occurrence of natural infection of the disease and artificial inoculation can be performed in the final stages (when less genotypes are evaluated), in the field or in the greenhouse (Sood et al. 2009). However, this type of selection demands space, time, trained staff, favorable environmental conditions for the occurrence of the disease and depends on the success of inoculations (Sood et al. 2013).

Molecular markers can be very useful in the allele transfer process, because if they are strongly linked to resistance alleles, they can be used in the marker assisted selection process (MAS - Marker-assisted selection) (Alzate-Marin et al. 2005). MAS is the use of markers that are tightly linked to genes of interest for selection of desirable genotypes instead, or in addition, to phenotypic screening (Pathania et al. 2017). The main advantages of using molecular markers in a breeding program are: time saving, consistency, efficiency and more accurate selection of complex traits (Jena and Mackill, 2008). The classification of genotypes using molecular techniques in sugarcane has been used for some diseases such as root rot caused by *Pachymetra*

chaunorhiza (Mcintyre et al. 2005) and brown rust (*Puccinia melanocephala*) (Barreto et al. 2017).

G1 was the first molecular marker associated with the resistance of sugarcane to orange rust. Its development was based on the study of 173 F1 progenies derived from crossing between clones CP95-1039 and CP88-1762 (Sugarcane Field Station at Canal Point, FL, United States) (Yang et al. 2018a). Three QTLs were identified, controlling resistance to orange rust (qORR109, qORR4 and qORR102), which separately explain 58, 12 and 8% of the phenotypic variation. Putative resistance (R) genes were characterized, and, together with the single sequence repeat (SSR) in the QTL qO109 intervals, were used for the development of the G1 diagnostic marker (Yang et al. 2018a). Other molecular markers associated with the resistance of sugarcane to orange rust were identified in an experiment using genotypes from the germplasm collection of the Sugarcane Research Station in Canal Point (FL, United States) in which, of the ten main molecular markers reported, the maximum amount of variation explained by a single marker was 10.7% (McCord et al. 2019).

The ideal for the use of molecular markers associated with disease resistance is that they explain most of the variation of the phenotypic characteristic (McCord et al. 2019). Considering this prerogative, the G1 marker has the potential for use in sugarcane breeding programs in other producer countries. However, for a molecular marker to be used in conditions different from those in which it was developed, its validation is necessary, confirming that, even in different genotypes and populations of the pathogen, there is still an association between the presence of the marker and resistance to the disease (Barreto et al. 2017).

Thus, the objective of the present study was to validate the molecular marker G1 in the detection of resistance to orange rust in Brazilian sugarcane genotypes.

3.2 Material and Methods

To validate the association between the G1 marker (Yang et al. 2018a) and the resistance of sugarcane genotypes to orange rust, two steps were performed. The first one consisting of molecular analysis to use the G1 marker to detect the orange rust resistant genotypes and the second, the artificial whorl inoculation and subsequent classification of genotypes in relation to their field resistance to orange rust.

The 80 genotypes used were chosen based on their economic and genetic importance within the breeding program (Table 1). In this group of genotypes are some of the main cultivars grown in Brazil and clones in different phases of the breeding program (PR001 to PR066) of the Sugarcane Genetic Improvement Program at the Federal University of Paraná

(PMGCA/UFPR), a program linked to RIDESA (Inter-University Network for the Development of the Sugarcane Industry).

Table 1. Background of the 80 genotypes evaluated for orange rust.

Genotype	Parental 1	Parental 2	Genotype	Parental 1*	Parental 2*
CB41-76	POJ2878	?	PR027	?	?
CTC4	SP83-5073	?	PR028	RB863129	SP832847
RB006970	RB855536	SP80-1816	PR029	RB935903	?
RB036066	SP70-1143	SP77-5181	PR030	SP801842	?
RB036088	RB855511	?	PR031	RB855002	?
RB036145	SP83-2847	TUC71-7	PR032	SP832847	RB855127
RB066498	RB966229	RB825548	PR033	RB956911	?
RB72454	CP53-76	?	PR034	RB961003	?
RB867515	RB72454	?	PR035	BJ7015	?
RB92579	RB75126	RB72199	PR036	RB855206	?
RB946903	RB765418	RB72454	PR037	RB855063	?
RB956911	RB855206	RB855035	PR038	RB855206	?
RB966928	RB855156	RB815690	PR039	SP801816	?
RB985476	H53-3989	RB855206	PR040	-	-
PR001	-	-	PR041	RB825548	RB931575
PR002	-	-	PR042	RB945099	?
PR003	-	-	PR043	RB966229	RB825548
PR004	-	-	PR044	RB835486	RB931556
PR005	-	-	PR045	CB2368	?
PR006	-	-	PR046	RB9655	?
PR007	-	-	PR047	SP703370	RB915079
PR008	-	-	PR048	SP701143	RB931555
PR009	-	-	PR049	RB855511	?
PR010	-	-	PR050	RB725143	RB931546
PR011	-	-	PR051	RB855511	RB966229
PR012	SP84-2066	SP80-165	PR052	RB867515	RB867515
PR013	-	-	PR053	RB867515	S1
PR014	RB855206	?	PR054	NA5679	?
PR015	SP80-1520	RB855536	PR055	RB931536	?
PR016	SP80-1816	RB855536	PR056	RB036066	?
PR017	SP80-1816	RB855536	PR057	RB867515	?
PR018	BJ7504	RB72454	PR58	L60-14	?
PR019	SP80-3280	RB855589	PR059	RB855113	?
PR020	-	-	PR060	F147	?
PR021	RB867515	SP80-3280	PR061	RB83102	RB72454
PR022	RB855589	?	PR062	CTC14	RB867515
PR023	SP83-5073	RB867515	PR063	RB988082	?
PR024	RB805203	?	PR064	RB912525	?
PR025	RB855546	RB962012	PR065	RB036066	?
PR026	SP931322	RB946903	PR066	Co62175	?

? = Unknown pedigree and - = Pedigree not available.

3.2.1 Analysis of G1 marker for orange rust resistance

Molecular analyses were performed at LAEM (Laboratory of Molecular Epidemiology) belonging to the Federal University of Paraná - UFPR, Sector of Agricultural Sciences, Curitiba, PR. Young leaves of each genotype were brought from the field and kept at -80 ° C until the moment of DNA extractions.

The total plant DNA was extracted according to the modified protocol proposed by Ferreira and Grattapaglia (1995) as described below: 250 mg of leaves without the central rib, were macerated in liquid nitrogen, and then 800 µL of CTAB buffer was added to the macerated tissue (CTAB 2%, NaCl 1.4M, PVP 10 -1.0%, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0 and 2% β-mercaptoethanol). The samples were incubated in a water bath at 65 ° C for one hour, shaking the tubes by inversion every 10 minutes. After removing the water bath, the samples were centrifuged at 5000 rpm at room temperature for 5 minutes. Then, a volume of approximately 500 µL of the supernatant was recovered, to which the same volume of chloroform: isoamyl alcohol (24: 1) was added. The samples were homogenized by inversion of the tubes and centrifuged at 12000 rpm at room temperature for 5 minutes. The upper phase was recovered, on average, a volume of 200 µL, to which 0.6 of the volume (120 µL) of chilled isopropyl alcohol was added, followed by homogenization by inversion of the tubes. The samples were stored in a freezer (-20 ° C) for one hour, and then centrifuged at 12000 rpm, at 6 ° C ± 1 ° C, for 20 minutes. Then, the supernatant was discarded, the DNAs were washed with 500 µL of cold 70% ethanol and centrifuged at 12000 rpm, at 6 ° C ± 1 ° C, for 10 minutes, after which the supernatant was discarded. The washing process was carried out three times, the last washing using absolute ethanol. Subsequently, the tubes were kept open in a flow chamber until the DNA dried. These were resuspended in 50 µL of autoclaved ultrapure water and the concentration of each sample was quantified in a spectrophotometer (Nanodrop® ND-1000 UV-VIS) where the concentration of 50 ng µL-1 was standardized. The samples were stored in a freezer at -20 ° C.

Each total DNA sample (150 ng) was subjected to touchdown PCR to amplify the G1 marker (Yang et al. 2018a) using the Forward: ACCATGGAAATCCATACGTC and Reverse: GGCCAACACTTAGGCCAATA primers. The reaction was carried out in a volume of 25 µL, according to the GoTaq® Master Mix protocol (Promega®), using the following concentrations: 1 X GoTaq® Master Mix (Promega®), 0.2 µM each of reverse and forward primers, 150 ng μL^{-1} of DNA template and ultrapure water up to 25 µL, and then following the touchdown PCR program described by Yang et al. (2018a). The PCR product was visualized on a 1% agarose gel stained with GelRed (Biotium). As positive controls, genotypes RB036088

and RB855156 (Fíer et al. 2020) were used, and as negative control, genotype RB066498 was used. The genotypes classified as positive (+) amplified a band corresponding to the G1 marker, with approximately 970 base pairs (bp) (Personal communication from Jianping Wang, 2019), while the genotypes identified as negative (-) did not present amplification of the 970 bp.

3.2.2 Evaluation of resistance to orange rust in the field

The experiment was carried out at the Experimental Station Paranavaí of the Federal University of Paraná (UFPR), in the municipality of Paranavaí, PR (23º05”S, 52º26” W, 470 m asl), in the period from 02/2019 to 02/2020. The soil is classified as a dystrophic Red Latosol, with smooth undulating relief (EMBRAPA 2006), the place has a Cfa-type climate, temperate, with at least 30 mm precipitation in the driest month, hot summer, average temperature of warmest month > 22 °C (Nitsche et al. 2019, FAO 2020).

The design used was randomized blocks with 80 genotypes and four replications. The experimental unit consisted of five seedlings per genotype, maintaining 0.5 m between seedlings and 1.5 m between rows. The seedlings used came from the planting of individualized buds in tubes containing commercial substrate: filter cake (1: 1). These were kept in a greenhouse with sprinkler irrigation until 60 days. After this period, the seedlings were transplanted to the field.

Urediniospores of *P. kuehnii* were collected in the experimental farm area with the aid of a vacuum pump and a glass collector on the day before inoculation by aspirating the abaxial face of symptomatic leaves from susceptible cultivars, as described by Klosowski et al. (2015). Spore viability was assessed in Petri dishes containing agar-water medium (2%). The plates were divided into four sections, accounting for 25 spores per section, totaling 100 spores per plate, according to Minchio et al. (2011). Spores whose length of the germ tube was greater than the diameter of the spore itself were considered “germinated” (Chapola et al. 2016).

To prepare the suspension of *P. kuehnii*, in distilled water, the spores were added and stirred, in order to homogenize the suspension. The concentration of this was adjusted to 1 x 10^4 viable spores mL-1 using a hemacytometer (Neubauer chamber, Optik Labor, Germany) (Sood et al. 2009). To the suspensions, 0.02% Tween20 in relation to the final volume was added.

The inoculation was done at 186 days after transplanting the seedlings in the field, in two stems per repetition, using 0.5 mL of the suspension, deposited with the aid of a pipette in the region of the apical meristem, called “leaf whorl”, as proposed by Sood et al. (2009). At 30 days after inoculation, genotypes were evaluated. To assign grades, a scale proposed by Sood

et al. (2009), according to symptoms verified in the symptomatic area of the leaves, being score 0 - absence of symptoms; score 1 - presence of chlorotic flecks; score 2 - orange colored lesions, without sporulation; score 3 - presence of one to five pustules with sporulation (production of urediniospores); and, score 4 - six or more pustules with sporulation joined, forming necrotic (coalescent) areas.

For the classification of genotypes, the average of the scores attributed to the culms of each of the treatments was calculated, categorizing the genotypes next, according to the classification of Sood et al. (2013), adapted by Yang et al (2018a), in which genotypes with scores of 0 - 2 were considered resistant; greater than 2, susceptible.

3.2.3 Statistical analysis

Efficiency of prediction of the resistant phenotype (selection accuracy) was obtained by dividing the number of both phenotypic and genotypic resistant genotypes by the number of genotypic resistant genotypes, and multiplying the result by 100 (Yang et al. 2018a). Furthermore, a bivariate analysis was performed to verify the association between the presence/absence of the molecular marker G1 and the resistance/susceptibility of the genotype to orange rust in the field. This association was verified using the Phi coefficient (ϕ), which can vary from zero to one, with values very close to zero indicating little association, and values closer to one, great association between variables. Chi-square (χ^2), with $\alpha = 5\%$ and degree of freedom = 1, was used as a test of statistical significance for the Phi coefficient (Lira and Chaves Neto 2008).

3.3 Results

The genotypes with the respective classification regarding resistance to orange rust and presence/absence of the amplification provided by the G1 molecular marker are shown in table 2. The genotypes classified as positive (+) for G1 were those in which the fragment relating to the molecular marker G1 was successfully amplified via PCR presenting an expected size of approximately 970 bp (Figure 1). The genotypes in which there was no amplification of the fragment corresponding to the marker were classified as negative (-) for G1.

Table 2. Classification of sugarcane genotypes according to the Sood et al. (2013), adapted by Yang et al (2018a), in which mean severity up to 2 was considered resistant and scores from 2.1 to 4 were called susceptible obtained by artificial inoculation of *Puccinia kuehnii* urediniospores in the leaf whorl and the presence (+) or absence (-) of the fragment amplified by the G1 marker.

Genotype	Average score	Field classification	G1	Genotype	Average score*	Field classification	G1
CB41-76	1.43 ± 0.33*	R	+	PR027	2.75 ± 0.44	S	-
CTC4	2.06 ± 0.09	R	-	PR028	3.38 ± 0.28	S	+
RB72454	3.09 ± 0.44	S	+	PR029	2.13 ± 0.14	S	+
RB867515	1.75 ± 0.38	R	+	PR030	2.14 ± 0.11	S	-
RB92579	3.00 ± 0.18	S	+	PR031	3.17 ± 0.20	S	-
RB946903	2.05 ± 0.53	R	-	PR032	2.19 ± 0.39	S	-
RB956911	3.62 ± 0.17	S	+	PR033	2.50 ± 0.27	S	+
RB966928	1.56 ± 0.28	R	+	PR034	2.37 ± 0.22	S	-
RB985476	1.81 ± 0.30	R	-	PR035	2.50 ± 0.53	S	-
RB006970	2.00 ± 0.27	R	-	PR036	2.94 ± 0.29	S	-
RB036066	1.67 ± 0.20	R	-	PR037	3.50 ± 0.27	S	-
RB036088	2.00 ± 0.09	R	+	PR038	2.43 ± 0.13	S	-
RB036145	3.71 ± 0.14	S	+	PR039	2.50 ± 0.22	S	-
RB066498	3.25 ± 0.13	S	-	PR040	2.75 ± 0.27	S	-
PR001	2.37 ± 0.25	S	+	PR041	2.63 ± 0.17	S	+
PR002	3.01 ± 0.20	S	-	PR042	1.94 ± 0.38	R	+
PR003	3.44 ± 0.25	S	-	PR043	3.02 ± 0.10	S	-
PR004	2.69 ± 0.14	S	-	PR044	2.44 ± 0.12	S	-
PR005	2.08 ± 0.11	S	-	PR045	3.07 ± 0.19	S	-
PR006	2.63 ± 0.25	S	-	PR046	3.19 ± 0.29	S	+
PR007	1.29 ± 0.42	R	-	PR047	3.21 ± 0.24	S	-
PR008	3.08 ± 0.32	S	+	PR048	2.44 ± 0.33	S	-
PR009	3.05 ± 0.12	S	-	PR049	1.87 ± 0.11	R	-
PR010	2.25 ± 0.18	S	-	PR050	3.62 ± 0.17	S	+
PR011	2.50 ± 0.22	S	-	PR051	3.62 ± 0.17	S	+
PR012	2.81 ± 0.24	S	-	PR052	2.71 ± 0.19	S	-
PR013	3.50 ± 0.18	S	-	PR053	3.50 ± 0.22	S	-
PR014	3.13 ± 0.23	S	+	PR054	2.56 ± 0.20	S	-
PR015	2.81 ± 0.24	S	+	PR055	3.09 ± 0.40	S	+
PR016	2.50 ± 0.13	S	+	PR056	2.43 ± 0.13	S	-
PR017	2.79 ± 0.37	S	-	PR057	2.75 ± 0.35	S	-
PR018	2.75 ± 0.33	S	+	PR058	3.81 ± 0.12	S	+
PR019	2.57 ± 0.23	S	-	PR059	3.14 ± 0.26	S	+
PR020	1.86 ± 0.12	R	+	PR060	2.50 ± 0.31	S	-
PR021	2.04 ± 0.27	R	-	PR061	2.19 ± 0.10	S	+
PR022	3.08 ± 0.22	S	-	PR062	2.02 ± 0.62	R	-
PR023	3.19 ± 0.29	S	+	PR063	2.81 ± 0.31	S	+

PR024	3.33 ± 0.16	S	+	PR064	2.31 ± 0.11	S	+
PR025	2.19 ± 0.38	S	+	PR065	1.69 ± 0.29	R	+
PR026	1.88 ± 0.23	R	+	PR066	2.10 ± 0.19	S	+

*Standand error

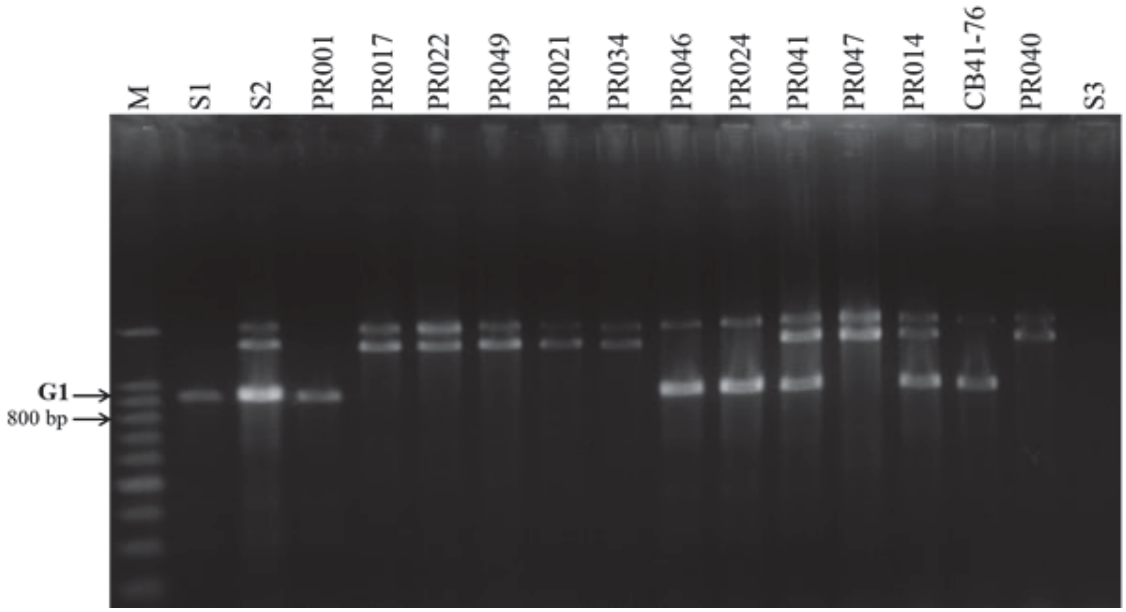

Figure 1. Electrophoretic pattern in agarose gel (1%) of the G1 molecular marker amplification product in sugarcane genotypes. **M,** 100 bp DNA Ladder (Sinapse); **S1,** positive standard 1 (RB036088); **S2,** positive standard 2 (RB855156); **S3,** negative standard (RB066498).

Of the 80 sugarcane genotypes analyzed, 35 (43.75%) were positive and 45 (56.25%) were negative for the presence of the molecular marker G1. This result shows that the presence of this marker is frequent among the analyzed genotypes. Of the 80 genotypes tested, 63 (about 79%) were classified as susceptible to orange rust and 17 (about 21%) were classified as resistant. Of the 63 susceptible genotypes, 28 (35%) showed a band relative to the molecular marker G1. Of the 17 genotypes classified as resistant through artificial inoculation in the leaf whorl, nine (about 53%) did not have the respective band (Figure 2). The number of sugarcane genotypes (frequency) in relation to the average scores for orange is in the Figure 3.

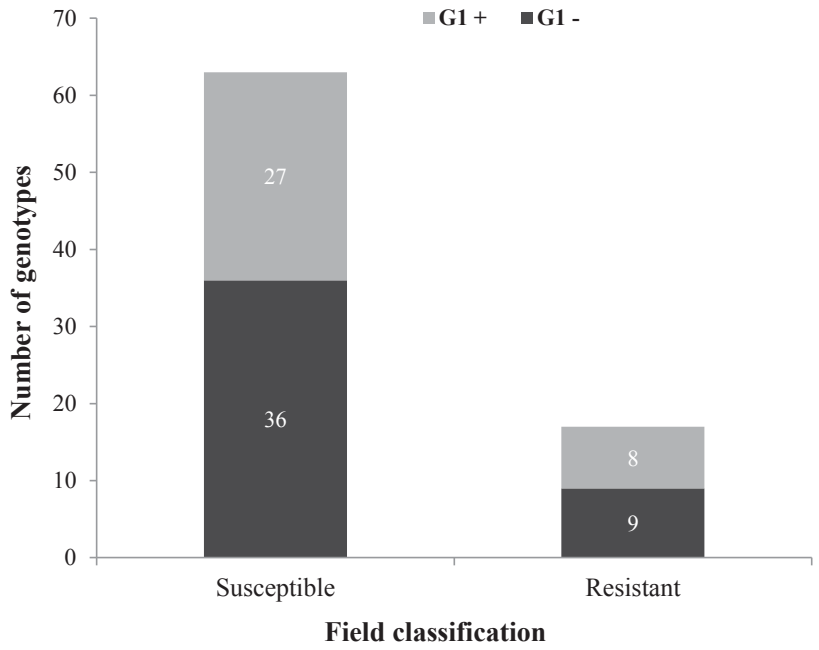

Figure 2. Classification of sugarcane genotypes to resistance to orange rust in the field and presence or absence of the G1 molecular marker.

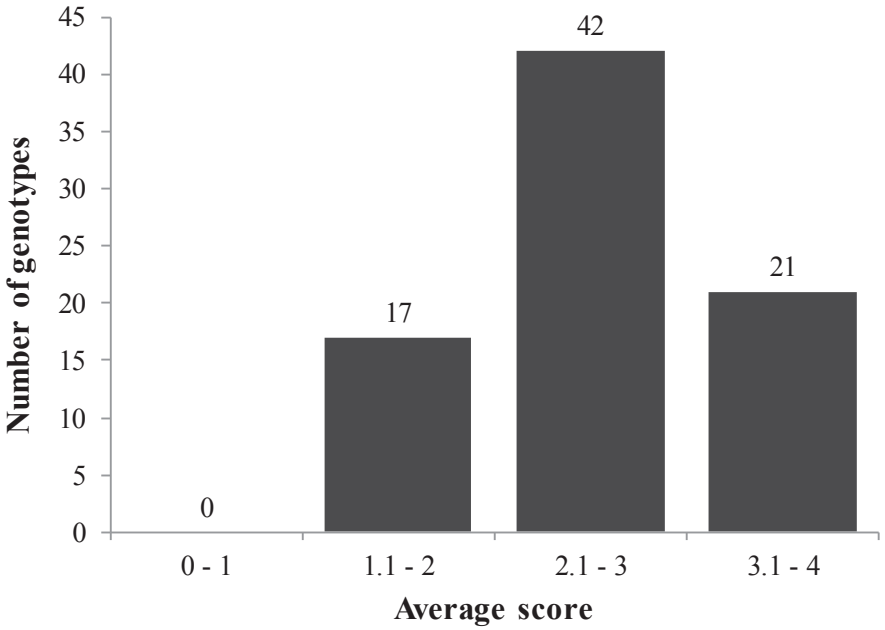

Figure 3. Number of sugarcane genotypes (frequency) in relation to the average scores for orange rust artificial inoculation obtained in the field.

The selection accuracy (efficiency in predicting the resistant phenotype) of G1 marker was 22.86% with 8 genotypes being resistant to orange rust (average score lower than 2) among the 35 genotypes that were positive to the presence of G1 marker. The mean score of resistance

among the genotype positive to G1 marker was 2.6 while the score was 2.7 among the G1 negative genotypes, a difference of 3.8% (Figure 4).

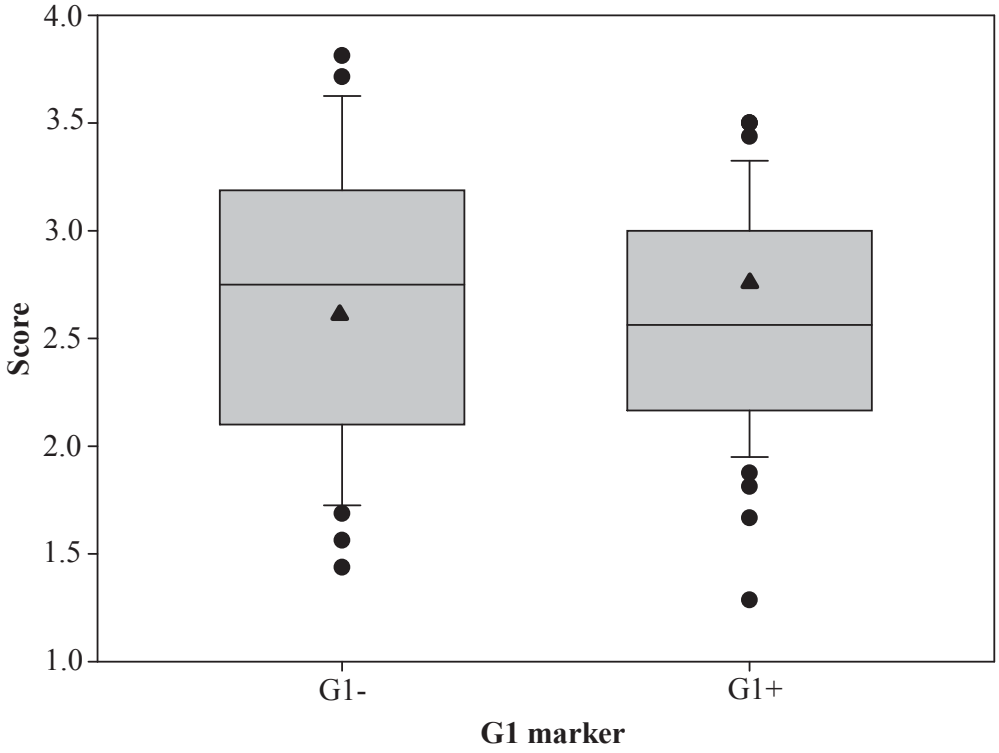

Figure 4- Boxplots of groups of sugarcane genotypes according to the presence (G1+) or absence (G1-) of G1 marker to orange rust. The triangle indicates the overall mean score of each group and the line inside boxplots indicates the median.

Table 3. Resistant and susceptible phenotype prediction in sugarcane genotypes, from G1 marker genotyping, considering two classes of phenotypes, according to the classification of Sood et al. (2013), adapted by Yang et al (2018a), used to evaluate orange rust in which mean severity up to 2 was considered resistant and scores from 2.1 to 4 were called susceptible.

Classification of phenotype	Total of genotypes (mean)	Number of genotypes with G1	Total of genotypes with G1	Prediction of G1 marker for resistant phenotype (%)	Number of genotypes without G1	Total of genotypes without G1	Prediction of G1 marker for susceptible phenotype (%)
0 - 2 (Resistant)	17 (1.8)	8	35	22.86			
2.1 - 4 (Susceptible)	63 (3.0)				36	45	80

The Phi coefficient found was $\phi = 0.035$, which indicates a low correlation between variables. Using the chi-square test, χ^2 was not significant at 5% probability, which indicates

that the variables (resistance/susceptibility to field and presence/absence of the G1 marker) are statistically independent, that is, there is no correlation between them in the conditions tested.

3.4 Discussion

The use of molecular markers as a tool in assisted selection for sugarcane resistance to diseases is very useful for crop improvement programs, allowing genotypes to be classified as resistant/susceptible in less than 24 hours, for example, through molecular techniques (Yang et al. 2018a). However, in the present study, the molecular marker G1 did not show good efficiency for MAS for the group of genotypes used in this work, since the selection accuracy was low and the correlation between the results of the molecular analysis and the field was not significant.

G1 was the first molecular marker associated with the resistance of sugarcane to orange rust (Yang et al. 2018a). As found by Yang et al. (2018b) through the observation of phenotypic segregation patterns, resistance to orange rust is quantitative in nature. In addition, multiple QTLs or markers associated with disease resistance have been identified, endorsing the theory that multiple genes are involved in the response of sugarcane to orange rust (Klosowski et al. 2013; Yang et al. 2018a). However, due to the high polyploidy, QTL studies in sugarcane are very laborious. The main difficulties of the process are the realization of a mapping that comprehends all homologous chromosomes and the need for field experiments with large populations and a high number of repetitions, so that the effects of quantitative traits alleles (QTA) detected (D'Hont et al. 2001). In order to identify molecular markers associated with resistance to orange rust in an experiment using genotypes from the germplasm collection of the Sugarcane Research Station in Canal Point (FL, United States) the three main ones, alone, explained less than 10.7% of the resistance variation (McCord et al. 2019). In the study, the authors point out that these markers may not be sufficient in the individual selection of clones for resistance to orange rust, however, their use may be efficient in choosing and combining parents with the same marker, seeking descendants less sensitive to the disease. In addition, the identification of markers in alleles with lesser effects is important in the breeding process. The combination of multiple resistant QTLs is important as a means of significantly reducing the disease, since several interactions between the alleles for orange rust have been observed, among them, additive effects (Yang et al. 2018b).

The methodology for assessing the resistance of the genotypes evaluated in this study was the same used by Yang et al. (2018a), based on the rating scale proposed by Sood et al. (2009). This methodology consists of depositing a suspension with a high load of

urediniospores of the fungus (10^4 urediniospores mL^{-1}) directly on the leaf whorl of plants in the field. The germination rate obtained for the spores used in the inoculations was 83%, which contributed to the efficiency of the test, providing that the viable inoculum came into contact with the plant tissue under suitable conditions for the development of the disease. The method of inoculation in the leaf whorl is pointed out as a way to take advantage of routine tests carried out in the field, exposing the genotypes to favorable conditions for the occurrence of the disease and classifying them according to their reaction, assisting researchers in the selection of promising genotypes for more advanced stages of the breeding programs (Sood et al. 2009). Considering the conditions to which the plants are submitted in this test - high inoculum load, high humidity, pathogen viability - and the careful evaluation of the symptoms shown by the plants, which involves the count of pustules with and without sporulation, it can be inferred that the genotypes classified as resistant after artificial inoculation, will commonly demonstrate reaction of resistance to the disease in evaluations in which only natural infection occurred in the field. However, Sood et al. (2009) point out that, in about 10% of the evaluations of the natural infection of brown rust (*Puccinia melanocephala*) escapes occurred, leading to false resistance reactions, which can be verified when carrying out artificial inoculation, and thus, genotypes considered resistant may show susceptibility reactions when subjected to artificial inoculation.

The cultivars evaluated in this study through inoculation in the leaf whorl showed similar results to published studies in which the resistance classification was made evaluating the natural infection. For example, cultivars RB72454 and RB956911 (Klosowski et al. 2015) were susceptible to orange rust and cultivars CTC-4 (Araújo et al. 2013), RB867515, RB946903, RB966928 (Klosowski et al. 2015), RB985476 (Fíer et al. 2020), RB036088 (Daros et al. 2017) were classified as resistant.

Under the conditions that the G1 marker was developed and validated, the efficiency in predicting resistant phenotypes for the F1 mapping population was 65.8%, where 165 genotypes were evaluated (Yang 2018a). Fíer et al. (2020), using the G1 marker observed an evaluating efficiency in predicting resistant of 71.43%, when evaluating 24 Brazilian commercial cultivars, which indicates that this marker would have potential for use for MAS in Brazil's sugarcane breeding programs. In that work, data from natural infection was used and resistance assessment was based on a diagrammatic scale by Amorim et al. (1987), which ranged from 1 (plants without symptoms) to 9 (highly susceptible plants), to evaluate the disease severity under natural field conditions, and cultivars with scores above 3 were considered susceptible. Of the 24 cultivars evaluated, 67; 21 and 12% were resistant,

intermediate and susceptible to orange rust, respectively. It was observed 10 resistant cultivars with the presence of G1 and 1 susceptible cultivar with the presence of G1. In our study, 80 genotypes were evaluated, with the percentage of resistant and susceptible genotypes being 21 and 79%, respectively. Five Brazilian commercial cultivars evaluated were the same and 9 were different to those evaluated by Fíer et al. (2020) and 66 clones under selection process. Among the 14 commercial cultivars, 64% were resistant, but when we evaluated the clones in the selection process (66), the percentage of resistant genotypes was 11%. The difference in resistance between commercial cultivars and clones is because clones are genotypes that can still be discarded during selection because if they do not show resistance to orange rust or other characteristics. The presence of the G1 marker was detected in 27 of susceptible genotypes (43%), and in 8 resistant genotypes. So, the efficiency in predicting resistant phenotype was 22.86%, a value much lower than that found by Fíer et al. (2020). Thus, the G1 marker was not efficient when both clones in the selection phase and commercial cultivars were analyzed.

The validation of a molecular marker may not occur when in conditions other than those in which the marker was developed. Silva et al. (2008), evaluating the potential of genetic markers associated with the *Lr1*, *Lr9*, *Lr10* and *Lr24* genes that give wheat plants resistance to leaf rust, observed that the marker region linked to the *Lr1* gene amplified in genotypes that do not have the respective gene, as was also observed by Chelkowski et al. (2003) and Urbanovich et al. (2006), when they tested this same marker under different conditions, which indicates that the marker was not efficient in identifying the gene in wheat genotypes under the conditions tested.

In sugarcane, MAS for disease resistance can be viable, as long as the marker is reliable and properly validated in the local conditions of each breeding program. An example are the markers associated with the gene with large effect is Bru1. This gene is described as a source of durable resistance of sugarcane to brown rust (*Puccinia melanocephala*). The two markers associated with Bru1, R12H16 and 9020-F4-RsaI have been validated under Brazilian conditions and are considered highly efficient in predicting resistant phenotypes. Bru1 is identified as the main source of resistance to brown rust in commercial sugarcane crops in Brazil (Barreto et al. 2017).

The results indicate that the G1 marker had a low efficiency to predict the resistance to orange rust in clones under selection in a Brazilian breeding program and Brazilian commercial cultivars of sugarcane. More studies are required to develop molecular markers that are significantly associated with the resistance of sugarcane to orange rust to be used during the sugarcane breeding program.

Conflict of interest

The authors declare that they have no conflict of interest.

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4. CHAPTER III - METHODOLOGY FOR ASSESSING RESISTANCE OF SUGARCANE GENOTYPES TO SMUT[3]

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[3] Prepared in accordance with the standards of Sugar Tech Journal.

ABSTRACT

Sugarcane smut caused by the fungus *Sporisorium scitamineum* is one of the major diseases that affect the culture. The main method of disease control is the usage of cultivars with genetic resistance. However, the processes of evaluation and classification genotypes regarding their resistance to sugarcane smut does not follow a standard. As such, the present study proposes a standardized methodology for classification of sugarcane genotypes concerning their resistance to smut and presents the reaction of 41 sugarcane genotypes to the disease. For this purpose, it is recommended for new trials the usage of the susceptible standard, RB935621 and at least one of the resistance standards (RB985476, CV6945 e RB956911), assess the incidence of whips in approximate 30-day intervals, starting from the emergence of the first whips, until the number of tillers tend to stabilize, obtain the AUDPC and consequently the relative percentage of the disease and in the end fit the genotypes regarding their resistance level according to the intervals of each level of resistance to sugarcane smut. Of the genotypes tested, three were classified as highly resistant (7.4%), eighteen as resistant (43.9%), eleven as moderately resistant (26.8%), one as moderately susceptible (2.4%) and eight as susceptible (19.5%).

Keywords: *Saccharum* spp., *Sporisorium scitamineum*, screening, reaction.

4.1 Introduction

Sugarcane smut is caused by the fungus *Sporisorium scitamineum* (Syd.) and was one of the first diseases reported in the culture, in the year 1877, in South Africa (Ferreira et al. 1989). Since then, the disease has spread to other sugarcane producing regions of the world,

arriving in Brazil in the year of 1946, infecting initially the cane fields of São Paulo state and dispersing to all producing regions of the country (Bergamin Filho et al. 1987). The damages to the culture caused by the disease occurs due to reduction of the quantity and quality of the cane juice, death of stalks and need to early renew the cane field, or yet making the planting of more productive cultivars unfeasible (Santos et al. 2004, Mansoor et al. 2016).

The disease symptomatology is the presence of a typical filiform structure, called "whip", this structure being the result of a modification of the plant meristem, induced by the causal agent. The whips vary in size and can reach more than one meter in length and are composed of a central column consisting parenchymal elements and vascular bundles, covered by the fungus teliospores, which are initially protected by a silvery membrane that breaks, exposing the teliospores, which are easily carried by the wind and rainwater (Ferreira et al. 1989). In susceptible cultivars, the disease symptoms can be observed before the appearance of the whip, through changes in the plant growth habit, which could present overgrowth of stools, thinning of the stalks, more upright tillers with leaf and internode shortening and discoloring of the internal culm tissues (Ferreira et al. 1989, Comstock 2000).

The whips can be generated at the apical or lateral shoots, being named apical whips and lateral whips, respectively. The apical whips usually stay above the culture height, which helps the teliospores originated from this place to be dispersed to greater distances, contributing to plant infection in neighboring areas (alloinfection). The lateral whips, in other hand, because they are often smaller, contribute to the autoinfection of the cane field contaminating the shoots of new tillers sent by neighboring clumps (Amorim 1989).

The most effective way of control of this disease is the use of resistant cultivars (Tokeshi and Rago 2005), in this way, during the development process of a cultivar, the sugarcane genetic improvement programs perform genotype evaluations to select those with the highest level of disease resistance. Besides that, after the cultivar release, it is of extreme importance that the producers know the level of resistance of the cultivars to sugarcane smut, so they can choose the most suitable for each situation. The resistance of cultivars planted is one of the determining factors in disease incidence, in addition to the presence of the initial inoculum and occurrence of favorable environmental conditions to pathogen development (Agrios 2004, Mansoor et al. 2016).

The intensity assessment of smut has used different variables in different studies about the disease, such as: % of infected stools (Latiza et al. 1980, Shen et al. 2014, Lemma et al. 2015) and % of diseased plants in the area (Santos et al. 2004, Bhuiyan et al. 2013, Mansoor et al. 2016, Sakaigaichi et al. 2018). The evaluation intervals also vary, from biweekly to yearly

(Bhuiyan et al. 2010, Shen et al. 2014). Different classification criteria have been adopted to characterize sugarcane genotypes regarding their resistance to smut. Latiza et al (1980) ranks genotypes, in nine levels, according to the % of infected stools, being level one, very highly resistant (1 a 2.5 % of infected stools), and level nine, very highly susceptible (>25.5% of infected stools). Shen et al. (2014) evaluating the number of infected stools, used a ranking with nine levels, from number one, highly resistant (0 to 3% of infected shoots) up to level nine, highly susceptible (>75% of infected shoots). Dalvi et al (2012) and Mansoor et al (2016), classified sugarcane genotypes in four levels of smut resistance, from resistant (0 to 5% of diseased plants) to susceptible (>30% of diseased plants). Sakaigaichi et al (2018) ranked wild genotypes and sugarcane cultivars in seven levels of resistance, from level one, highly resistant (no infected plants) to level seven, susceptible with values higher than 63.2% of diseased plants. Santos et al (2004) graded Brazilian cultivars regarding their resistance to sugarcane smut using a scale wherein genotypes with 0 to 5% of diseased plants were considered resistant, from 5.1 to 15%, intermediate, and >15% susceptible.

This situation creates a lack of standardization in classification of genotypes regarding their resistance to smut. The proposal for a standardized methodology for evaluation and classification is of extreme importance to allow a reproducible comparison of genotypes under different conditions of resistance evaluation, since due to the quantitative standard of sugarcane genetic resistance to smut, the availability of inoculum in the area, and variable conditions of the environment (relative humidity, temperature, winds), associated to the plant genetics, determine the reaction of genotypes to the disease (Mansoor et al. 2016).

Thus, the present study proposes a standardized methodology for classification of sugarcane genotypes regarding their resistance to smut (*S. scitamineum*) under Brazilian conditions and presents the reaction of 41 sugarcane genotypes to the disease.

4.2 Material and Methods

4.2.1 General Crop Management

The experiment was carried out at the Experimental Station of Paranavaí of the Federal University of Paraná (UFPR), in the municipality of Paranavaí, PR (23º05”S, 52º26” W, 470 m asl), in the period from 02/2019 to 02/2020. The soil is classified as a dystrophic Red Latosol, with smooth undulating relief (EMBRAPA 2006), the place has a Cfa-type climate, temperate, with at least 30 mm precipitation in the driest month, hot summer, average temperature of warmest month > 22 °C (Nitsche et al. 2019, FAO 2020).

4.2.2 Experimental Design and Treatments

The design used was randomized blocks with 41 genotypes and four replications. The experimental unit consisted of five seedlings per genotype, maintaining 0.5 m between seedlings and 1.5 m between rows. The 41 genotypes used were chosen based on their economic and genetic importance within the breeding program (Table 1). In this group of genotypes are some of the main cultivars grown in Brazil and clones in different phases of the breeding program of the Sugarcane Genetic Improvement Program (PMGCA), a program linked to RIDESA (Inter-University Network for the Development of the Sugarcane Industry).

4.2.3 Seedling preparation and inoculation procedure

The seedlings used came from individualized buds. Before planting, heat treatment was carried out by immersing the buds in water at 52 °C for 30 minutes (Comstock 2000), followed by fungus inoculation through immersion of the shoots in a suspension containing 1.0 x 10^6 viable teliospores mL^{-1} inside a plastic container with a volume equal to 500 L. The immersion time was 30 minutes, with agitation being performed approximately every five minutes. After this period, the inoculated shoots were stored in closed black plastic bags and maintained at an approximate temperature of 30 ºC for 24 hours, being afterwards planted in tubes containing commercial substrate: filter cake (1: 1). These were kept in a greenhouse with sprinkler irrigation until 60 days. After this period, the seedlings were transplanted to the field.

The inoculum was collected approximately 60 days before inoculation. Young whips (covered by the film) were collected, avoiding uncovered whips, in which part of the spores have been washed, which could compromise their viability. The whips were spread out on a canvas placed over a plastic tarp in a well ventilated room to dry for 4 to 5 days. After complete drying of the collected whips, the leaf cover and the film of the whips were removed, and a gentle scraping was performed to remove the teliospores, which were then sieved with the help of a sieve attached to a vacuum cleaner. The teliospores obtained were packed in paper bags containing 10g of spore/bag and identified, being stored in bottles containing silica gel and kept in refrigerator until the moment of inoculation. The viability of the teliospores was evaluated in Petri dishes containing water-agar medium (2%). The dishes were divided into four sections, and 25 teliospores were counted per section, totaling 100 teliospores per dish. Teliospores whose germ tube length was greater than the diameter of the spore itself were counted as "germinated" (Minchio et al. 2011). The average germination obtained was 80%.

4.2.4 Evaluation and classification of genotypes for resistance to sugarcane smut

The evaluations started at 48 days after the seedlings were planted in the field, when the first whips appeared, being performed eight evaluations at approximately every 30 days after the appearance of the whips. The number of tillers with young whips (presence of full or partial silvery membrane) emitted in the period between evaluations and the number of healthy tillers per plot were evaluated at the field. The total number of tillers per plot was calculated adding the number of tillers with whips (diseased) and the number of healthy tillers. From the collected data, for each genotype, it was obtained the disease incidence (I) according to the equation below:

$$I = (\frac{number\ of\ tillers\ with\ whips}{total\ number\ of\ tillers}) * 100$$

With the disease incidence data over time, the area under the disease progress curve (AUDPC) was calculated as proposed by Shaner and Finney (1977). Afterwards, the genotypes were sorted from lowest to highest AUDPC value, so that the genotype that had the lowest and the highest value were considered standards of resistance and susceptibility, respectively. After the sorting of the averages, a relativization of the genotypes AUDPC was performed to obtain values from 0 to 100, being the zero value the resistance standard cultivar and the value 100 the susceptibility standard cultivar. The relativized AUDPC (%) was obtained by the division of AUDPC value of the genotype by the AUDPC value of the susceptible standard and multiplying by 100.

Once the % values of relativized AUDPC for each genotype was obtained, they were classified in 5 levels of resistance according to the following ranges: highly resistant (HR) – 0; resistant (R) – values between 0.1 to 15%; moderately resistant (MR) – values between 15.1 to 30%; moderately susceptible (MS) – values between 30.1 to 45% and susceptible (S) – values greater than 45%.

After the genotypes were classified according to each resistance level, progress curves over time with average incidence values for each resistance level were obtained.

4.3 Results

The genotypes classification and their respective AUDPC values and relative percentages (%) are presented in table 2. Of the 41 tested genotypes, three were classified as HR (7.4%), eighteen as R (43.9%), eleven as MR (26.8%), one as MS (2.4%) and eight as S (19.5%). The genotype that had the highest AUDPC number was the genotype RB935621, which was considered the susceptibility standard for classification of other genotypes.

The sugarcane smut epidemic started in 48 days after transplanting the seedlings in the field, when the observation of the first whips were possible. The peaks of incidence curves were observed in the fourth evaluation (94 days after the start of the epidemic) for all classification groups. Except for genotypes classified as HR, which whips were not observed in the evaluated period, the curves showed similar pattern (Figure 1). The highest observed incidence value was 20,9% in the RB935621 genotype (susceptibility genotype).

4.4 Discussion

In the present study, it was proposed a standard evaluation methodology based on the incidence of smut for classification of sugarcane genotypes regarding the plant resistance to the disease through the usage of resistance and susceptibility standards, allowing more reproducible results of genotype reactions in different evaluation conditions. Furthermore, it was possible to observe a variability in smut resistance levels in the different genotypes, being this knowledge of disease resistance of extreme importance to the culture of sugarcane for recommending the use of cultivars with the highest levels of resistance in area where smut is an issue, assuring high productivity.

The disease quantification in plants can be performed in different ways, as for assessing severity and incidence. Severity (percentage of the organ or plant area affected by the disease) is the measurement used to quantify diseases, such as leaf spots, rusts, downy and powdery mildew. However, for diseases in which the infection is responsible for the unfeasibility of the affected organ or plant, as is the case with smuts, incidence is used, which represents the percentage of affected organ or plant by the disease (Lopes et al. 2014, Amorim and Bergamin Filho, 2018). Incidence is the quantification measure used in resistance experiments of barley genotypes to true loose smut [*Ustilago nuda* (Jens.) Rostr.] and to covered smut [*U. hordei* (Pers.) Lagerh.] in which the level of the disease is represented by the percentage of infected plants (Grewal et al. 2008). Besides barley, the classification of corn genotypes (Pataky et al. 1995), oat and wheat regarding their resistance to smut are also done based in incidence, usually represented by the percentage of infected plants (Menzies et al. 2009).

The sugarcane smut can be assessed by the incidence of diseased plants and/or stools in the area (Latiza et al. 1980, Santos et al. 2004, Bhuiyan et al. 2013, Shen et al. 2014, Lemma et al. 2015, Mansoor et al. 2016, Sakaigaichi et al. 2018). The incidence of tillers with whips was assessed in the present study by the usage of counting of the number of healthy tillers and diseased tillers, and not the incidence of diseased stools, in order to also ease the usage of the method in culm planting, and in ratoon, where is difficult to delimit the start and end of each

stool. Thereby, the proposed methodology could be used in different types of planting (pre-sprouted seedlings or culms) and in plant-cane and ratoon.

Besides the evaluation methodology, another important point to be highlighted is the evaluation numbers and intervals. In the present study, eight monthly evaluations were performed from the start of the epidemic to 268 days of culture implantation in the field, with approximately 30-day intervals. In sugarcane, changes in the number of tillers occur naturally until around 250 days of culture, when this number tends to stabilize, being for its own tillering capability of the genotype, or yet climatic factor, fertilizing and cultural practices like spacing and depth of planting and, consequently, the disease incidence varies in a similar manner, with whips being emitted during the whole tillering period of the culture (Machado et al. 1982).

The variations that occur in the culture with the emission of new diseased tillers (whips) over time demonstrates the importance of periodic evaluations of the disease, as if only one or two evaluation are done in culture cycle, these values can be under or overestimated, depending on the epidemic phase in which they were observed and, consequently, the genotype classification accuracy can be compromised. Thus, the usage of a variable that encompass the whole period of the epidemic can be more efficient for genotype classification, as is the case of area under the disease progress curve (AUDPC). The usage of AUDPC as a genotype classification criterion is widely used in different pathosystems as apple x *Erwinia amylovora* (Momol et al. 1996), *Chenopodium quinoa* x *Peronospora farinosa* f. sp. *chenopodii* (Kumar et al. 2006), rice x *Pyricularia grisea* (Mohapatra et al. 2008), potato x *Phytophthora infestans* (Duarte et al. 2012), potato x *Alternaria grandis* (Duarte et al. 2014) and cocoa x *Phytophthora palmivora* (Ling et al. 2017), for example.

In literature, in different genotype classification systems to smut, the interval between evaluations or yet the ideal number of evaluations is not a consensus. Lemma et al. (2015) performed evaluations every ten days, until the ten months of the culture. Other sugarcane genotype classification studies regarding smut resistance mentioned 30-day evaluation intervals, for a seven-to-eleven-month period starting from the emergence of the symptoms (Mansoor et al. 2016, Sakaigaichi et al. 2018), while in another study with the same purpose, the genotypes were evaluated three times during the epidemic, once every two months (Co et al. 2008). In a study with sugarcane genotypes, Bhuyian et al (2010) evaluated the incidence of smut just once a year, resulting in an evaluation of plant-cane and one in ratoon. Therefore, we opted to standardize the resistance classification of sugarcane genotype to smut assessing every month over the culture cycle according to Mansoor et al. (2016) and Sakaigaichi et al. (2018) to make the process feasible, since evaluations every 10 to 15 days of a great number of

genotypes, as it happens in breeding programs, would make the process too laborious and time consuming. Besides, the values of the performed evaluations in a 15-day interval did not contribute to big changes in the incidence curves of genotypes over time (data not shown).

The usage of AUDPC and the genotype relativization according to a susceptibility standard proposed in this study emerges as an important methodology in standardizing genotype classification to smut. For instance, the RB935621 genotype, which was the genotype with the highest AUDPC, consequently used as susceptibility standard showed maximum incidence of 20.9% during the epidemic. In this case, this genotype would be classified as moderately susceptible according to Mansoor et al. (2016) and Sakaigaichi et al (2018), and as susceptible according to Santos et al. (2004) and Dalvi et al. (2012).

Besides this lack of standardization, it is important to highlight that the incidence of smut greatly varies concerning the amount of initial inoculum and the environment. For evaluation of resistance to smut in this study the artificial inoculation was used, which allows a standardization of the initial inoculum, and the cultivation site allowed maximum incidence values of 20% to be observed. However, when the experimental conditions are changed, incidence values can be greater or lesser. If incidence values in specific ranges are used for the different resistance levels, the reproducibility in classification of smut resistance can be affected, making it difficult to apply in different trials. Therefore, the proposed methodology aims to standardize the resistance of sugarcane genotypes to smut looking for a way to ease the issue of lack of reproducibility of classification of genotypes due to the presence of different quantities of viable inoculum in the area and environmental factors being decisive in development of an epidemic and generate variable incidence values between cultivation sites. Similar studies have been proposed for other diseases (Duarte et al. 2012, 2014). It is possible that the genotype level of resistance changes, in future trials in different sites, but this is due to the genetic variability of the fungus (Benevenuto et al. 2016), and not the change of initial inoculum or environment.

Most of the tested genotypes in this study (~78%) were classified as HR, R or MR to the disease, this shows that most of the evaluated genotypes has some level of resistance to smut. This is justified due to the evaluation of cultivars which have already undergone a selection process. Among these genotypes, three (RB985476, CV6945 e RB956911) did not show any symptom of the disease throughout the evaluations, which makes it important that they are used in trials as resistance standards.

Regarding the ranges of relative percentages for setting the resistance levels, these were defined through type of disease, researcher's experience and average AUDPC incidence values

tests. The level considered highly resistant was set as those genotypes in which no symptoms of the disease were observed throughout the experiment. Because smut is a disease in which the infection is responsible for making the affected plant unviable, unlike leaf disease which makes only a part of the plant unviable, we opted not to split the ranges in equidistant intervals of relative percentage (25%) for other resistance levels. Therefore, we opted to use intervals of 15% to be stricter about the classification, being considered susceptible genotypes above 45% the relative percentage to the susceptibility standard. The Scott-Knott test of average at 5% probability with the AUDPC values of incidence showed that with 15% separated the genotypes well at different levels in addition to the experience of researchers who know well the behavior of the genotype in the field in different locations. For instance, genotypes that are cultivars and with their resistance well known fit in the proposed levels of this methodology, as is the case of cultivars RB92579 and RB966928 that has an intermediate resistant to smut (Arcoverde et al. 2018, Cursi et al. 2021), as well as the cultivars RB867515, which is considered resistant to the disease (Daros et al. 2015).

The resistance evaluation of genotypes to the disease is a routine process in sugarcane breeding programs, however, the protocol often does not follow a defined standard. Thus, the most reproducible method to obtaining results regarding the resistance classification of sugarcane genotypes to smut is recommended for new trials in Brazilian conditions the usage of the susceptible standard genotype RB935621 and at least one of the resistance standards (RB985476, CV6945 and RB956911), evaluate the incidence of whips in approximate 30-day intervals, starting from the emergence of the first whips, until around 250 days of culture, when the number of tillers tends to stabilize, obtain the AUDPC and consequently the relative percentage of the disease and in the end fit the genotypes regarding their level of resistance according to the intervals on each resistance levels to sugarcane smut. Taking in consideration that the planted genotypes vary in other countries, this methodology can be adapted changing just the susceptibility standard which should be the most well-known susceptible genotype.

4.5 References

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4.6 Tables e Figures

Table 1. Background of the 41 genotypes of sugarcane evaluated for smut

Genotype	Parental 1	Parental 2	Genotype	Parental 1	Parental 2
RB985476	H53-3989	RB855206	RB006995	SP80-1816	RB855536
CV6945	-	-	RB106814	RB867515	RB867515
RB956911	RB855206	RB855035	RB046209	RB855063	MP
RB041443	RB805203	?	RB106832	RB931536	?
RB056363	RB855063	?	RB006629	SP80-1816	RB855536
RB011941	BJ7504	RB72454	RB966928	RB855156	RB815690
RB006970	RB855536	SP80-1816	RB056349	RB855206	MP
RB106822	NA5679	?	RB056300	RB912525	?
RB056351	RB855206	?	RB056301	RB956911	?
RB126202	RB036066	?	RB036066	SP70-1143	SP77-5181
RB127825	CTC14	RB867515	RB92579	RB75126	RB72199
RB046258	RB855002	?	RB036145	SP83-2847	TUC71-7
RB946903	RB765418	RB72454	RB136301	RB956911	?
RB046215	SP-931322	RB946903	RB835486	L60-14	?
RB066498	RB966229	RB825548	RB006984	SP80-1816	RB855536
RB066484	RB966229	RB825548	RB046299	SP83-2847	RB855127
RB867515	RB72454	?	RB126276	?	?
RB056396	SP-801816	?	RB016916	SP80-3280	RB855589
RB046221	RB935903	?	RB126201	RB036066	?
RB046210	RB855546	RB962012	RB935621	RB835089	SP70-1143
RB056320	BJ7015	?			

? = Unknown pedigree and - = Pedigree not available.

Table 2. Ranking of sugarcane genotypes to resistance to smut (*Sporisorium scitamineum*) using the relative percentage of disease in comparing to susceptible standard, RB935621

Genotype	AACPD incidence	Percentage	Resistance level	Highest incidence* (%)	Genotype	AACPD incidence	Percentage	Resistance level	Highest incidence* (%)
RB985476	0.0	0.0	HR	0.00	RB006995	335.7	16.9	MR	13.33
CV6945	0.0	0.0	HR	0.00	RB106814	369.4	18.6	MR	11.36
RB956911	0.0	0.0	HR	0.00	RB046209	377.6	19.0	MR	7.02
RB041443	23.0	1.2	R	2.02	RB106832	382.3	19.3	MR	6.56
RB056363	35.1	1.8	R	2.99	RB006629	419.8	21.2	MR	8.60
RB011941	45.3	2.3	R	3.08	RB966928	450.7	22.7	MR	14.14
RB006970	73.4	3.7	R	3.08	RB056349	484.5	24.4	MR	10.14
RB106822	101.3	5.1	R	6.12	RB056300	531.2	26.8	MR	9.90
RB056351	102.0	5.1	R	7.69	RB056301	534.2	26.9	MR	9.43
RB126202	158.2	8.0	R	6.67	RB036066	563.5	28.4	MR	20.51
RB127825	183.5	9.3	R	8.57	RB92579	578.5	29.2	MR	12.38
RB046258	195.8	9.9	R	6.56	RB036145	685.0	34.5	MS	16.48
RB946903	209.4	10.6	R	6.78	RB136301	1098.2	55.4	S	26.67
RB046215	210.0	10.6	R	6.35	RB835486	1346.3	67.9	S	17.54
RB066498	211.0	10.6	R	8.62	RB006984	1354.4	68.3	S	20.31
RB066484	219.4	11.1	R	15.38	RB046299	1682.3	84.8	S	30.77
RB867515	232.4	11.7	R	8.33	RB126276	1713.8	86.4	S	35.94
RB056396	235.4	11.9	R	9.23	RB016916	1836.4	92.6	S	39.02
RB046221	236.0	11.9	R	8.70	RB126201	1892.7	95.4	S	39.47
RB046210	248.5	12.5	R	16.67	RB935621	1983.5	100.0	S	30.56
RB056320	259.9	13.1	R	9.89					

*: the highest incidence was not obtained at the same time for all genotypes; ** HR - highly resistant, R - resistant, MR - moderately resistant, MS – moderately susceptible and S- susceptible

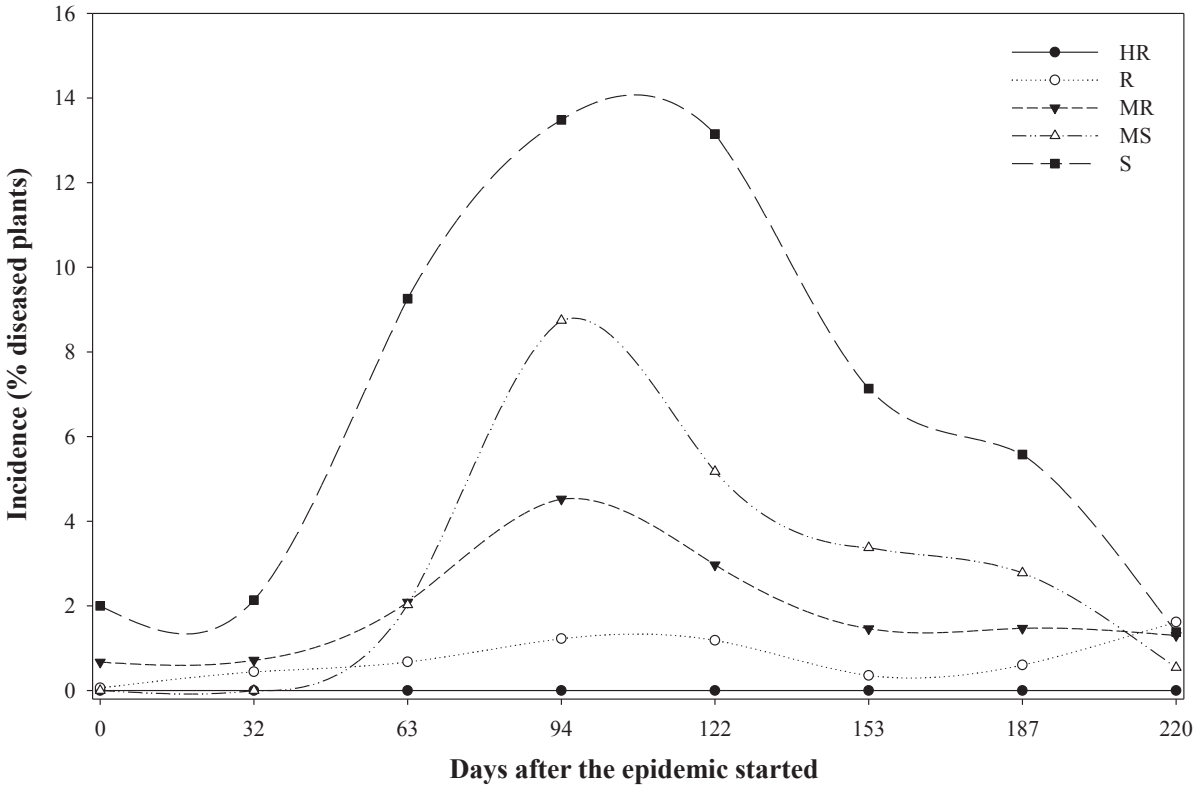

Figure 1. Incidence of smut (%) over time in sugarcane genotypes to resistance levels, HR – highly resistance, R – resistance, MR – moderately resistance, MS – moderately susceptible and S – susceptible.

5. GENERAL CONCLUSIONS

Of the genotypes tested for brown rust, four were classified as susceptible, six as moderately susceptible and only one showed moderate resistance. For orange rust, three of the tested genotypes were classified as susceptible, seven as moderately susceptible and one as moderately resistant. The screening of those genotypes will assist breeding programs in preliminary trials and the selection of promising genotypes for more advanced phases of the programs, and the classification provides information for producers on the choice of cultivars to be planted.

The G1 marker had a low efficiency to predict the resistance to orange rust in clones under selection in a Brazilian breeding program and Brazilian commercial cultivars of sugarcane.

The proposed methodology recommends for new trials the usage of the susceptible standard genotype RB935621 and at least one of the standard resistance genotypes RB985476, CV6945 and RB956911), assess the incidence of whips in approximate 30-day interval, starting from the emergence of the first whips, until when the number of tillers tends to stabilize, obtain the AUDPC and consequently the relative percentage of the disease and in the end fit the genotypes regarding their resistance level according to the interval on each level of resistance to sugarcane smut. Of the 41 tested genotypes, three were classified as highly resistant (7.4%), eighteen as resistant (43.9%), eleven as moderately resistant (26.8%), one as moderately susceptible (2.4%) and eight as susceptible (19.5%).

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