Field resistance and molecular detection of the orange rust resistance gene linked to G1 marker in Brazilian cultivars of sugarcane

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ABSTRACT

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Sugarcane (*Saccharum* spp.), an important crop for tropical and subtropical countries, is used in the production of sugar and biofuel. Orange rust, a disease caused by the fungus *Puccinia kuehnii*, can reduce the yield and harm the sugarcane industry. Molecular markers linked to resistance genes can help breeding programs confirm introgression of favorable alleles, find new resistance sources and release new cultivars that have durable resistance. In the current study, the aims were (i) to evaluate in the field the resistance to orange rust of 24 Brazilian commercial cultivars; (ii) to assess the frequency of the allele at G1 marker locus in the set of cultivars, and (iii) to study the usefulness of G1 marker to predict the resistant phenotype and its potential for marker assisted selection. A diagrammatic scale, which ranged from 1 (plants without symptoms) to 9 (highly susceptible plants), was used to determine the disease severity. Considering resistant cultivars those with mean severity up to 3, G1 marker efficiency in predicting the resistant phenotype was 71.43%. In addition, there was a reduction of 35% in the overall mean severity when G1 marker was present. G1 marker is an important molecular tool that can be used by breeding programs in the search for sugarcane cultivars resistant to orange rust.

Keywords: *Puccinia kuehnii, Sacharum* spp., breeding, disease

RESUMO

Fier, I.; Balsalobre, T.W.A.; Chapola, R.G. Hoffmann, H.P.; Carneiro, M.S.. Resistência no campo e detecção molecular do gene de resistência à ferrugem alaranjada associado ao marcador G1 em cultivares brasileiros de cana-de açúcar. ***Summa Phytopathologica,*** v.46, n.2, p.92-97, 2020.

A cana-de-açúcar (*Saccharum* spp.) é uma cultura importante para países tropicais e subtropicais, utilizada principalmente para produção de açúcar e biocombustível. A ferrugem alranjada, doença causada pelo fungo *Puccinia kuehnii*, pode reduzir a produtividade e prejudicar toda a cadeia industrial. Marcadores moleculares associados com genes de resistência podem auxiliar programas de melhoramento a confirmar introgressão de alelos favoráveis, encontrar novas fontes de resistência e liberar novas cultivares com resistência durável. No presente estudo os objetivos foram: (i) avaliar em campo a resistência a ferrugem alaranjada de 24 cultivares comerciais brasileiras; (ii) acessar a frequência do alelo do marcador G1 no conjunto de cultivares; e (iii) avaliar a utilidade do marcador G1 na predição de fenótipo resistente e seu pontencial na seleção assistida por marcadores. Uma escala diagramática, a qual varia de 1 (plantas sem sintomas) até 9 (plantas altamente suscetíveis), foi utilizada para determinar a severidade da doença. Quando consideramos como cultivares resistentes àquelas que tiveram média de severidade até 3, a eficiência do marcador G1 na predição do fenótipo resistente foi de 71.43%. Em adição, houve uma redução de 35% na média geral de severidade quando o marcador G1 foi presente. O marcador G1 é uma importante ferrramenta molecular que pode ser usada por programas de melhoramento na busca por cultivares de cana-de-açúcar resistentes a ferrugem alaranjada.

Palavras-chave: *Puccinia kuehnii, Sacharum* spp., melhoramento, doença

Sugarcane orange rust is caused by the biotrophic fungal pathogen *Puccinia kuehnii*, an economically important pathogen worldwide. Since its first report causing endemic disease in Australia in 2000, a new race of the pathogen devastated the high-performing sugarcane cultivar Q124, and caused Aus$150–210 million in yield losses (1). In susceptible cultivars, the pathogen has been responsible for important yield losses reaching up to 50% cane yield (2, 3, 4). In 2007, the disease was discovered in the United States (5) and rapidly spread to other countries of America, such as Guatemala (6), Mexico, El Salvador, Panama (7), Costa Rica and Nicaragua (8). It was also detected in Colombia (9) and Ecuador (10). In Brazil, orange rust was first reported in 2009 and is currently present in all cultivated areas of the country (11). Brazil is the world leader in sugarcane production, presenting approximately nine million cultivated hectares and productivity was estimated at 73.22 ton.ha^{-1} in the 2018/19 crop season (12, 13).

Typical symptoms of orange rust are pustules (uredinial lesions) on the underside of the leaves showing cinnamon to orange coloration (14). In severe infections, coalescing pustules can cause premature death of leaf tissue (15). This disease causes reduction in the photosynthetic rate and carbon capture, due to the formation of pustules, thus reducing the growth and tillering of the plant (16, 17, 18, 19). Spores of the orange rust pathogen are extremely numerous on infected leaves of susceptible plants and are easily dispersed by the wind and rain (15). Chemical control of orange rust with the application of fungicides can be used in the

short term in cases of severe pathogen infection (1, 7). However, the use of resistant cultivars is the most effective control method and does not burden farmers with additional charges to maintain crop productivity (14).

Despite the relevance to the sugarcane industry, few studies have been conducted to identify genetic variability and presence of races for *P. kuehnii*. Moreira et al. (20) showed that *P. kuehnii* isolates did not compose different races, but the isolate from one site (Araras, São Paulo State, Brazil) was the most aggressive race. There are also few studies to understand the genetic inheritance of this disease.

Diagrammatic scales developed for brown rust assessment (21, 22) were used to verify the incidence and severity of orange rust (19). More recent methods have been proposed specifically for orange rust, both for artificial inoculation (23) and for natural field incidence (18, 24). Klosowski et al. (25), using the diagrammatic scale developed by Klosowski et al. (24) and considering resistant the asymptomatic genotypes, studied the inheritance of orange rust resistance and results suggested that resistance is controlled by a major gene in conjunction with several other genes of minor effects. The results also suggested that resistant genotypes can be obtained from the crossing between two susceptible genotypes. In another study, using the diagrammatic scale developed by Sood et al. (23) and considering resistant the genotypes with scores between 0 and 2 (presence of lesions but without sporulation), Yang et al. (26) developed a molecular marker associated with a resistance gene, called G1, from QTL mapping of F1 population derived from a cross between CP95-1039 (resistant) and CP88-1762 (susceptible). G1 molecular marker was capable of predicting 65.8% resistant phenotypes in the mapping population. G1 marker genotyping is easily performed through agarose gel with a fragment of approximately 950 base pairs.

To develop resistant cultivars, trials for phenotypic evaluation and identification of genetic sources of resistance should be conducted. In this same line, the molecular markers associated with genes responsible for resistance to orange rust can be used in the screening of resistant clones in early stages of breeding programs. In addition, they may indicate potential parents for future crosses and help establish resistance alleles. As is the case for brown rust, in which molecular markers associated with the *Bru1* resistance gene have been used by breeding programs to increase the level of resistance to this disease (17, 27, 28), development of molecular markers associated with resistance to orange rust is expected to also contribute to changing the current scenario and accelerating the availability of resistant cultivars.

The aims of the present study were (i) to evaluate the field resistance to orange rust of 24 Brazilian commercial cultivars of sugarcane; (ii) to assess the frequency of the allele at G1 marker locus in these cultivars, and (iii) to study the usefulness of G1 marker to predict resistant phenotype and its potential application in marker assisted selection (MAS). This is the first report of the use of G1 molecular marker in Brazilian sugarcane germplasm.

MATERIAL AND METHODS

Plant material

Field evaluation and G1 marker analysis were carried out for 24 Brazilian cultivars of sugarcane (Table S1). These cultivars represent an important genetic background for Brazilian breeding programs, especially regarding two factors: i) they have been or are still among the most cultivated cultivars in the country (29) and; ii) they are used as main parents in Brazilian breeding programs.

Severity of natural orange rust infection in the field

All data of field assays were obtained under conditions of natural infection. The phenotypic response of cultivars to orange rust was based on historical data of several trials conducted between 2011 and 2018 (Table S1) at the Agricultural Sciences Center of the Federal University of São Carlos (UFSCar), located in Araras, São Paulo State, Brazil (22º 21' 25" S; 47º 23' 03" W and average altitude of 611 m). Araras Municipality is located in a region classified to have moderate to high risk of orange rust epidemics (30). The climate is classified as Cwa mesothermic (Köppen classification), showing hot and humid summers and dry winters. The annual average temperature in the experimental area is 21.5°C, ranging from 17.9°C in the coldest month (July) to 24.2°C in the hottest month (February), and average annual precipitation is 1,435 mm. Experimental design and assessment of orange rust resistance were described by Chapola et al. (19). Briefly, the trials were conducted in randomized complete block design with four replicates, and plots consisted of two rows of 2 m length spaced 1.4 m apart. The susceptible cultivar SP89-1115 was planted as border/spreader rows between replicates to increase the inoculum pressure within the experimental area. The disease severity was determined on the +3 leaf, in ten plants per replicate, by estimating the percentage of leaf area affected by symptoms based on the diagrammatic scale of Amorim et al. (22), in which a score of 1 indicates absence of sporulating pustules (uredospores), a score of 2 indicates very rare sporulating pustules and scores from 2 to 9 indicate increasing density of sporulating pustules. A mean value of disease severity was obtained for each cultivar considering all the performed observations. Based on mean disease severity, cultivars with scores of 1 to 3 were classified as resistant, cultivars with scores from 4 to 6 were classified as intermediate and cultivars with scores from 7 to 9 were classified as susceptible.

G1 marker analysis

Total genomic DNA samples were extracted from the 1+ internode (leaf primordia) of the 24 sugarcane cultivars, as proposed by Al-Janabi et al. (31). Samples were quantified with NanoDrop One equipment (Thermo Scientific) and stored at -20°C. PCR amplifications were performed in 20µl reaction containing 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 µM each forward and reverse primer, 0.5 U Taq DNA polymerase (Promega), 40 ng template DNA and ultrapure water to complete volume. Touchdown PCR was performed, as proposed by Yang et al. (26). Briefly, after initial denaturation at 95°C for 5 min, four steps were carry out: i) five cycles of 1 min denaturing at 96°C, 5 min annealing at 68°C with a decrease of 2°C in each subsequent cycle, and 1 min extension at 72°C; ii) five cycles of 1 min denaturing at 96°C, 2 min annealing at 58°C with a decrease of 2°C in each subsequent cycle, 1 min extension at 72°C; iii) 25 cycles of 1 min denaturing at 96°C, 1 min annealing at 50°C and 1 min extension at 72°C; and iv) a final extension at 72°C for 5 min. PCR products were run on 1% agarose gel in horizontal electrophoresis. Considering the genotyping results, the cultivars with presence of G1 maker, a fragment of approximately

950 base pairs, were encoded with 'P' and cultivars with absence of this fragment were encoded with 'A'. Prediction of the resistant phenotype was obtained by dividing the number of both phenotypic and genotypic resistant cultivars by the number of genotypic resistant cultivars, and multiplying the result by 100.

RESULTS

The results regarding orange rust mean severity, reaction to the disease and genotyping of G1 marker for each cultivar are summarized in Table 1. Orange rust mean severity, based on historical data, showed that 16 (66.67%), 5 (20.83%) and 3 (12.50%) sugarcane cultivars can be grouped in resistant, intermediate and susceptible reaction classes, respectively.

Of the 24 evaluated cultivars, 14 (58.33%) had the G1 marker and showed overall mean severity of 2.62. Four cultivars presenting G1 were asymptomatic (SP91-1049, SP80-3280, RB966928 and RB855536). On the other hand, G1 marker was also present in cultivars showing mean severity higher than 1, which ranged from 1.35 (RB855453) to 7.44 (RB72454). Although susceptible to the disease, the cultivar RB72454 is one of the parents to the resistant cultivars RB855536 (SP70-1143 x RB72454), RB867515 (RB72454 x ?) and RB835054 (RB72454 x NA56-79), as well as to the intermediate cultivar RB855156 (RB72454 x TUC71-7), all presenting G1 marker (Table 1).

Regarding resistant cultivars, those with mean severity scores between 1 and 3 had G1 marker efficiency in predicting resistant phenotype of 71.43% (Table 2). Such prediction of efficiency decreased to 28.57% and 57.14% when cultivars showing mean severity scores equal to 1 and 1 to 2, respectively, were considered resistant (Table 2).

On the other hand, G1 marker was absent in ten cultivars (Table 1) which had overall mean severity of 3.55. For these cultivars, mean severity scores were greater than 1, ranging from 1.19 (SP80-1842) to 7.56 (SP79-2233). G1 marker efficiency in predicting susceptible phenotype was 100%, 70% and 40% when cultivars showing mean severity scores greater than 1, 2 and 3, respectively, were considered susceptible (Table 2).

Considering the evaluated cultivars, there was a reduction of 35% in the overall mean severity when G1 marker was present, decreasing from 3.55 to 2.62 (Figure 1).

Table S1. Twenty-four Brazilian commercial cultivars of sugarcane evaluated for orange rust severity, their parental, growing area in hectares (ha) and number of field trials that participated between 2011 and 2018.

Cultivar	Parental 1	Parental 2	Growing area (ha)[1]	Number of trials
RB72454	CP53-76	?	1,056	7
RB835054	RB72454	NA56-79	44,803	5
RB835486	L60-14	?	9,907	7
RB855035	L60-14	SP70-1284	15,893	5
RB855156	RB72454	TUC71-7	215,570	11
RB855453	TUC71-7	?	186,432	8
RB855536	SP70-1143	RB72454	98,586	8
RB867515	RB72454	?	875,692	7
RB925211	RB855206	?	2,422	4
RB92579	RB75126	RB72199	284,713	11
RB935744	RB835089	RB765418	17,662	6
RB965902	RB855536	RB855453	35,849	3
RB966928	RB855156	RB815690	518,152	8
RB975201	RB855113	?	35,519	4
RB975242	F147	?	10,838	4
RB975952	RB835486	RB825548	13,063	4
RB985476	H53-3989	RB855206	6,775	4
SP79-2233	H56-2954	?	0	4
SP80-1842	SP71-1088	H57-5028	56,589	9
SP80-3280	SP71-1088	H57-5028	50,262	4
SP81-3250	SP71-1279	CP70-1547	150,667	11
SP83-2847	HJ5741	SP70-1143	133,219	8
SP89-1115	CP73-1547	?	551	13
SP91-1049	SP80-3328	SP81-3250	9,828	4

[1] Growing area in hectares, concerning the states of São Paulo and Mato Grosso do Sul, according to 2018/19 varietal census (RIDESA, 2018). São Paulo and Mato Grosso do Sul accounted for 60% Brazilian sugarcane production in 2018 (CONAB, 2018).

Table 1. Groups of sugarcane cultivars according to the presence or absence of G1 molecular marker, orange rust mean severity[1] and reaction to disease of each cultivar, and overall mean severity by groups of cultivars.

Cultivars	Mean severity[1]	Reaction to disease
G1 Presence		
SP91-1049	1.00	Resistant
SP80-3280	1.00	Resistant
RB966928	1.00	Resistant
RB855536	1.00	Resistant
RB855453	1.35	Resistant
RB867515	1.36	Resistant
RB835054	2.00	Resistant
RB965902	2.00	Resistant
RB935744	2.18	Resistant
RB855035	2.80	Resistant
RB92579	3.67	Intermediate
RB855156	4.08	Intermediate
RB925211	5.81	Intermediate
RB72454	7.44	Susceptible
Overall mean severity	2.62	
G1 Absence		
SP80-1842	1.19	Resistant
RB975242	1.83	Resistant
RB975201	2.00	Resistant
RB835486	2.14	Resistant
RB985476	2.16	Resistant
RB975952	2.33	Resistant
SP83-2847	3.35	Intermediate
SP81-3250	5.59	Intermediate
SP89-1115	7.37	Susceptible
SP79-2233	7.56	Susceptible
Overall mean severity	3.55	

[1]Orange rust mean severity was obtained from several field trials conducted between 2011 and 2018.

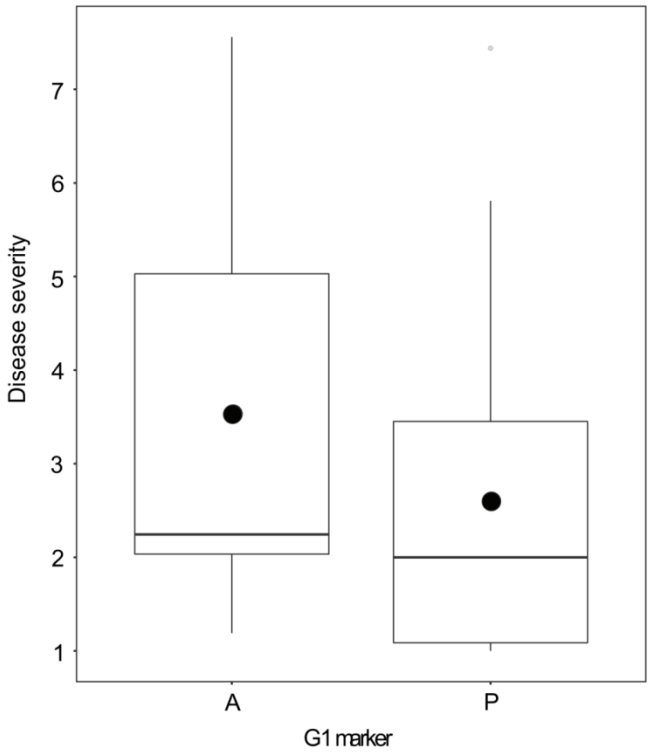

Figure 1. Boxplots of groups of sugarcane cultivars according to the presence (P) or absence (A) of G1 marker to orange rust severity. The black point indicates the overall mean severity of each group and the line inside boxplots indicates the median.

DISCUSSION

Sugarcane is one of the most important crops around the world, widely used for sugar and ethanol production (12, 32). However, diseases such as orange rust are related to yield reduction. Development of molecular tools could help find resistance sources and consequently contribute to the release of new cultivars with satisfactory resistance levels. In a recent study, Yang et al. (26) developed a molecular marker associated with a resistance gene to orange rust, called G1, which was evaluated in the present study for a set of sugarcane cultivars from Brazilian breeding programs.

The field trials conducted between 2011 and 2018 showed that 66.67% evaluated cultivars were resistant to orange rust, indicating that although this disease was recent in Brazil, some resistant cultivars were already available before the arrival of the pathogen. This was important to avoid great yield losses due to orange rust in the Brazilian growing

Table 2. Resistant and susceptible phenotype prediction in sugarcane cultivars, from G1 marker genotyping, considering three classes of resistant phenotypes, according to the diagrammatic scale used to evaluate orange rust severity: mean severity =1, mean severity ≤ 2 and mean severity ≤ 3.

Classification of resistant phenotype	Number of resistant cultivars (mean)	Number of resistant cultivars with G1	Total of cultivars with G1	Prediction of G1 marker for resistant phenotype	Number of susceptible cultivars (mean)	Number of susceptible cultivars without G1	Total of cultivars without G1	Prediction of G1 marker for susceptible phenotype
Mean severity = 1	04 (1.00)	04	14	28.57%	20 (3.41)	10	10	100.00%
Mean severity ≤ 2	11 (1.21)	08	14	57.14%	13 (4.34)	07	10	70.00%
Mean severity ≤ 3	16 (1.71)	10	14	71.43%	08 (5.60)	04	10	40.00%

areas, allowing rapid substitution of susceptible cultivars. Nevertheless, the number of asymptomatic or resistant cultivars to orange rust is still small (18, 19, 20), especially because the breeding programs did not practice selection against the disease until its emergence.

G1 molecular marker, associated with the resistance gene, was present in 58.33% evaluated Brazilian cultivars, showing 71.43% efficiency in predicting resistant phenotype when cultivars with mean severity scores up to 3 were considered resistant, according to the diagrammatic scale proposed by Amorim et al. (22). This result was slightly higher than that obtained by Yang et al. (26), who indicated 65.8% efficiency in predicting resistant phenotype for the F1 mapping population evaluated in their study. In addition, similar to that reported by Yang et al. (26), in the present study the presence of G1 marker reduced the overall mean severity by 35%.

On the other hand, the disease severity evaluation methodology and the phenotypic classification into resistant and susceptible were different between the studies: Yang et al. (26) used a diagrammatic scale developed by Sood et al. (23), which ranged from 0 (plants without symptoms) to 4 (highly susceptible plants), to evaluate the disease severity after artificial inoculation and classified the genotypes of the F1 mapping population as resistant if their score was between 0 and 2 and as susceptible if their score was 3 and 4; in the present study, we used the diagrammatic scale by Amorim et al. (22), 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. Originally, the diagrammatic scale of Amorim et al. (22) was developed to evaluate brown rust severity. However, the differences between studies are mainly due to the type of test: artificial inoculation of the pathogen carried out by Yang et al. (26) and natural infection in the present study. Anyway, the resistance gene identified by G1 marker showed to be effective in decreasing the disease severity for both the biparental progeny (26) and the set of cultivars evaluated in the present study, which do not have the same pedigree. Thus, G1 marker demonstrates great potential to be used in MAS of orange rust resistance in sugarcane.

Moreira et al. (20) showed that *P. kuehnii* isolate from Araras Municipality, São Paulo State, Brazil, presented the greatest aggressiveness and, in the present study, ten cultivars with G1 marker showed mean severity above 1 (RB855453, RB867515, RB835054, RB965902, RB935744, RB855035, RB92579, RB855156, RB925211, RB72454), also suggesting that the pathogen has high aggressiveness and that the resistance gene associated with G1 marker could act together with other genes to cause the asymptomatic phenotypic effect. Similarly, Klosowski et al. (25) showed that resistance to orange rust is controlled by a major gene and several other accessory genes, indicating possible transgressive segregation.

Although there is still no evidence of different virulent races of *P. kuehnii*, Yang et al. (26) suggests that the resistance gene associated with G1 marker is responsible for durable resistance (horizontal resistance) rather than single race-specific resistance (vertical resistance). To demonstrate durable resistance, the disease reaction in the field trials should be evaluated for several crop years, considering the ideal age of the plants and the favorable climate conditions for the pathogen occurrence (30). These conditions were met in the present study, providing reliable data for breeders in the search for increasing resistance levels to orange rust. However, the presence of G1 marker in intermediate and susceptible cultivars, such as RB92579, RB855156, RB925211 and RB72454, also indicates that more studies are necessary to elucidate the inheritance of the resistance gene linked to G1 and to understand which cellular mechanisms are responsible for decreasing the disease severity. Furthermore, the half-sib cultivars RB855536, RB835054 and RB855156 suggest that more than one copy of the resistance gene linked to G1 marker may be needed to increase the resistance levels to orange rust. Finally, investigation to find new sources of resistance to orange rust is fundamental, since resistant cultivars without the resistance gene linked to G1 marker, as indicated for SP80-1842, RB975242, RB975201, RB835486, RB985476 and RB975952, may be very useful for breeding programs in the maintenance of horizontal resistance.

This study is the first to report the use of G1 molecular marker in Brazilian sugarcane germplasm, showing its capacity to predict the resistant phenotype and potential to be used in MAS of orange rust resistance in sugarcane. The resistance gene linked to G1 marker was effective in decreasing the disease severity and was present in important cultivars grown in Brazil. The G1 marker is a valuable tool for breeding programs in the search for increasing resistance levels to sugarcane orange rust.

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