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ARTICLE

Caninde2/Milan: promising wheat line to discover novel genes for resistance to wheat blast

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Abstract: *Wheat blast disease is responsible for severe production losses. The disease resistance associated with the 2NS/2AS translocation is effective, but its level can be variable. In this study, we evaluated the presence of 2NS/2AS translocation in 310 advanced breeding lines from six crosses (Caninde 2/Milan, Milan/Caninde 2, Maringa/Milan, Milan/Maringa, Ciano79/Milan and Milan/Ciano79), and also studied their wheat blast reaction in the field to three virulent pathogen strains (P13-009, P14-031 and P14-039) collected in Paraguay. Advanced lines of two crosses (Caninde 2/Milan and Milan/Caninde 2) yielded the highest number of blast-resistant entries without 2NS/2AS translocation. Earlier studies have shown Caninde 2 to be a moderately susceptible line, which in combination with Milan, is probably adding non-2NS/2AS type resistance to these crosses. Our result indicates that such a resistance is based on several additive factors derived from multiple sources, which need to be explored further and also used to develop more durable wheat blast-resistant germplasm in the future.*

Keywords: *2NS/2AS translocation, wheat, blast,* Pyricularia oryzae

INTRODUCTION

Wheat blast disease (or brusone) is caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) pathotype *Triticum* Catt (MoT) (Couch et al. 2005, Tosa and Chuma 2014). This pathogen bleaches a portion of the spike above the site of infection, thereby rendering it sterile and causing severe damage to grain production. Under severe infection conditions, production losses can reach up to 100% (Igarashi 1991, Kohli et al. 2011). Wheat blast (WB) was first identified in Brazil in 1985 (Igarashi et al. 1986), later extended to Bolivia in 1996 (Barea and Toledo 1996), Paraguay in 2002 (Viedma and Morel 2002), Argentina in 2007 (Cabrera and Gutiérrez 2007, Perelló et al. 2015), Bangladesh in 2016 (Malaker et al. 2016), India in 2017 (Bhattacharya and Pal 2017) and more recently in Zambia (Tembo et al. 2020). The chemical control of WB is considered inefficient due to either the high disease pressure (Fernandes et al. 2017) or the appearance of new biotypes resistant to fungicides (Castroagudín et al. 2015). Therefore, the genetic resistance in the wheat germplasm remains the most cost-effective strategy.

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Since the early identification of the disease, Brazilian cultivars such as BH 1146, CNT 8, several IAC and OCEPAR selections were credited as showing different levels of field resistance, while other cultivars such as BR18, IPR 85, CD 113 etc. have shown moderate levels of wheat blast resistance over the years in many locations (Kohli et al. 2011). However, the identification of Milan, a CIMMYT wheat advanced line carrying the 2NS/2AS translocation, was a game changer and widely used as a source of resistance to the disease worldwide. The presence of the translocated 2NS segment in the germplasm confers resistance to WB (Cruz et al. 2016). While none of the Brazilian cultivars mentioned above carry 2NS segment, other authors have indicated that a major QTL of resistance is present in the 2AS chromosome, thereby reinforcing the potency of the 2NS translocation (He et al. 2020, Ferreira et al. 2020).

It has also been shown that the effectiveness of 2NS-based resistance can be variable depending on the genetic background (Cruz et al. 2016, Cardozo et al 2018). Therefore, it is essential to identify additional sources of non 2NS type resistance to broaden the germplasm base and strengthen the wheat blast resistance widely. Further, five genes conferring resistance to the MoT pathotype have been identified: *Rmg2*, *Rmg3*, *Rmg7*, *Rmg8* and *Rmg GR119* (Zhan et al. 2008, Tagle et al. 2015, Anh et al. 2015, Anh et al. 2018, Wang et al. 2018). However, the resistance conferred by *Rmg2*, *Rmg3* and *Rmg7* was rendered ineffective by aggressive MoT isolates (Cruz and Valent 2017). Additional QTLs for resistance to WB have been identified in chromosomes: 5B and 7B (Ferreira et al. 2020), 1AS, 2BL, 3AL, 4BS, 4DL and 7BS (He et al. 2020) and 2B, 4B, 5A, 6A, 1A, 4A and 5A (Goddard et al. 2020). As mentioned earlier and due to unstable performance of 2NS sources to WB in Paraguay, the objective of this study was to identify non-2NS germplasm which would enhance the genetic resistance of the combined sources and most likely provide a better protection against the disease.

MATERIAL AND METHODS

Plant materials

In total, 310 lines from different crosses (Caninde 2/Milan, Milan/Caninde 2, Maringa/Milan, Milan/Maringa, Ciano 79/Milan and Milan/Ciano 79), forming part of a wheat blast collection received from CIMMYT, Mexico, were sown in the field and inoculated artificially with WB pathogen during 2018 and 2019 crop cycles (Table 1). These lines were selected from a much larger collection (about 3000 lines), based on their agronomic type and local adaptation. Milan (2NS/2AS translocation present) and Caninde 11 (2NS/2AS translocation absent) were used as resistant and susceptible controls, respectively.

Field inoculations

Genotypes under study were sown in late April (rainy season), in two rows, one-meter-long and 20 cm apart from each other. The field tests were conducted in Caacupé (Central region of Paraguay), under artificially inoculated conditions, as natural wheat blast occurrence is restricted at this location due to unfavorable climatic conditions. In order to identify a higher degree of resistance in the germplasm, the inoculum comprised three virulent *Magnaporthe oryzae* pathotype *Triticum* strains (P13-009, P14-031 and P14-039). These strains were isolated from diseased wheat spikes (collected from different regions of the country) and identified microbiologically using the Klaubauf et al. (2014) code. Partial sequencing of the ITS (internal transcribed spacer) regions and intervening 5.8S nuclear ribosomal RNA (nrRNA) genes of P13-009 and P14-039 were registered at the GenBank (ID: MN947529 and MN947534.1, respectively). Partial RNA polymerase II largest subunit gene was also sequenced for P13-009 and P14-039 and registered at the GenBank (MN984718 and MN984725, respectively). The strains are being maintained in the National Collection of *Pyricularia* on wheat. For the preparation of the inoculum, strains were plated in an Oat-Agar culture medium, multiplied for 10 days at 25 ± 3 °C and a photoperiod of 12 hours. Subsequently, mycelium was crushed with an L-shaped glass rod, and the plates were exposed to continuous fluorescent light for 3 days. Conidia were removed with the help of a brush and sterilized with distilled water (Marangoni et al. 2013). Two plates per strain were used to prepare the inoculum suspension. The conidia concentration was adjusted using a Neubauer hemacytometer, to 5.10^4 conidia mL^{-1} (Chavez et al. 2015).

Field inoculations were carried out when spikes were completely outside of the flag leaf, stage 61 to 65 (anthesis), on the BBCH scale (Lancashire et al. 1991). To raise environmental humidity and favor the pathogen growth, sprinkler irrigation was applied two hours before inoculation. Subsequently, 10 spikes per row of each material were selected,

and each spike was sprinkled with the conidia suspension. Immediately after inoculation, each spike was covered with a transparent polyethylene bag to maintain humidity. The bags were removed 16 hours after inoculation. In the days following inoculation, irrigation was continued on daily basis to maintain soil moisture and environmental humidity.

Evaluation of disease symptoms in the spike was carried out 15 days after inoculation, using a modified version of the scale proposed by Tagle et al. (2015). This scale classifies symptoms as follows: 0 = No spike infection; 1 = Small lesions on glumes, <1.5 mm; 2 = Lesions of intermediate size, <3 mm; 3 = Mixture of green and white glumes, without apparent necrosis, caused by a hypersensitivity reaction; 4 = Completely necrotic spike. The main disease symptom is present in the reproductive stage, and there is a low correlation between observations in the reproductive and vegetative stages. Therefore, evaluation was made only in the spike (Arruda et al. 2005, Chávez et al. 2017).

DNA extraction and molecular markers analysis

DNA was extracted from the leaves of the infected plants in the field, or grains obtained from the infected lines. Liquid nitrogen was used to grind the leaves and DNA was extracted following Gilbertson et al. (1991) protocol. Polymerase Chain Reaction (PCR) was performed with the following primers: Ventriup (5'-AGG GGC TAC TGA CCA AGG CT- 3'), LN2 (5'-TGC AGC TAC AGC AGT ATG TAC ACA AAA-3') (Helguera et al. 2003) and Yr17neg-F (5'-GAT CCA TGA CGC GCA TTT G-3'). PCR conditions were: 94 °C (3 min) followed by 35 cycles (94 °C 45 sec, 58 °C 30 sec, 72 °C 30 sec), and a final extension at 72 °C (7 min). Ventriup/LN2 amplifies a 259bp fragment used to detect 2NS/2AS translocation. Yr17neg-F/LN2 amplifies a 163bp fragment that indicates the absence of Yr17, therefore absence of 2NS/2AS translocation. Amplicons were visualized with UV light in 2% agarose gel stained with ethidium bromide. Milan was used as positive control for the identification of the 2NS/2AS translocation.

Statistical analysis

For data analysis, all the lines were tested for the presence or absence of the 2NS/2AS translocation, or the heterozygous status of 2NS/2AS translocation in the cross. The infection score classification, based on the lesion size on the spike described above, allowed considering lines with score from 0 to 2 as resistant and 3 to 4 as susceptible. Other factors associated with the susceptible lines such as the presence or absence of the 2NS/2AS translocation, the heterozygous translocation condition of 2NS/2AS, and the Parentage of the cross were evaluated. Generalized Linear Models (Venables and Ripley 2002) were used to evaluate which factor or combination of factors better explains the susceptibility or resistance in the lines. A binomial family with a logistic link function was implemented to contrast the models focused on predicting susceptibility based on the aforementioned factors. These analyses were performed using the 'glm' function implemented in the Stats Package in R v3.6.2 (Team 2019).

To evaluate and contrast the statistical support of each model, the following workflow was used. First, the goodness of fit and statistical significance ($\alpha = 0.05$) of each model were evaluated using a likelihood ratio test contrasting with a null model. This test reports whether a particular model is at least better than random in predicting susceptibility. Then, the competing models were compared using the Akaike Information Criterion corrected for second-order bias (AICc) (Hurvich and Tsai 1991). In this information theory-based decision criteria, the best model is the one with the lowest AICc value. Such a model minimizes the loss of predictive performance of a simpler model versus a more complex one. The AIC and Bayesian Inference Criterion values (Schwarz 1978) were also compared using the 'compareGLM' function in the rcompanion package (Mangiafico 2015), in R. Finally, to obtain the performance of the models, we calculated the Area Under the Receiver Operating Characteristics Curve (Fawcett 2006), commonly known as AUC. A receiver operating characteristic curve is a graphical plot that illustrates the diagnostic ability of a binary classifier system as its discrimination threshold is varied. A model with random predictions reports AUC values of ≈ 0.5, equivalent to flipping the coin. On the other hand, when the performance of a model in a binary classification is very good, the AUC is close to 1.

Once the best model was determined, we used the probability that a line was susceptible given its factor profile pondered between 0 and 1 according to Pr (S|factors profile)/max{Pr (S|factors profile)} to establish a ranking of candidate profiles for sources of genetic resistance. In addition, an Alluvium plot and a Mosaic plot with Pearson residuals were generated to descriptively visualize the frequency relationships between the best model factors and resistance or susceptibility.

RESULTS AND DISCUSSION

Molecular marker analysis

All 310 lines representing different crosses were analyzed for the presence or absence of the 2NS/2AS translocation. PCR with the three primers (Ventriup/LN2/Yr17neg-F) yielded a 259 bp amplicon in the samples positive for 2NS/2AS translocation, or a 163 bp amplicon in samples without the 2NS/2AS translocation. These markers behave as co-dominant when used together (Figure 1). Only 26 samples were positive for both amplicons, indicating heterozygosity.

Principal primers (Ventriup/LN2), used to confirm the presence of segment 2NS/2AS in a genotype, amplify the N-allele of molecular marker *Xcmwg682*. Ventriup targets the first exon of the N-genome homolog of TraesCS2A02G010200 and is N-genome specific; while LN2 has specificity to the A and N genomes and is in the 3'UTR of the gene. The second primer (Yr17neg) was designed to amplify with LN2 and is specific to the 3'UTR of the A and B genomes, due to its position overlapping 2 indels 5 bp and 2 bp in length compared to the other genomes. The interactive specificity of the LN2 and Yr17neg primers helps to amplify only the A genome. Therefore, the presence of the 163 bp fragment, resulting from the amplification of Yr17negF/LN2, indicates the absence of 2NS translocation.

Screening germplasm for wheat blast infection

Based on their field infection score, the lines with low infection (0-2) were classified as resistant, and those with infection scores of 3 or 4 were categorized as susceptible. WB infection response and the presence or absence of the 2NS/2AS translocation were evaluated in 310 lines (Table 1), using Milan as the positive control and Caninde 11 as the negative control for the 2NS/2AS translocation. Both control cultivars behaved as expected: Milan was categorized as resistant while Caninde 11 as susceptible. Logistic models were calculated to explain the source of resistance to wheat blast (Table 2). The AIC allows a comparison of different models and evaluates how the model can explain the data. This decision criterion shows that the 2NS/2AS translocation and the cross behave independently to obtain the best AICc value (289.9), thereby contributing separately to the wheat blast resistance observed in the germplasm. The performance of the best model by the AUC acts as a good model for predicting susceptibility (0.75, 0.9).

In terms of resistance to wheat blast infection, Caninde 2/Milan and Milan/Caninde 2 stand out as the crosses with the highest number of lines without 2NS/2AS translocation (Figures 2a, 2b). The ranking of the crosses based on the

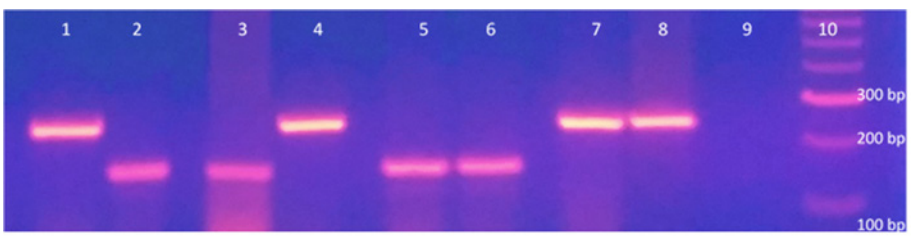

Figure 1. Results of the 3-primer PCR (Ventriup/LN2/Yr17negF). Lanes 1-7: filial lines from the cross Caninde2/Milan (763-768-769-776-779-785-786), lane 8: Milan (positive control), lane 9: PCR negative control (mix without DNA), lane 10: Marker (Hypper ladder 50bp).

Table 1. Number of lines of each cross with/without the 2NS/2AS translocation and their respective wheat blast infection response (resistant/susceptible)

Cross	2NS Translocation Absent		2NS Translocation Present		Total
	Resistant	Susceptible	Resistant	Susceptible	
Maringa/Milan	7	10	25	3	45
Milan/Maringa	9	3	17	0	29
Ciano79/Milan	9	9	15	4	37
Milan/Ciano79	19	19	30	7	75
Caninde 2/Milan	29	5	31	0	65
Milan/Caninde 2	23	8	26	2	59
Total	96	54	144	16	310

Table 2. Compared statistics for Generalized Linear Models (GLM)

Model	Compared GLM						
	Rank	Df. Res	AIC	AICc	BIC	AUC	p-value
Null model	1	309	335	335.2	342.7	0.5	-
Only Translocation 2NS/2AS	2	308	306	306.1	317.3	0.686	,
Only Cross	6	304	323	323.2	349	0.652	<0.001
Heterozygosity of 2NS/2AS translocation	3	307	307	307.3	322.1	0.692	<0.001
2NS/2AS translocation and cross with no interaction	7	303	289	289.9	319.3	0.763	<0.001
Synergy between 2NS/2AS translocation and cross	12	298	296	297.8	348.6	0.763	<0.001
Independent contribution of 2NS/2AS translocation, cross and heterozygosity	8	302	293	293.5	330.1	0.767	<0.001

Df.Res: Residual difference
AIC: Akaike Information Criterion
AICc: Akaike Information Criterion corrected for second-order bias
BIC: Bayesian Information Criterion
AUC: Area Under the Receiver Operating Characteristic Curve

probability of their susceptibility to wheat blast infection is presented in Figure 2c. It is well known that, in the presence of 2NS/2AS translocation, some lines (Caninde 2/Milan, Milan/Maringa and Milan/Caninde 2) show a lower level of susceptibility to the disease. However, in the absence of the 2NS/2AS translocation, Caninde 2/Milan lines show a higher probability of being resistant to the wheat blast disease as compared with the filial lines of other crosses (Maringa/Milan, Milan/Ciano79 and Ciano79/Milan), carrying the translocation.

In order to confirm the wheat blast resistance in non 2NS germplasm, 21 lines of the crosses Caninde2/Milan and Milan/Caninde2 were screened under both field and greenhouse conditions. Eighty one percent of these lines were classified as resistant in the field, where control cultivars Milan and Caninde11 behaved as expected: resistant and susceptible, respectively. However, under greenhouse conditions both control cultivars (Caninde 11 and Milan) were classified as susceptible and only 33% of the filial lines under test were classified as resistant (unpublished data). We believe that this discrepancy was caused by external factors, such as higher temperature, affecting plant growth conditions in the greenhouse. Higher temperatures have been reported to lower the resistance to WB (Silva et al. 2021). In this study, the conditions in the greenhouse even resulted in overcoming the resistance in Milan (used as control).

Soon after the identification of WB disease in 1985 (Igarashi et al. 1986), Brazilian researchers evaluated larger sets of germplasm to locate the sources of resistance (Barros et al. 1989, Igarashi 1991, Urashima et al. 2004). While many commercial varieties such as BH1146, IAC8, IAC24, IAC27, IAC28, IAC162, Ocepar6, Ocepar12, CEP7780, CEP8066, and Iapar1 etc. were selected for low infection in the field, most of these were found to be susceptible in the subsequent studies (Igarashi 1991, Urashima and Kato 1994). It must be pointed out that such differences in results may be attributed to the pathogenic variability and/or early artificial inoculation work being done based on leaf infection, which did not translate into spike resistance (Chavez et al. 2017). The moderate level of field resistance of the cultivar BR18-Terena to the wheat blast disease was recently explained based on several quantitative trait loci (Goddard et al. 2020). However, all these sources of resistance provided only a low level of protection, especially under severe disease conditions.

The first excellent source of wheat blast resistance was identified in a CIMMYT advanced line, Milan, based on a regional network of screening and evaluation in the epidemic region (Kohli et al. 2011). The resistance of Milan was identified to be based on the presence of 2NS/2AS translocation in it and all germplasms carrying it performed better than their counterpart without the translocated fragment vis-à-vis the pathogen (Cruz et al. 2016, Ferreira et al. 2020, Juliana et al. 2020, Cruppe et al. 2021). However, it has been observed that the presence of 2NS/2AS segment does not always lead to wheat blast resistance but is also dependent on the background of the germplasm (Cruz et al. 2016, Cardozo Téllez et al. 2018). The genetic background probably explains the differences found in the infection scores and resistance of the germplasm under study.

It was observed that the resistance displayed by some crosses (e.g., Maringa/Milan) depends more on the presence of the 2NS/2AS fragment than others (e.g., Caninde 2/Milan). On the other hand, even with the presence of the translocation in some crosses (e.g., Caninde 2/Milan and Ciano 79/Milan), the genetic background explains the difference in the severity of the infection scores.

Our results reveal that despite the absence of 2NS/2AS translocation in many of the lines pertaining to different crosses under study, several sister lines from the crosses Caninde 2/Milan, Milan/Caninde 2 and Milan/Ciano 79 etc., show a high degree of resistance to the wheat blast disease, which can be considered coming from novel sources. We visualize three possibilities for such a reaction: a) the factor/s for genetic resistance to WB being transferred outside the 2NS segment into the wheat genome, b) an undetected major resistance gene, and c) the additive effect of other minor resistance sources of blast resistance in the second parent, thereby enhancing the contribution of the background. Although Cruppe et al. (2021) did not find additional sources to be contributing much to the resistance of 2NS/2AS

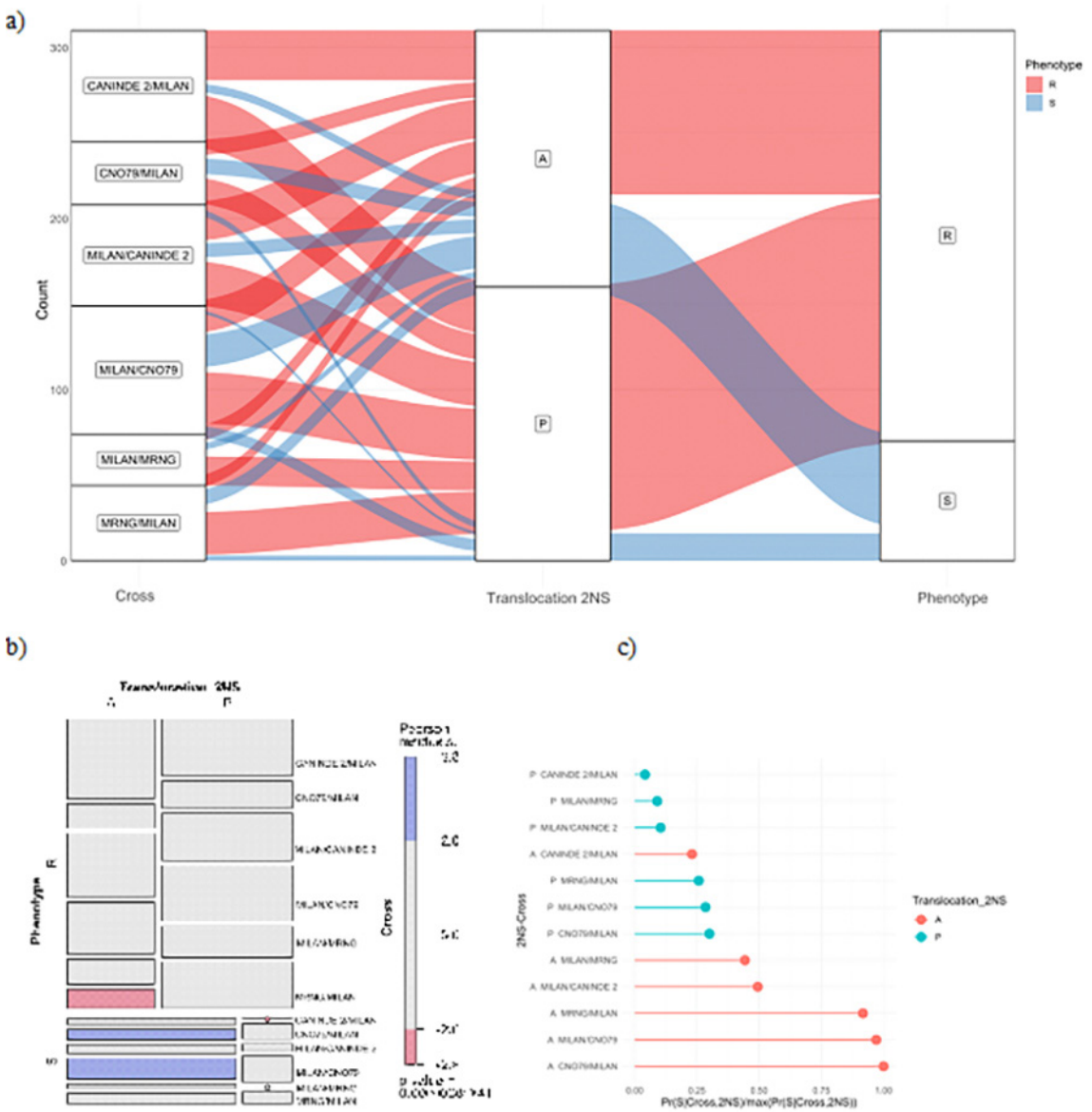

Figure 2. a) Alluvial plot of crosses with (P)/without (A) the translocation 2NS/2AS and their response to wheat blast infection: resistant (R) or susceptible (S). Scores of 0-2 were considered as resistant (represented by red lines) and 3-4 as susceptible (represented by blue lines). b) Mosaic plot of crosses with (P)/without (A) the 2NS/2AS translocation associated with the level of resistance (R) / susceptibility (S) to wheat blast. The cell sizes are associated with the frequency obtained. Pearson – residuals are on the right side of the image. The sign of this value indicates whether the observed frequency is greater/lower than the expected value. c) Ranking of crosses according to their susceptibility to wheat blast. Blue lines indicate the presence of 2NS/2AS and red lines the absence.

base, we assert that these differences can be due to the germplasm under study and/or caused by the pathogen strains being used to evaluate the resistance.

In the case of the lines from a cross Caninde 2/Milan, Caninde 2 is the progeny of a Paraguayan cross (ITAPUA35/PF84432//CORDILLERA 4), deriving its gene pool from large and diverse sources. Itapua 35 (CMH74A.754//PEL72380/ARTHUR 71) is a CIMMYT advanced line selected to incorporate an excellent level of field resistance to *Stagnospora nodorum* blotch from Brazilian germplasm Pel 72380 and Arthur 7, and was also classified as moderately susceptible to wheat blast infection (Chavez and Kohli 2018); Cordillera 4 (MN 72131 = AEPOGLOM/II64.27) is an advanced line from Minnesota, USA, introduced to incorporate leaf rust resistance from an unknown source from Russia (Aepoglom) and PF 84432 is a Brazilian advanced line (LD*2/ALD//2*HAD/7/ALZ110/2*IAS54/6/TP/4/TZPP/SON64//NAPO/3/CIANO /5/ PEL 11319-61// IAS20/ND81 (PF6968), developed for an overall excellent resistance to wheat diseases. It must be pointed out that several parents of this line, Londrina, Hadden, Alvarez 110, Toropi, Tezanos Pintos Precoz (TZPP) and PF 6968 etc., have been widely used in the Southern Cone region and globally, for carrying resistance to multiple diseases of wheat. Although the resistance of Itapua 35, PF 84432 and Aepoglom has not been tested for wheat blast disease, we propose them to be the carriers of several genes (or quantitative trait loci), which solely or in combination with other parents of Caninde 2 are responsible for this non-2NS/2AS basis of resistance.

Therefore, we consider that the low infection scores to wheat blast in a large proportion of non-2NS/2AS lines, in Caninde2/Milan and Milan/Caninde 2 crosses, result from the additive effects for resistance derived from multiple sources present in these lines and they complement the 2NS/2AS translocation resistance when the fragment is present. These combined sources of genetic resistance to wheat blast disease will most likely be more durable and serve to develop newer germplasm in the future. However, further research is needed to better understand the genetic basis of these promising sources of non-2NS-based resistance to wheat blast disease.

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