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ORIGINAL ARTICLE

Fusarium ear rot resistance in South American popcorn lines

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Abstract

To contribute to increasing ear rot resistance in new popcorn cultivars, the present study was aimed to identify germplasm with resistance to Fusarium ear rot (FER) in popcorn inbred lines. The study was conducted in Campos dos Goytacazes, Rio de Janeiro, Brazil. Ear rot severity was evaluated in 176 lines of different populations of popcorn from South America. In the first stage, the lines were evaluated in two seasons under natural infection conditions in the field, in a design of groups of experiments arranged in randomized blocks. In the second stage, the genotypes selected with potential FER resistance had their kernels evaluated in a blotter test to confirm the absence of the pathogen. Finally, in the third stage, the lines selected as candidates for resistance in the previous stages were again evaluated under artificial inoculation conditions in a protected environment. The selection of genotypes with potential use for the breeding of the crop considered the possibilities of gains in heterosis and the local phytosanitary requirements for the sale of popcorn grains. Of the 176 evaluated lines, nine exhibited resistance to FER and will be used for the composition of crossing blocks aiming at the generation of resistant hybrids. The field evaluation stages and confirmation of the presence of the pathogen via the blotter test were essential for saving resources and optimizing the selection process.

Keywords *Fusarium verticillioides* · Genetic resistance · Genetic resources · Popcorn breeding

Introduction

Popcorn (*Zea mays* var. Everta) is a special type of maize intended specifically for human consumption. In addition to its thicker pericarp and popping expansion features, popcorn also differs from common maize in its greater susceptibility to diseases. Fungal leaf and ear diseases are among the main limitations for maintaining grain yield and phytosanitary quality (Magan et al. 2003; Di Domenico et al. 2015; Likhayo et al. 2018). In particular, the kernel rot caused by fungi of the genera *Fusarium*, *Aspergillus*, and *Penicillium* has been the disease of greatest concern in terms of phytosanitary and food safety risks (Duncan and Howard 2010; Ekwomadu et al. 2018). In Brazil, studies about ear rot show that *F. verticillioides* is the most predominant pathogen associated with ear rot in maize (Stumpf et al. 2013). Other studies have reported other species such as *F. graminearum*, *F. meridionale*, and *F. cortaderiae* (Kuhnem et al. 2016; Machado et al. 2021). These *Fusarium* species can produce toxins such as fumonisins, beauvericin, moniliformin, and enniatins in different cereal crops (Rosa Junior et al. 2019; Nicolli et al. 2020).

Fungicides have been employed as a form of control (Andriolli et al. 2016; Eli et al. 2021); however, there are difficulties in controlling the development of fungi, especially in storage stages where the increase in mycotoxin production is even riskier (Munkvold 2003). Additionally, the indiscriminate use of fungicides reduces the sustainability of production systems, since it jeopardizes both the maintenance of the fauna and the health of workers and consumers if used incorrectly and indiscriminately (Cullen et al. 2019), besides enabling the selection of fungi variants resistant to the active principles of fungicides. In this context, the use of cultivars with higher levels of ear rot resistance is considered one of the most efficient and sustainable forms of control.

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Besides Fusarium ear rot being the most worrisome problem for maize grain production in Brazil, Robertson-Hoyt et al. (2007) report that *Fusarium* and *Aspergillus* infections in maize grains share the same genetic resistance mechanism. Therefore, the development of Fusarium ear rot (FER) resistant cultivars can opportunistically reduce *Aspergillus* infection in maize grains. In popcorn, despite the efforts of breeding for the development of resistant cultivars, the number of studies on ear rot pales compared to those with common maize (Solalinde et al. 2014; Schwantes et al. 2017; Kurosawa et al. 2017; Almeida et al. 2021). Although there is potential for the development of hybrids resistant to ear rot, limitations in identifying sources of genetic resistance are, therefore, a counterproductive factor to the development of new cultivars.

Some South American countries have considerable popcorn production, e.g., Argentina and Brazil, which account for 8.9% and 8.3% of the export market (Tridge 2021). The predominant tropical and subtropical climate in producing regions, mainly in Brazil, contributes to a greater occurrence of diseases such as the kernel rot (Renfro and Ullstrup 1976; Luna et al. 2016). Identifying germplasm with resistance alleles in these regions can contribute both to the agricultural development of these countries and to advances in the development of resistant cultivars in other regions of the world.

In recent decades, Brazilian research institutions have been working with different populations of popcorn collected in South America (Miranda et al. 2008; Ematné et al. 2012; Ribeiro et al. 2016; Almeida Silva et al. 2017). It is believed that despite the challenges for producing hybrids with high popping expansion and yield, the use of lines extracted from local populations may contribute ear rot resistance alleles and thus enhance the productivity of popcorn hybrids by reducing the dependency on imported seeds in South American agriculture. The objective of this study was to identify germplasm resistant to Fusarium ear rot in a panel of lines extracted from different popcorn populations in South America.

Material and methods

This study examined 176 inbred popcorn lines (Table 1) at the seventh generation of selfing (S_7).

The identification of FER-resistant lines consisted of three steps: a) selection of candidate lines for resistance in field trials under natural pathogen infection; b) elimination of asymptomatic susceptible lines through germination tests in a controlled environment, and; c) confirmation of resistance of the lines selected from the inoculation of the pathogen in a controlled environment. All procedures for each step are described below:

Selection of candidate lines for resistance in field trials

Field trials were conducted in two different seasons (winter and summer) to assess the incidence and severity of FER in 176 popcorn lines. The tests were carried out at the experimental station of the Antônio Sarlo State Agricultural School, located in Campos dos Goytacazes, Brazil (21°42′56″ S and 41°20′34″ W).

All lines were evaluated twice, first in the winter season and then in the summer season. In the winter (May to August 2019) and summer (October 2019 to January 2020) seasons, the lines were evaluated in five and six smaller trials, respectively. The trials were arranged in this way due to the unavailability of a single area sufficient for the establishment of a single trial with all lines.

Each minor trial received an *n* number of regular lines (*n* differed for each trial due to loss of plots and treatments) and three common lines. Common lines were used to correct trial effects within each of the seasons. In each minor trial, treatments were arranged in randomized blocks with three replications. Common lines were randomized and inserted within the minor trial. The experimental plot consisted of a single planting row with 16 plants. A spacing of 0.2 m between plants and 0.9 m between rows was adopted, corresponding to a density of 55,555 plants ha^{-1}.

As a fertilizer treatment, 32 kg ha^{-1} nitrogen, 112 kg ha^{-1} phosphorus, and 64 kg ha^{-1} potassium were applied at planting. During plant development, the area was also fertilized with 150 kg ha^{-1} nitrogen, split into two applications (at 30 and 40 days after planting, respectively). Irrigation was provided throughout the plant's growth cycle, and weeds were controlled through mechanized weeding. The control of fall armyworm was achieved by applying an insecticide (Engeo Pleno™ S) during the first 60 days of growing. The experimental station where the trials were conducted does not apply fungicides to any maize crop. Similar growth techniques were applied in both seasons.

During the winter season, the average air temperature was 23 ℃, average relative humidity was 76.7%, and accumulated precipitation was 221.6 mm. In the summer, the respective values were 26 ℃, 77.6%, and 657.6 mm.

Data collection in the field trials

After harvesting, all plots were evaluated for Fusarium ear rot (FER) severity. This variable was individually assessed in each ear of the plot affected by rot, by assigning a score based on the percentage of visibly symptomatic kernels. The value for each plot was the mean of all ears evaluated.

Table 1 Description of the S_7 lines evaluated in the study and their respective populations and collection countries

Lines	Population	Reaction to FER*
L54—L55—L59	Beija-Flor	Unknown
L61 – L63 – L66 – L70 – L71	Angela	Resistant [a]
L74 – L75 – L76 – L80 – L88	Viçosa	Resistant [b]
L201 – L202 – L203 – L204 – L205 – L206 – L207 – L208 – L209 – L212 – L213 – L214 – L215 – L216 – L217 – L220 – L221	IAC 125	Resistant [b]
L231 – L232 – L234 – L236 – L238 – L240 – L241	BOZM 260	Unknown
L261 – L263 – L265 – L266 – L268 – L270 – L271 – L272 – L273 – L274	PARA 172	Resistant [c]
L291 – L292 – L294 – L295 – L297 – L298 – L300	URUG 298	Unknown
L321 – L322 – L324 – L325 – L326 – L328 – L329 – L330 – L331 – L332	Barão de Viçosa	Unknown
L351 – L352 – L353 – L354 – L355 – L357 – L358 – L359 – L360 – L361 – L363 – L364 – L365 – L366 – L367 – L368 – L369	PR 023	Unknown
L381 – L382 – L383 – L385 – L387 – L388 – L389 – L390 – L391 – L392 – L393 – L394 – L395 – L396	SAM	Unknown
L411	CHZM 13 134	Unknown
L441 – L443 – L444	BOYA 462	Unknown
L471 – L472 – L473 – L474 – L475 – L477 – L478 – L480 – L482 – L483	SE 013	Unknown
L501 – L502 – L503 – L506 – L507 – L508 – L509 – L510 – L511 – L512 – L513	PA 170 ROXO	Unknown
L531 – L533 – L534 – L535	ARZM 07 049	Unknown
L561 – L562 – L563	ARZM 05 083	Unknown
L591 – L592 – L593 – L594 – L596	RS 20	Unknown
L623 – L624 – L625 – L626 – L627 – L628	PA 091	Unknown
L651 – L652 – L653 – L654 – L655 – L656	ARZM 13 050	Unknown
L682 – L683 – L684 – L685 – L686 – L688 – L690 – L691 – L692 – L693 – L694 – L695 – L699	UENF 14	Unknown
P1	Zélia	Resistant [a]
P2 – P3	CMS-42	Unknown
P4	SAM	Unknown
P5 – P6 – P7	Zaeli	Unknown
P8 – P9 – P10	IAC 112	Unknown

* Populations with reports of FER resistance or at least one descendant line identified as resistant

[a] reported by Schwantes et al. (2017); [b] reported by Kurosawa et al. (2017); [c] reported by CIMMYT (2022)

Initially, our proposal was to evaluate 194 inbred lines, but due to low fertility in some lines, we considered 176 lines for a more precise assessment of FER severity. The severity evaluation was standardized using a scale adapted from CIMMYT's (1994) proposal, with scores ranging from 0 to 100, in 5-point intervals. All ears harvested in the plot were included in the evaluation, except those severely affected by pests, which were not considered for FER evaluation. The entire assessment was performed by a single evaluator. Throughout the two seasons, a total of 11,742 ears were evaluated. The evaluation respected the order of repetition within each day, ensuring that any influence from evaluator fatigue was accounted for as a block (repetition) effect.

Data from each of the harvests were subjected to O'nneill-Mathews' analysis of variance and Shapiro–Wilk's test of normal distribution of residuals at a significance level of 5%. Then, for the data from each of the seasons, an analysis of variance (ANOVA) was undertaken following the model of groups of experiments in complete randomized blocks (GERBD), as proposed by Gomes and Guimarães (1958), according the model:

$$Y_{ijk} = \mu + B/T_{k(j)} + T_j + L_{i(adj)} + cLT_j + \varepsilon_{ijk}$$

where Y_{ijk} is the value observed in plot from i-th genotype in the j-th trias (minor trial) within the k-th repetition; μ is the constant effect or overall mean; $B/T_{k(j)}$ is the effect of k-th repetition within the j-th experiment; T_j is the effect of j-th experiment; cLT_j is the effect of interaction between common genotypes and the j-th experiments; $L_{i(adj)}$ is the effect of i-th genotype (line) considering the adjustment from j-th experiment where the genotype were evaluated; and ε_{ijk} is the aleatory error associated at Y_{ijk} observation (NID, 0, $\sigma = 1$).

After identifying significant effects between the different minor trials in each season, the mean of the common lines was used to adjust the mean of the regular lines and thus allow a fairer comparison between all lines. The adjustment was achieved by summing the mean value of each regular line with the value of the correction factor (CF) estimated from the mean of the lines common to the minor trials:

$$CF = \overline{X}CL_i - \overline{X}CL,$$

where CLi is the mean value of the common lines in minor trial i; and CL is the overall mean of each line common to all minor trials of the respective season. This method results in adjustments ranging from 0.5% to 2.0% in the plot values between the experiments within each season.

Selection of candidate lines for resistance to Fusarium ear rot

In the selection stage, we considered the genetic nature of the germplasm evaluated in the field and the use of this material for the breeding program in the future. In maize breeding programs, lines are used to produce hybrids. Studies conducted up to the present date on the development of hybrids based on part of our germplasm showed that the highest obtained value of relative heterosis for FER severity was -20.7% (Schwantes et al. 2017; Almeida et al. 2021). The entire selection was based on FER severity values.

To define a reference value for selection, we adopted the threshold of 5% of moldy kernels (the category into which kernels affected by FER fall) accepted for samples of popcorn grains sold in Brazil (Anvisa 2011). Based on this information, lines with up to 25.7% FER severity in the ears were deemed promising for the generation of hybrids that meet the phytosanitary requirements of the Brazilian market. Thus, based on the estimated residual variances for each season based on analysis of variance, we estimated the confidence interval for determining the reference value for selection of lines in each season. The confidence interval was estimated using Student's t-test, based on the equation below:

$$\mu \geq \overline{X} + t_\alpha \frac{s}{\sqrt{n}}$$

where μ is the overall mean of the set of lines in the season; $\overline{X}$ is the value of the sample mean, here considered the value of the pre-established reference point (25.7); t_α is the preset value in Student's t distribution for probability value α and n-1 observations; s is the standard deviation of the sample, considering the variance associated with the ANOVA error; and n is the number of observations made in the respective season.

Lines that showed a FER severity mean below the reference value for the two seasons were selected as interesting to ear rot resistance exploration.

Identification of susceptible asymptomatic lines by the blotter test

After harvesting and evaluating the ears in the field trials, kernel samples from each of the plots were stored in paper bags and kept at a temperature of 25 ºC. After selecting the candidate lines for the respective season, 50 kernels from each plot of these lines were immersed in a sodium hypochlorite solution (1%) for 1 min and subsequently washed in distilled water as described by Schwantes et al. (2017). This disinfection procedure aimed to eliminate microorganisms present on the outside of the kernels. Additionally, kernels from two lines evaluated as susceptible were also included in the evaluation panel as blotter test controls.

The kernels were placed on paper in acrylic boxes measuring 10 × 10 and 7 cm deep. The boxes and papers were previously sterilized. After allocating the kernels, the boxes were moistened with distilled water and placed in a growth chamber at 25 °C, under controlled light (16 h of light and 8 h of dark), for seven days (sufficient period for the germination of all grains).

After the incubation period, the kernels were visually assessed to identify the development of *Fusarium* colonies. This assessment consisted of counting kernels with the presence of the colony relative to the total number of kernels evaluated.

Data from the two seasons were considered jointly (average of the two seasons in each plot) for this stage of the evaluation. The data were subjected to analysis of variance by the F-test at a significance level of 5%. Line means were compared using Tukey's test at the 5% significance level.

Evaluation of selected lines in a controlled environment

To confirm the resistance of the lines, those selected in the field trials and which were not identified as susceptible asymptomatic in the blotter test stage were evaluated in a protected environment (Fig. 1-A).

The test was conducted in an environment covered with a shade net and protected by a screen. The lines were grown in 18-L pots, in substrate based on earth and sand (2:1). The experiment was laid out in a completely randomized block design with five replications. Two plants grown in a single pot composed the experimental plot.

Each pot received 2 g of nitrogen, 4 g of phosphorus, and 2 g of potassium at planting. Additionally, during cultivation, the plants received 6 g of nitrogen, split into two applications at 25 and 35 days after planting. The plants received approximately 300 mL of water via drip irrigation daily.

Fourteen days after the release of the stigma on the ears, the plants were inoculated with the application of 5 mL of *F. verticillioides* isolates spore suspension (Fig. 1 – B) at a concentration of 1.5×10^6 spores mL^{-1}. The suspension was

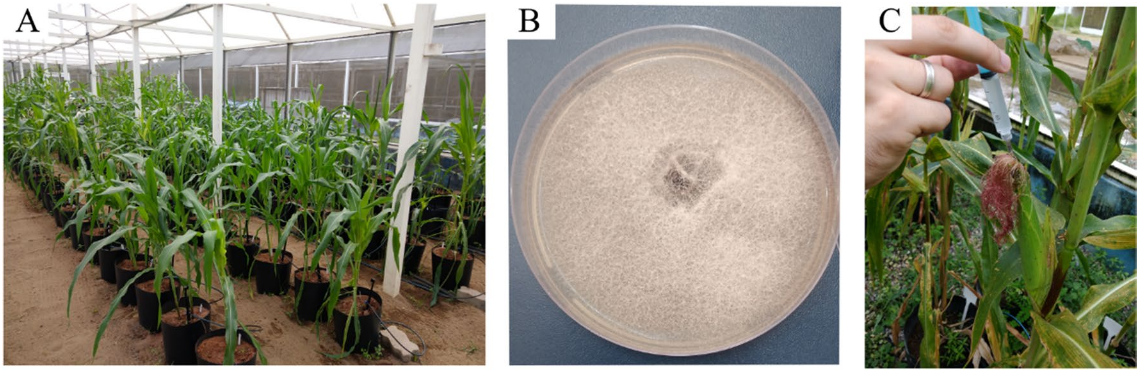

Fig. 1 Images of the experiment in a controlled environment (**A**), colony of the *Fusarium verticillioides* isolate used in the inoculation (**B**), and procedure for inoculating the popcorn ears (**C**)

applied on the stigma of the ears with a graduated syringe (Fig. 1 – C). The isolate used was a mixture of isolates CF/UENF 501 and CF/UENF 502. These isolates are maintained at the Plant Pathology Clinic of the Universidade Estadual do Norte Fluminense (UENF).

At 120 days after planting, the ears were harvested and visually assessed for FER severity. The evaluation was performed using a diagrammatic scale adapted to that proposed by CIMMYT (1994), with scores from 0 to 100 at 5-point intervals, corresponding to the percentage of visibly symptomatic kernels.

Data on FER severity on the ears were subjected to O'nneill-Mathews' analysis of variance and Shapiro–Wilk's test of normal distribution of residuals. Then, the F-test was applied and means were compared by Tukey's test. The significance level of 5% was adopted for all tests applied.

Results

Analysis of variance revealed significant differences between minor trials, between lines for FER severity in both seasons, as well as significant differences for the Line × Season interaction (Table 2).

Disease severity was more intense in the summer season, when the average percentage of infected kernels more than doubled that observed in the winter. The significant interaction between seasons was due to the low severity observed, even in susceptible lines, during the winter. Thus, the greater intensity of the disease in the summer made it possible to better discriminate the susceptible genotypes.

Of the evaluated panel, 48 lines (27.2% of the evaluated lines) that exhibited severity values lower than 25% in the winter showed higher values in the summer (Fig. 2). Only three lines (L322, P2, and P8) showed a higher severity of infected kernels in the summer season, compared with the winter.

Fusarium ear rot severity was higher than the selection values of two seasons in 104 lines of the evaluated panel. Finally, only 11 lines showed less than 25.7% of grains infected by *Fusarium*, value that was used to section criteria in this case. These 11 lines are, therefore, those with the best potential for generating hybrids that can meet the phytosanitary requirements of the local grain market.

The count of the number of kernels infected by *Fusarium* in the selected lines revealed that despite not displaying symptoms on the kernels, line L688 showed the fungus in

Table 2 Summary of analysis of variance for Fusarium ear rot severity in inbred popcorn lines, considering the group of experiments in complete randomized blocks (GERBD) within season and complete randomized block designs to jointly analyses (RBD) between the seasons from adjusted values to popcorn inbred lines

GERBD across minor Trials (T) within the Seasons						RBD across Seasons (S)			
S.V	d.f	Winter		Summer		S.V	d.f	MS	*P*
		MS	*P*	MS	*P*				
Block/T	4	228.2	0.155	166.5	< 0.001	Block/S	4	98.5	0.647
T	10	2090.7	< 0.001	2924.6	< 0.001	S	1	214,154.4	< 0.001
L_{adj}	175	1802.2	< 0.001	2187.1	< 0.001	L_{adj}	175	2280.6	< 0.001
$L_{com.}$ x T	8	542.8	0.005	281.6	0.145	L x S	175	893.7	< 0.001
Error	366	148.4		169.0		Error	700	158.4	
Mean		36.1		63.3		Mean		50.9	

S.V. source of variation, *d.f.* degrees of freedom, *MS* mean square, *P* probability value (p-value), *T* Trial (minor trial), *Ladj* lines with value adjusted, *Lcom* common lines from minor trials, *S* season, *L* lines

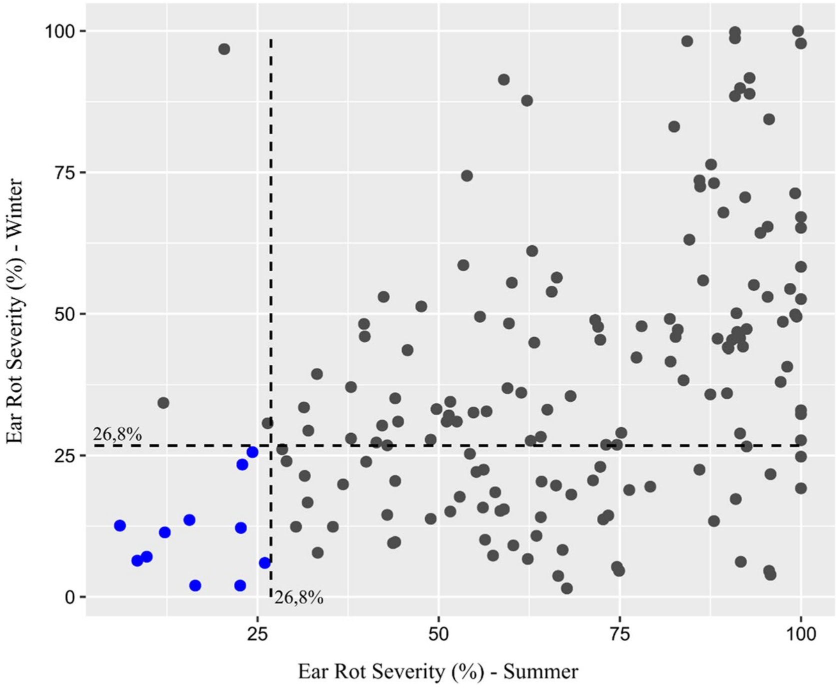

Fig. 2 Dispersion of 176 inbred popcorn lines regarding mean Fusarium ear rot (FER) severity in different seasons (heritability estimated = 0.7). Black points indicate lines with ear rot severity between 26.8% and 100% in summer or between 26.8% and 100% in the winter – not should be exploited for FER resistance. Blue points indicate lines with ear rot severity between 5.2% and 25.6% in summer and between 6% and 26.3% in the winter – that should be exploited for FER resistance

percentages significantly similar to those of lines L353 and L390, which were selected as susceptible controls for comparison purposes in that stage (Fig. 3).

Line L688 showed an average of 3% and 20% of infected kernels in the evaluations of the winter and summer harvests, respectively. However, after germination, there was an average of 78% of kernels with the presence of the pathogen (76.1% in winter and 81.9% in summer). Because of this, this line was discarded as a candidate genotype for FER resistance.

Despite the previously mentioned divergence, for the other lines, the amount of infected kernels approached the values assigned through the visual assessments of the ears in the field trials.

Inoculation of the pathogen in a controlled environment revealed that of the 10 candidate lines for FER resistance, nine showed values below 25.7% (established as the desired maximum threshold for lines). Only line L503 was not confirmed as resistant when subjected to artificial inoculation (Fig. 4).

Lines L503 showed FER severity means of 11.4% and 12.2% in the winter and summer crops, respectively. However, the blotter test revealed an average of 21% of kernels infected by *Fusarium*. Under artificial inoculation, L503 showed a mean FER severity of 29.6%. The discrepancy between the values observed in the field and in the controlled environment stage may have occurred due to the action of uncontrolled factors in the field. The amount of natural

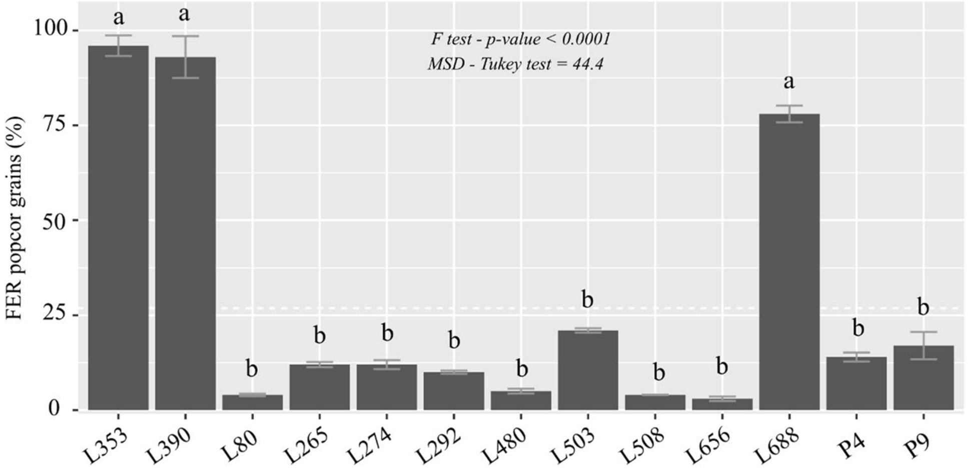

Fig. 3 Comparison between popcorn lines regarding the mean percentage of kernels infected by *Fusarium* after germination after two seasons of evaluation in field (intervals up the bars indicate the standard deviation among seasons). Common letters indicate means without significant differences at 5% probability by Tukey's test

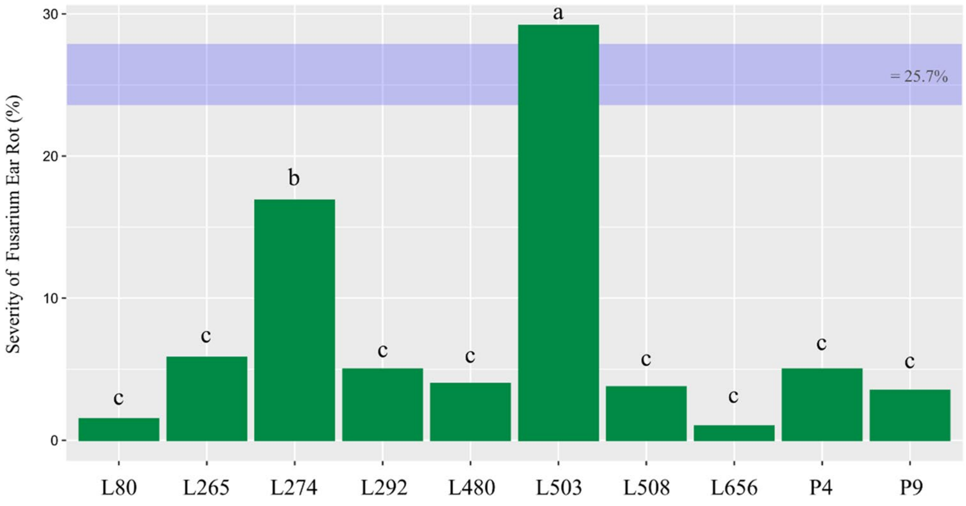

Fig. 4 Mean *Fusarium* ear rot severity in popcorn lines under artificial inoculation of the pathogen. Common letters indicate equal means according to Tukey's test at the 5% significance level. The blue band indicates the confidence interval where the means do not differ by 25.7% according to Student's t-test at the 5% level

inoculum available in the evaluation fields, as well as the amount of spores deposited on each ear, may be some of these factors that interfered with the occurrence of greater rot in line L503.

A comparison of means between lines revealed that lines L80, L265, L292, L480, L508, L656, P4, and P9 did not differ from each other, showing a mean severity below 10%. Despite exhibiting severity below the established maximum value, line L274 differed from the others by having a higher percentage of infected kernels. Breeders should consider the possibility of developing hybrids using L274 as a parent, as it presents high levels of mycotoxins.

Discussion

A total of 176 popcorn lines were evaluated in field trials during two seasons. Despite the natural infection of the disease, the natural occurrence of the pathogen was sufficient to distinguish genotypes with different levels of severity in both seasons. Moreover, in a study evaluating resistance to Fusarium ear rot in 24 lines of common maize in South Africa, Small et al. (2012) demonstrated how environments with a history of the disease can contribute to selection of materials with potential resistance. In the present study, the field evaluation stages made it possible to identify 165 lines with susceptibility to ear rot, which were discarded as a potential source of resistance alleles. The estimated heritability (h^2) was 0.7, considering the two seasons. This demonstrates that it is possible to select lines with genetic resistance based on phenotypic data. Other studies have reported heritabilities of up to 0.6 for ear rot in corn or popcorn (Horne et al. 2016; Almeida et al. 2021; Gaikpa et al. 2021).

Despite the possibility of distinguishing the lines in the two seasons, disease severity was notably higher during the summer season, and consequently so was the discrepancy between treatments. The increase in average daily temperature and the greater accumulated volume of rainfall possibly favored the progress of the epidemic. Rossi et al. (2009) demonstrated that daily temperature fluctuations in the range of 17 to 27 ºC associated with high moisture in the ears favor the reproduction of spores of *F. verticillioides*, the main causative agent of ear rot in maize. Although the average daily temperature was 23 ºC in winter season, there were daily fluctuations in the range of 11 to 35 ºC and an accumulated precipitation volume 50% lower than in the summer season (Fig. 5).

In the winter season (Fig. 5-a), lower temperatures and less rainfall were observed after the vegetative stage. These conditions are not optimal for *Fusarium* development. However, the occurrence of the disease during the winter season may have been influenced by the sprinkler irrigation used during the trials. This irrigation method was utilized in both seasons, suggesting that rainfall, in conjunction with irrigation, may have contributed to an increased disease incidence in the plants. Nonetheless, the use of irrigation can be an important factor in maximizing disease occurrence during the winter.

Overall, ear rot severity varied from 1.6% to 100% across the lines. From a genetic point of view, in lines at the seventh generation of inbreeding, all the variation observed between individuals is expected to be predominantly due to the additive effect (Hallauer et al. 2010). Although different studies have shown the possibility of exploiting heterosis to reduce ear rot with part of the lines tested in this study, we observed that the variation due to additive effects was greater than the maximum deviation value observed in the hybrids tested in these studies (Schwantes et al. 2017; Almeida et al. 2021). This result corroborates those of studies that indicate that despite the possibility of gains by exploiting non-additive genetic effects, the exploitation of additive effects in the first stages is either equally important or even more advantageous

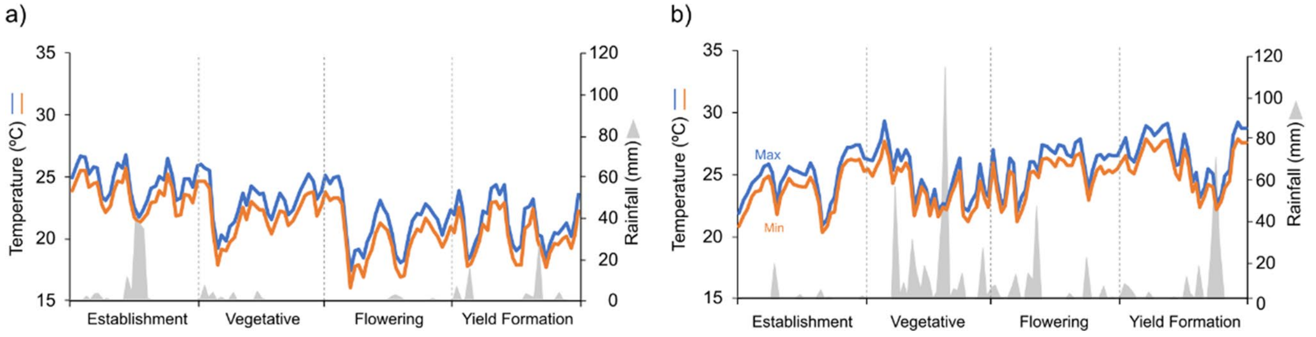

Fig. 5 Dairy temperature maximum (blue line), minimum (red line) and rainfall (blue sequence) registered in different plant growth stages on the field experiments with 176 popcorn lines during winter (**a**) and summer (**b**) season in Rio de Janeiro - Brazil

(Lanubile et al. 2017; Netshifhefhe et al. 2018; Tembo et al. 2022). In other words, the choice of parental lines with a lower degree of susceptibility to FER is essential to increase the resistance of hybrids to be generated from the crossing of these parents.

Thus, the inclusion of the ear rot severity trait as one of the selection criteria during the line production stages is a strategy capable of allowing the development of hybrids with higher grain yield and phytosanitary quality at the end of the process.

In this study, we considered lines resistant when they showed FER severity below 25.7%. This criterion was established based on an expected reduction value in the hybrid production stages based on information from studies that used part of these lines, added to the maximum value of 5%, established by Brazilian legislation (Anvisa 2011). This reference value may be inadequate if used in a set of lines from different germplasm or even for programs aimed at the development of hybrids for other countries, where the limits of injured kernels may differ from the one used here. In addition, the lack of further studies on the potential reduction of FER when hybrids are created poses a limitation to the precision in selecting lines. Therefore, the values considering selection criteria may change based on future studies focused on the development of new hybrids.

Another option for selecting superior lines is to utilize resistance classification systems proposed by other studies. However, the threshold values for the classification of varieties regarding the degree of susceptibility to ear rot are not well established among researchers. Small et al. (2012) classified maize lines with 17.7% severity as highly susceptible; with up to 5% severity as resistant; and with up to 2% severity as very resistant to FER. Chen et al. (2012) studied segregating populations for the detection of quantitative trait loci (QTL) and considered a line with a mean FER severity lower than 1.4% as resistant and a line with a mean of 6.5 to 7.0% as susceptible. Horne et al. (2016) used crosses between contrasting lines to obtain a segregating population and indicated lines with up to 47% as more resistant and lines with 80 to 88% as more susceptible to FER.

Therefore, the choice of this selection strategy adopted in this study was not aimed at the definition of a criterion for classifying lines into levels of susceptibility. The objective with the established value was to identify the maximum number of lines with some probability of use in the breeding program in order to meet the local demand. Other plant breeders are expected to be able to use this initial idea to identify values of interest for selecting plants that are more resistant to FER according to the demand and reality of their respective breeding programs.

In studies evaluating genotypes for disease resistance, particularly those involving the association of molecular markers, the evaluation process typically includes initial development and assessment of plants in a controlled environment, followed by verification of plant response in the field (Mutlu et al. 2005; García-Ruiz et al. 2014) or simultaneously (Kim and Reinke 2019).

In this study, we used the reverse path of the steps. This is because evaluating a high number of genotypes demands larger spaces in controlled environments, a resource that we did not have available during our research. Therefore, when evaluating a large number of genotypes, it is common practice to initially select promising resistant genotypes through field trials, and subsequently reduce the number of genotypes for evaluation in controlled environments based on available resources (Silvar et al. 2010; Rebouças et al. 2018; Imerovski et al. 2019).

The evaluation stages in the field based on the natural occurrence of the pathogen contributed to saving resources during the process of identifying resistant lines. The use of the blotter test also made it possible to identify asymptomatic lines or lines with symptoms that were difficult to detect visually, as was the case with L688. This line has a matte yellow pericarp, which makes it difficult to visualize the *Fusarium* hyphae. However, laboratory germination tests showed that despite the asymptomatic appearance, the kernels were colonized with the fungus. Czembor and Ochodski (2009) demonstrated that despite the potential of the blotter test technique for distinguishing resistant genotypes, this step should always be used as a complement to field evaluations, since the virulence of the pathogen to infect kernels may be lower if inoculated only in a controlled environment.

Another divergence of results regarding the field stage occurred with line L503, which showed a mean rot severity on the ears below the reference value (25.7%) during the evaluation harvests in the field and in the germination tests. Nonetheless, its severity was greater than the reference value in the confirmatory test in a controlled environment. Our hypothesis is that this response difference was due to a higher inoculum pressure applied to this line in the resistance confirmation step when compared with natural occurrence in the field. It is important to highlight that although line L503 showed values below the reference value, in the group selected as candidates for resistance, this was the line that exhibited absolute values closest to 25.7%, both in the field stages and in the germination tests.

The use of the three steps to eliminate non-resistant lines provided greater safety in the selection process of genetic materials that could be used in popcorn breeding programs.

Among the nine lines selected at the end of the trials, only L265 and L274 were obtained from the same population, i.e., variety PARA 172. This population was collected in Paraguay and has been reported as resistant to ear rot since its collection (CIMMYT 2022). This demonstrates that FER resistance alleles were selected more frequently in these two lines among those obtained from this same population.

The fact that there are lines from eight different genealogies (Viçosa, PARA 172, URUG 298, SE 013, PA 170 Roxo, ARZM 13 050, SAM, and IAC 112) points to possible advantages in investing in crossbreeding strategies aimed at exploiting gains in heterosis for *Fusarium* resistance, since the genetic distance between the parents is one of the premises to obtain high heterosis values. However, the heterotic potential of these crosses requires crossing stages for the future evaluation of hybrids. In addition to the lines identified as resistant, those also confirmed as susceptible may support gene identification and inheritance studies related to *Fusarium* resistance in popcorn.

In studies on Fusarium ear rot (FER) resistance, the evaluation of mycotoxin production is often associated with severity evaluations to assess potential risks to human health (Butrón et al. 2015; Njeru et al. 2020; Bennett et al. 2023). *Fusarium* species can produce several mycotoxins, with fumonisin B1 being one of the most abundant (Parsons and Munkvold 2010; Nicolli et al. 2020). However, the mycotoxin evaluation process becomes costly when a large number of genotypes need to be assessed (Bolduan et al. 2009). Therefore, mycotoxins are typically evaluated in the final stages of breeding, such as during the performance evaluation of hybrids in the case of corn (Löffler et al. 2010; Stagnati et al. 2020).

Furthermore, the correlation between FER severity and fumonisin accumulation in grains has been studied to explore the possibility of indirectly reducing mycotoxin levels by selecting genotypes more resistant to FER. Correlations ranging from 0.6 to 0.9 have been reported by Robertson et al. (2006) across different generations of corn. Bolduan et al. (2009) found correlations between 0.8 and 0.9 between visual evaluation of ear rot and deoxynivalenol and fumonisin concentrations using two evaluation methods. Studies by Vandicke et al. (2019) and Birr et al. (2021) indicate that there is no significant linear correlation between mycotoxin concentration and the amount of *Fusarium* DNA, or between different mycotoxins in kernel samples of corn.

The evaluation of mycotoxins in hybrids developed from lines selected in this research will help further investigate correlations between mycotoxins and visual ear rot evaluation, as well as correlations between parental lines and hybrids. In this study, the evaluation of ear rot under the conditions of natural occurrence in the field, as well as the evaluation of seeds after germination, contributed to the elimination of symptomatic and asymptomatic susceptible lines. The best discrimination of genotypes under naturally occurring conditions occurred in the hotter and rainier season. Lines L80, L265, L274, L292, L480, L508, L656, P4, and P9 are sources of resistance to FER and can be exploited in popcorn breeding programs to obtain resistant cultivars.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests On behalf of all authors, the corresponding author states that there is no conflict of interest.

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