Crop Protection 142 (2021) 105504

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Using anthesis date as a covariate to accurately assessing type II resistance to Fusarium head blight in field-grown bread wheat [1]

M.F. Franco [a,b,1], G.A. Lori [c,d], M.G. Cendoya [a], J.S. Panelo [a,c], M.P. Alonso [a,b], N.E. Mirabella [a], I. Malbrán [b,d], A.C. Pontaroli [a,b,*]

[a] Unidad Integrada Balcarce (Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata – Estación Experimental Agropecuaria Balcarce, Instituto Nacional de Tecnología Agropecuaria), CC 276 (7620), Balcarce, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CC 276 (7620) Balcarce, Argentina
[c] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), CC 276 (7620) Balcarce, Argentina
[d] Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CC 31 (1900) La Plata, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Anthesis date
Environmental conditions
Severity
Area under the disease progress curve
QTL mapping

ABSTRACT

The identification of sources of resistance to Fusarium head blight (FHB) in bread wheat is key to the sustainable management of the disease. An accurate characterization of resistance is critical; however, the experimental designs commonly used disregard germplasm variability in anthesis date (moment of highest disease susceptibility). Here, an accurate methodology for assessing type II resistance to FHB in the field was developed. Individual spikes of 126 RILs were point-inoculated at their optimum moment. The effect of anthesis date and environmental conditions on the prediction of bread wheat lines' performance was established. Anthesis date explained 26% of the total phenotypic variation for Severity at 21 days post-inoculation (dpi). Including this factor in the model increased the accuracy of the best linear unbiased predictors through a reduction of the residual and genotype by environment interaction variances. In addition, the genotypic variance and heritability of FHB severity at 21 dpi were increased. In summary, including the anthesis date effect in the model lead to a more precise and objective characterization of the level of genetic type II resistance to FHB under field conditions.

1. Introduction

Fusarium head blight (FHB) is one of the most important fungal diseases affecting bread wheat (*Triticum aestivum* L.) in all cropping areas of the world, including Argentina (Lori et al., 2003; Mazzilli et al., 2007; Gilbert and Haber, 2013). This disease is frequent in regions where wet and warm weather coincide with the heading, flowering and grain filling of wheat (Sutton, 1982; Bai and Shaner, 1994; Anderson, 2007). Although many *Fusarium* species can cause FHB, *F. graminearum* Schwabe is the main pathogen in many countries (Schroeder and Christensen, 1963; Bai and Shaner, 1994). This fungus penetrates through anthers or natural openings at flowering, colonizes the rachis, and disperses in the spike. As a result, FHB reduces yield, as well as seed and grain quality, decreasing germination percentage and flour baking properties (McMullen et al., 1997). Furthermore, the damages induced by the pathogen are further aggravated by the presence of mycotoxins in infected grains, which affect human and animal health (Kendrick, 1992).

Control of FHB by chemical, cultural or biological methods is ineffective and/or not feasible (Bai and Shaner, 1994; Galich, 1997) due to the ubiquitous nature and wide host range of the pathogen (Sutton, 1982; Mourelos et al., 2014) and the dependence of the disease upon environmental conditions prevailing during the flowering stage (Parry et al., 1995; Lori et al., 2009). For this reason, the development of resistant cultivars is one of the most important strategies to diminish losses due to this disease (Anderson, 2007; Buerstmayr et al., 2009). Resistance to FHB is a complex trait, with no single gene providing complete protection (Bai and Shaner, 2004). Most authors agree on the existence of two main types of resistance to FHB: resistance to initial infection and resistance to spread

* Corresponding author. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CC 276 (7620) Balcarce, Argentina.
E-mail address: pontaroli.ana@inta.gob.ar (A.C. Pontaroli).

[1] This work is part of a thesis by M.F. Franco in partial fulfillment of the requirements for the Doctor's degree (Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina).

https://doi.org/10.1016/j.cropro.2020.105504
Received 8 November 2019; Received in revised form 11 November 2020; Accepted 9 December 2020
Available online 15 December 2020

of the pathogen within the spike, referred to as type I and type II resistance, respectively (Schroeder and Christensen, 1963). To date, several genetic sources for FHB resistance have been identified (Rudd et al., 2001; Buerstmayr et al., 2009). However, most of these are in backgrounds of exotic origin, with poor agronomic and quality characteristics (Anderson, 2007; Comeau et al., 2008).

Identifying and characterizing new sources of resistance in locally adapted germplasm is a promising tool for developing disease-resistant cultivars. Thus, the mapping of quantitative trait loci (QTL) for FHB resistance, the development of molecular markers to facilitate marker-assisted selection (MAS), and the pyramidization of diverse resistance QTL from adapted sources might enable to substantially enhance the level of FHB resistance in adapted and high-performance germplasm (Buerstmayr et al., 2003; Anderson, 2007; Liu et al., 2013). However, precise phenotypic evaluation is a necessary precondition for any QTL mapping study (Buerstmayr et al., 2009, 2019).

Type I resistance is typically evaluated by measuring the disease incidence (percentage of the total number of heads that show disease symptoms) or disease severity (percentage of diseased spikelets per unit area) after spray inoculation or scattering of *Fusarium*-infected grains. In contrast, Type II resistance is usually measured in point-inoculated spikes by following the amount (severity) and/or speed of spread (Area under the Disease Progress Curve -AUDPC-) of the typical FHB symptoms along the head from the inoculated spikelets (Yang et al., 2005; Buerstmayr et al., 2009). In general, while the resistance to initial infection is evaluated in disease nurseries, screening for resistance to the spread of the fungus within the head is evaluated under both controlled and field conditions (Anderson, 2007). However, as screening for type II resistance is both labor and time-intensive, the few studies including field evaluations were generally conducted by the spawn of infected grains or spray inoculations, generating confusing effects of both types of resistance and often leading to inaccurate conclusions.

Furthermore, the prevailing environmental conditions during the moment of inoculation can drastically affect the degree of fungal penetration and spread, and mycotoxin accumulation. It is widely reported that the disease's development is mainly dependent on the combination of rainfall, humidity, and temperature conditions around the inoculation moment (Andersen, 1948; Sutton, 1982; Parry et al., 1995; Hooker et al., 2002). To date, different types of approaches have been investigated using heading date as a covariate into the analysis in order to dissect the effect of passive mechanism of resistance and obtain a high prediction for FHB severity after spray inoculation (Emrich et al., 2008; Moreno-Amores et al., 2020). However, the limited number of studies in which the screening of type II resistance was carried out under field conditions using the point inoculation technique did not consider the effect of the environmental conditions at the anthesis date (or the inoculation day) for the prediction of the trait. Therefore, the meteorological conditions when the plant host meets the pathogen, which may reduce the reliability of the FHB performance estimates, were not considered. The aims of the present study were (i) to determine the effect of the anthesis date on FHB reaction evaluated under field conditions and (ii) to analyze the implications of considering this information when the genetic values of the individuals are predicted.

2. Methods

2.1. Plant materials and inoculum

A biparental population of 126 recombinant inbred lines (RILs) developed by Alonso et al. (2018) was used in all field experiments. The RIL population was derived from the cross between 'Baguette 10' (B10) and 'Klein Chajá' (KCJ), two Argentinean spring bread wheat cultivars of different germplasm origin. Both parental cultivars exhibit intermediate resistance against FHB. In all the experiments, a susceptible cultivar with asynchronous flowering, 'SY300', was included as a check.

Wheat spikes were inoculated with a suspension of macroconidia of *F. graminearum* strain SP1 (belonging to the collection of the CIDEFI-UNLP-CICBA) (Malbrán et al., 2012, 2014). The isolate was prepared as described by Malbrán et al. (2012); the concentration was adjusted to ~100,000 spores ml^{-1} using a hemocytometer.

2.2. Field experiments

Field experiments were carried out on a typical Argiudol soil at the experimental station of the Instituto Nacional de Tecnología Agropecuaria (INTA) Balcarce (37°46′ 14″ S; 58°18′ 23″ W; 113 m.a.s.l.), Buenos Aires Province, Argentina, during the 2016 and 2017 cropping seasons. In each year, two experiments were carried out. The combination of year and experiments provided four environments. Sowing dates were June 23rd (Environment 1) and July 07th (Environment 2) in 2016 and June 19th (Environment 3) and July 20th (Environment 4) in 2017. In each environment, all RILs, parents and the check cultivar were grown in a randomized complete block design with two replications. Plots consisted of a single 1 m-long row with 30 cm row spacing. Sowing density was adjusted to 300 plants m^{-2}. All experiments were rainfed and conducted under optimal nutritional conditions, with chemical control of weeds and pests. Meteorological data such as temperature, rainfall, and relative humidity (RH) were measured by a standard weather station located at the experimental station.

2.3. Inoculation procedure

Anthesis date [Zadoks growth stage 65 (Zadoks et al., 1974);] was recorded for each plot at the moment when around 50% of its spikes flowered. At this point, ten homogeneous flowering spikes per plot were numbered and identified with self-adhesive paper labels. A floret from each of the two middle spikelets was labeled with a permanent non-toxic marker and inoculated by means of the point inoculation (PI) technique. To do so, a 5 μl drop was placed next to the ovary of each marked floret using a micropipette. Five of the selected spikes were inoculated with the macroconidial suspension, while the remaining five spikes were inoculated with sterilized water to be used as controls. At each inoculation date, three spikes of SY300 in anthesis were also inoculated with the macroconidial suspension.

Immediately after inoculation, spikes were individually covered with wet polyethylene bags to keep ~100% relative humidity for 48 h; paper bags were placed over the polyethylene bags to prevent sun burning. During grain filling, plots were covered with a net to protect spikes from damage by birds.

2.4. Fusarium head blight phenotyping

Fusarium head blight development was followed on each inoculated spike. Spikelets were considered symptomatic when they developed a lesion that had the ability to produce spores. The number of symptomatic spikelets was recorded for each spike at 12, 17 and 21 dpi. Disease severity was calculated as the proportion of spikelets on each spike that showed symptoms (Stack and McMullen, 1995).

The AUDPC was calculated for each treatment, according to Shaner and Finney (1977) as:

$$AUDPC = \sum_{i=1}^{n} \frac{(S_i + S_{i+1})}{2} * (t_{i+1} - t_i) \quad (1)$$

where Si = disease severity at the i*th* observation, ti = days at the i*th* observation, and n = total number of observations.

2.5. Statistical analysis

Pearson correlation coefficients were calculated to estimate the correlation between FHB severity at 21 dpi (SEV21) and the Area under the Disease Progress Curve (AUDPC). Because these two variables were

tightly correlated (r = 0.94, Fig. A1), only Models for SEV21 were fitted.

Data analysis was performed using Linear Mixed Models (LMM). First, a naive model (M_N) was fitted considering only genotypes and genotype × environment interaction as random factors:

$$log(y_{ijk}) = \mu + \tau_i + \gamma_{j(i)} + \varepsilon_{ijk} \quad (2)$$

Where $\log(y_{ijk})$ is the natural logarithm of the response variable (SEV21) on replication "*k*" of line "*i*" in the environment "*j*", μ is the mean value of the logarithm of the response variable, τ_i is the random effect of line "*i*" on the log of the response, $\gamma_{j(i)}$ is the random interaction effect between line "*i*" and environment "*j*", and ε_{ijk} is the random error of the observation on replication "*k*" of line "*i*" in the environment "*j*".

Assumptions on this model are, $\tau_i \sim N(0;\sigma_g^2)$, $\gamma_{j(i)} \sim N(0;\sigma_{ge}^2)$ and $\varepsilon_{ijk} \sim N(0;\sigma_{res}^2)$. All random effects are independent of each other.

Then, a full model (M_F) was fitted including anthesis date as a fixed factor in addition to the random effects:

$$log(y_{ijk(s)}) = \mu + \delta_s + \tau_i + \delta\gamma_{j(i)} + \varepsilon_{ijk(s)} \quad (3)$$

Where $\log(y_{ijk(s)})$ is the natural logarithm of the response variable (SEV21) on replication "*k*" of line "*i*" in the environment "*j*" that have reached anthesis on day "s", μ is the mean value of the logarithm of the response variable, δ_s is the fixed effect of anthesis date "s", τ_i is the random effect of line "*i*" on the log of the response, $\gamma_{j(i)}$ is the random interaction effect between line "*i*" and environment "*j*", and $\varepsilon_{ijk(s)}$ is the random error of the observation on replication "*k*" of line "*i*" in the environment "*j*" that have reached anthesis on day "s".

Assumptions on this model are, $\sum_1^d \delta_s = 0$; $\tau_i \sim N(0;\sigma_g^2)$, $\gamma_{j(i)} \sim N(0;\sigma_{ge}^2)$ and $\varepsilon_{ijk} \sim N(0;\sigma_{res}^2)$. All random effects are independent of each other.

Subsequently, the data were individually analyzed for each year. To do so, two LMM considering only genotypes and genotype × environment interaction as random factors (M_{N16} and M_{N17}), and two LMM_F including anthesis date as a fixed factor in addition to the random effects (M_{F16} and M_{F17}) were fitted. Additionally, SEV21 was relativized to the SY300 performance in each inoculation day, and a M_F was fitted.

Data were log-transformed prior to analysis to achieve normality, and distributions of residuals in each model were tested for homogeneity of variance. Linear Mixed Models were fitted using the *lme* function from package *nlme* (Pinheiro et al., 2013) of the R software (R Core Team, 2013), and variance components were estimated from the *lme* models by the restricted maximum likelihood (REML) method (Milliken and Johnson, 2001). Broad sense heritabilities (H^2) were estimated from variance components, according to Hallauer et al. (2010), as follows:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \left(\frac{\sigma_{ge}^2}{e}\right) + \left(\frac{\sigma_{res}^2}{re}\right)} \quad (4)$$

where σ_g^2 is the genotypic variance, σ_{ge}^2 is the genotype × environment interaction variance, σ_{res}^2 is the error variance; e is the number of environments, and r is the number of replications per experiment.

In addition, best linear unbiased predictors (BLUPs) for SEV21 of the RIL were obtained from the M_N and M_F models and Pearson correlation coefficients were calculated to estimate the correlation between the BLUPs of these models and between SEV21 and anthesis date with and without adjustment.

3. Results

3.1. Weather conditions and phenology

In all the experiments, the flowering of the RIL population occurred within ~10 days; it was a little bit more concentrated in Environments 1 and 2 than in Environments 3 and 4, due to higher mean temperatures during 2016 as compared to those in 2017 (Fig. 1).

In 2016, weather conditions were more favorable for FHB spread within a spike. Mean temperature was higher in Environments 1 ($\overline{X}$ = 17.8°C) and 2 ($\overline{X}$ = 17.3°C) than in Environments 3 ($\overline{X}$ = 14.2°C) and 4 ($\overline{X}$ = 15.4°C). Also, the mean temperature showed greater variability in Environments 1 (S = 3.6) and 2 (S = 3.6) compared with Environments 3 (S = 2.6) and 4 (S = 1.6) (Fig. A2). No differences in the mean minimum temperature were observed between environments ($\overline{X}$ = 10 ± 1.1°C). However, in 2016, the mean maximum temperature was higher in Environments 1 ($\overline{X}$ = 25°C) and 2 ($\overline{X}$ = 24.5°C) than in Environments 3 ($\overline{X}$ = 20.2°C) and 4 ($\overline{X}$ = 20.6°C).

The air relative humidity around anthesis showed similar mean values in all the environments, with greater variability in Environments 1 (S = 13.7) and 2 (S = 12.8) than in Environments 3 (S = 9) and 4 (S = 10.1). On the other hand, total rainfall was more abundant and more distributed in Environments 3 (31.4 mm) and 4 (61.8 mm) than in Environments 1 (21 mm) and 2 (15.4 mm) (Fig. A3).

3.2. Fusarium head blight resistance

Fusarium head blight was present in all field experiments and all evaluated genotypes showed FHB symptoms when inoculated. The average infection efficiency of the inoculated spikes for all the environments was 69.7%, and large differences in the reaction to FHB were observed between RILs. Only 4% of the control spikes showed natural infection.

Both FHB SEV21 and AUDPC were continuous variables. The RIL population's frequency distribution for the two variables was asymmetric in the four environments. Average FHB SEV21 was higher in Environments 1 ($\overline{X}$ = 0.38) and 2 ($\overline{X}$ = 0.51) than in Environments 3 ($\overline{X}$ = 0.24) and 4 ($\overline{X}$ = 0.27). For both variables, transgressive segregation (i.e., RILs with more extreme values than those of the parents) was detected in all the environments ($p < 0.05$) (Fig. 2).

Meanwhile, cultivar SY300 showed a highly variable performance in all the environments (Fig. 3). SEV21 ranged from 0 to 0.2 in Environment 1, from 0.22 to 0.39 in Environment 2, from 0.05 to 0.36 in Environment 3 and from 0.06 to 0.17 in Environment 4. In addition, SY300 showed a different performance in relation to the RIL average in the different days of inoculation in all the environments.

3.3. Comparison of statistical models

The analysis of variance (ANOVA) of the M_F showed a significant effect of the anthesis date for the logarithm of SEV21 ($F = 16.49$, $p < 0.0001$). For this variable, anthesis date explained 26.04% of the phenotypic variation.

Also, when the anthesis date was included in the model (*i.e.,* in M_F), the error and genotype × environment interaction variances were reduced, improving the accuracy of the model predictions (Table 1). Thus, despite the high correlation between the BLUPs predicted by both models (ρ = 0.96, data not shown), the differences between the means of the lines became more detectable, increasing the genotypic variance.

Fig. 4 shows BLUPs expansion when the anthesis date was considered as a source of variation in the analysis. The fit of the model was improved by including anthesis date: the genotypic variance for SEV21 raised from 9.71% to 24.25%. At the same time, the heritability of this trait increased markedly.

In addition, by incorporating the anthesis date into the adjustment, the correlation between SEV21 and anthesis date became no significant ($r = 0.11$, $p < 0.05$ in the M_N and $r = -0.10$, $p > 0.05$ in the M_F). These results show that, when the precision of the prediction was improved, a greater portion of the observed phenotypic variation was caused by the differences between genotypes rather than by the effect of the anthesis

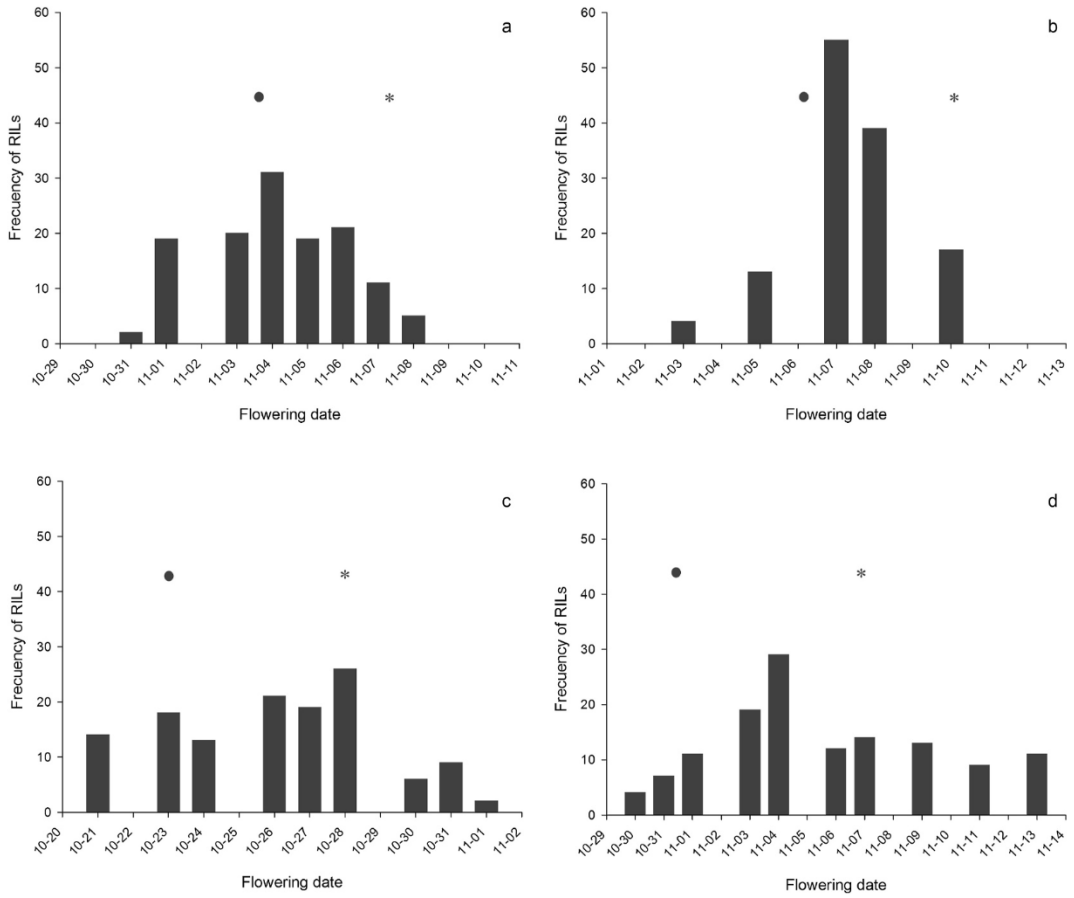

Fig. 1. Frequency distribution of flowering date of 126 RILs evaluated in Environment 1 (a), Environment 2 (b), Environment 3 (c), and Environment 4 (d). The stars and circles indicate the flowering date of parental cultivars Baguette 10 and Klein Chajá, respectively.

date.

Furthermore, when the models were adjusted individually for each year, the correlation between BLUPs from each model was increased by incorporating the anthesis date as a source of variation (Table 2). This increase was greater in 2016 due to the greater environmental variability observed during flowering in Environments 1 and 2 in relation to that in Environments 3 and 4. However, incorporation of anthesis date with only one year of evaluation was not enough to improve the accuracy of the model predictions.

Finally, when the M_F was fitted for SEV21 relative to the SY300 performance on each inoculation day, heritability was also increased ($H^2 = 0.7$), improving the prediction of the genetic values of the RILs. However, the effect of the anthesis date for the relative SEV21 ($F = 25.84$, $p < 0.0001$) remained significant.

4. Discussion

One of the main objectives of QTL mapping studies associated with FHB resistance is to estimate the level of genetically determined resistance on each individual in the population as precisely as possible (Buerstmayr et al., 2009). Hence, screening methodologies that accurately characterize the reaction to this disease are required (Fuentes et al., 2005). One of the main problems with FHB resistance phenotyping is the high environmental influence on disease establishment and progress, causing significant genotype × environment interactions which can significantly bias QTL mapping (Miedaner et al., 2001; Gervais et al., 2003; Buerstmayr et al., 2009; Lori et al., 2009). For this reason, the aim of our study was to develop an accurate method to characterize type II resistance to FHB under different growing conditions, considering the effect of the environment to which the materials are exposed during flowering.

Our experiments were conducted under natural field conditions. As it was observed, the flowering period for the RIL population was concentrated in ~10 days. Although the difference in the flowering date for the parents varied between 4 and 7 days in the different experiments, the greater amplitude of flowering date in the population was due to the fact that it is a quantitative trait, governed by both a few genes with a large effect with environmental influence (Yan et al., 2004; Vanzetti et al., 2013) and numerous small-effect quantitative trait loci (Würschum et al., 2018). Previous knowledge of the genetic constitution of the parental cultivars (Vanzetti et al., 2013) indicates that the RIL population segregates for some flowering genes in a transgressive way.

The environmental conditions that affected the expression of FHB varied among the different flowering dates in all the environments. Consequently, our results differ from most of the research on type II resistance, which is generally conducted in greenhouses where the environment is carefully controlled (Buerstmayr et al., 2009; Liu et al., 2009). However, since FHB resistance is a complex, polygenically inherited trait significantly affected by the environment (Bai and Shaner, 1994; Kolb et al., 2001; Buerstmayr et al., 2003), it is usual that a high proportion of the QTLs found in experiments conducted under uniform environments are environment-specific (Klahr et al., 2007; Buerstmayr et al., 2009). For this reason, the assessment of FHB resistance expressed under different conditions is more accurate at estimating the repeatability of the trait and the stability of QTL estimates across environments (Mesterhazy, 1995a; Buerstmayr et al., 2009; Liu et al., 2013).

We point inoculated wheat spikes at anthesis to specifically determine the reaction of the lines to the disease progression. This technique has been adopted as a standard methodology for evaluating type II

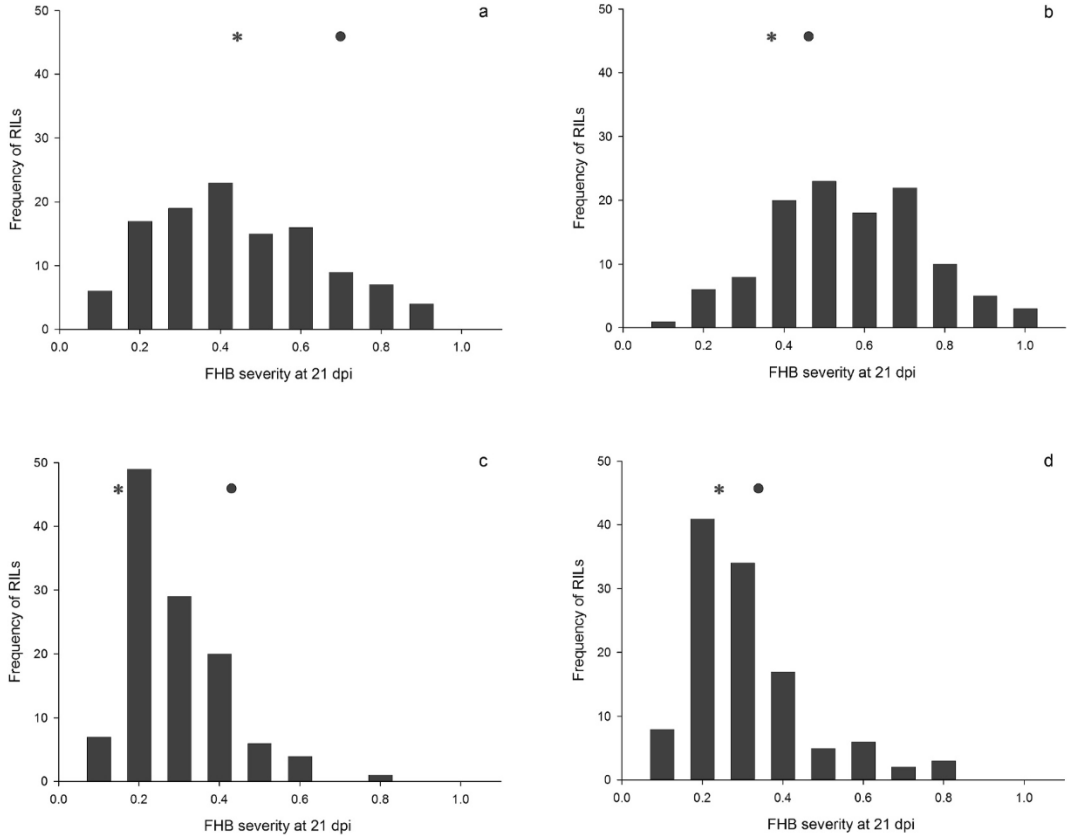

Fig. 2. Frequency distribution of FHB severity at 21 dpi for 126 RILs derived from Baguette 10 x Klein Chajá evaluated in Environment 1 (a), Environment 2 (b), Environment 3 (c), Environment 4 (d). The stars and circles indicate the performance of parental cultivars Baguette 10 and Klein Chajá, respectively.

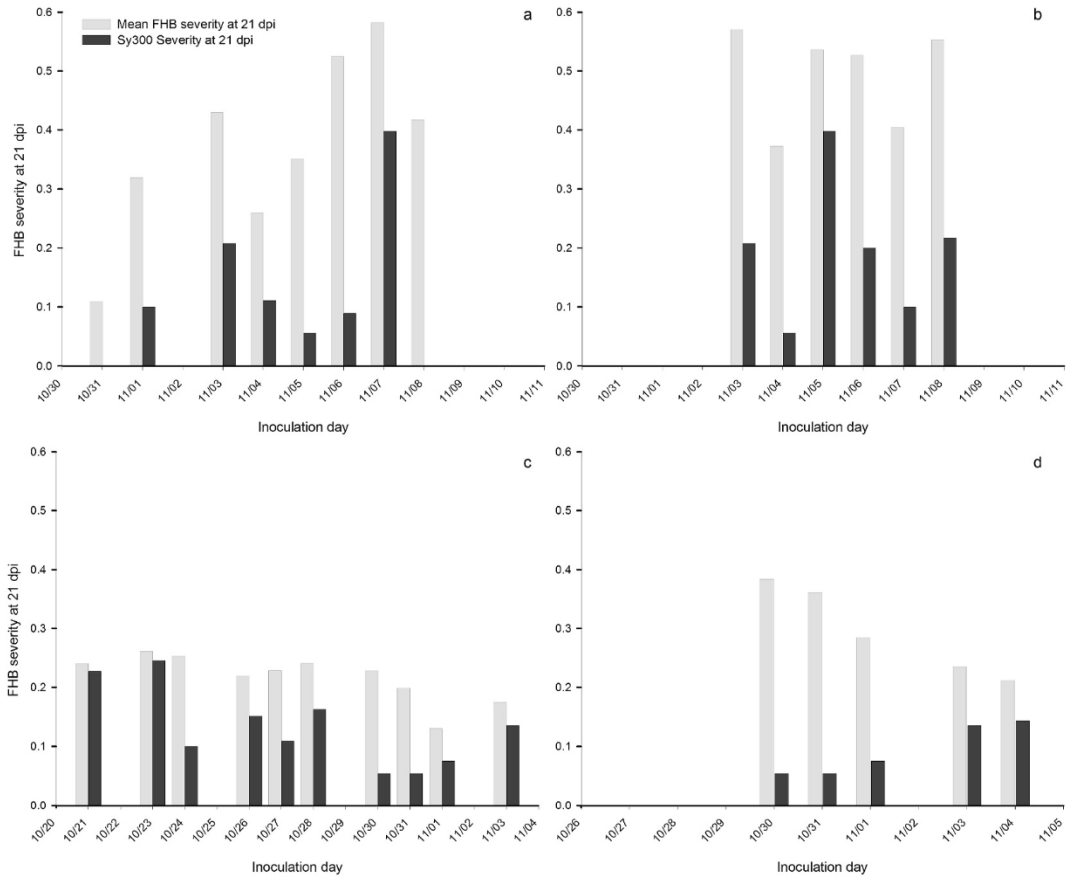

Fig. 3. FHB severity at 21 dpi of cultivar SY300 (check) and mean severity of the RIL population in each inoculation day in Environment 1 (a), Environment 2 (b), Environment 3 (c), Environment 4 (d).

Table 1
Variance components and heritabilities from the Naive model (M_N) and Full Model (M_F) for FHB severity at 21 dpi. Numbers in parenthesis indicate the percentage of the total variation.

	Genotypic variance (σ_g^2)	Genotype × environment interaction variance (σ_{ge}^2)	Error variance (σ_{res}^2)	Heritability (H^2)
M_N	0.049 (9.71)	0.190 (37.40)	0.269 (52.89)	0.378
M_F	0.091 (24.25)	0.042 (11.09)	0.244 (64.66)	0.691

resistance to FHB in experiments conducted under greenhouse (Liu et al., 2013; Foulkes et al., 2015). However, as the methodology is labor-intensive and time-consuming, field experiments aimed at evaluating resistance to FHB are generally conducted using grain spawn and/or spray inoculations in mist-irrigated nurseries (Dill-Macky, 2003; Buerstmayr et al., 2009). This increases the differences in disease incidence among lines and hinders the separation between the different types of resistance to FHB. In our experiments, the inoculation of the spikes in anthesis using point inoculation assured a high efficiency of infection (~70%) and allowed for the development of FHB in all the experiments and genotypes. In addition, we ensured the same number of sites of entry of the fungus in each spike to avoid confounding effects of type I resistance.

In the few investigations in which the evaluation of type II resistance was carried out under field conditions using the point inoculation method (Buerstmayr et al., 2002), the effect of the anthesis date was not generally considered in the prediction of the genetic values of individuals. In this sense, numerous studies have demonstrated than the

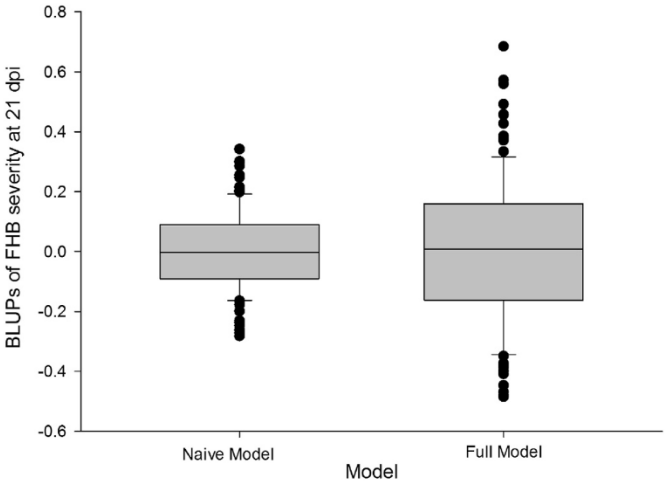

Fig. 4. Boxplot distributions of the BLUPs for FHB severity at 21 dpi of the 126 RILs by the two fitted models (Naive Model and Full Model). Boxes indicate the middle 50% of the data and the median (solid line). The whiskers show the range of adjacent values; dots indicate outliers.

prevailing environmental conditions during the inoculation (temperature, duration of canopy wetness, humidity, and rainfall) affect the development of FHB in wheat since they alter the incidence of infection, incubation periods, rates of colonization of spikes and mycotoxin content (Pugh et al., 1933; Andersen, 1948; Hooker et al., 2002). In our experiments, anthesis date explained 26% of the total phenotypic variation for SEV21, and its incorporation as a source of variation in the model reduced the residual and the genotype × environment interaction

Table 2
Pearson correlation coefficients (r) between the BLUPs of the Full Model and the BLUPs of the individual models of each year for FHB severity at 21 dpi.

Statistical model	r
M_{N16}	0.769
M_{F16}	0.829
M_{N17}	0.866
M_{F17}	0.883

variances, made the correlation between SEV21 and anthesis date no significant and increased the accuracy of genotypic BLUPs. Simultaneously, the genotypic variance and heritability of this trait were increased, maximizing the probability of finding differences between genotypes.

This is a relevant result because the FHB reaction is strongly correlated with morphological and plant development characteristics. As infection occurs mainly at anthesis, differences between flowering dates can be a barrier to distinguish 'true' FHB resistance from 'apparent' resistance that is caused by host escape (Kolb et al., 2001). By distinguishing the effects of the anthesis date, we achieved greater confidence in the characterization of the FHB reaction of the genotypes. In the same sense, several alternatives aimed at dissecting the effect of passive resistance mechanisms such as the development stage (*i.e.*, flowering date) or morphological traits (*i.e.*, plant height) have been investigated. Klahr et al. (2007), for instance, found that when the effects of plant height and heading date were eliminated by covariance analysis, many of the QTLs previously mapped for FHB resistance were not identified, suggesting that the effects conferred by these *loci* were not based on true resistance mechanisms. Likewise, Miedaner et al. (2006) adjusted the FHB ratings to the effect of heading date and studied the effect of QTL introgression from resistant donors in European elite spring wheat. Similarly, other covariance considerations evidence additional advantages in relation to traditional mapping methods when trying to identify QTL. Thus, He et al. (2016) included plant height and heading date as covariates into the QTL mapping. In the same way, several studies have used the strategy proposed by Emrich et al. (2008) and included the heading date as a quantitative covariate in a mixed linear model, ensuring to select genotypes with good resistance to *Fusarium* and avoiding at the same time the influence of heading date. Recently, a research carried out by Moreno-Amores et al. (2020) compared different types of approaches to include heading date and plant height as covariates into the analysis in order to obtain a high prediction for FHB severity and adequate reduction of undesired trade-offs.

In our research, no association between FHB resistance and plant height was found ($p > 0.1$, data not shown). However, when the data were analyzed individually for each year, we observed that the inclusion of the anthesis date as a source of variation in the model significantly improved the estimation of BLUPs, mainly in environmentally unstable years such as it was 2016 in our study. However, to maximize the accuracy of the prediction, it is still necessary to consider more than one year in the evaluation. These results coincide with previous reports, which suggest that at least three independent biological experiments (locations or years) are necessary for obtaining stability in the reaction of a genotype (Mesterhazy, 1995b).

On the other hand, the few available reports on experiments conducted in the field for the evaluation of type II resistance usually include resistant and/or susceptible controls with a broad range of flowering dates in order to separate and interpret the environmental effects due to different flowering dates (Buerstmayr et al., 2009). In our work, we selected SY300 for being susceptible to *F. graminearum* infection and for having an asynchronous flowering that spans for approximately 10 days. This allowed us to have flowering spikes of this cultivar during the entire flowering period of the RIL population. In our experiments, SY300 exhibited intermediate resistance (type II) against FHB and showed marked differences in its performance for the different dates of inoculation. We relativized the reaction of the 126 RILs to that of cultivar SY300 for each inoculation day. However, when the models were fitted for the relativized variable, the effect of anthesis date continued to be significant, even if the heritability of the trait improved significantly. These results show that the relativization of the data to a control may improve the prediction of the response but does not allow to completely remove either the environmental effects or the genotype × environment interaction in the estimation of the trait.

Finally, some considerations should be made about the methodology proposed here. Firstly, it should be emphasized that our work is oriented to scientific research studies (for instance, QTL mapping studies) in which accurate phenotyping of type II resistance to FHB is required. However, it should be considered that for routine screening of FHB resistance in large breeding populations, a faster inoculation method is preferable. In this sense, according to Miedaner et al. (2003), spray inoculation would be advantageous because it requires less time and labor for inoculation and rating. Secondly, it is important to highlight again that our research is mainly focused only on the type II resistance characterization. However, this type of resistance is only a part of FHB resistance, and most genotypes combine quantitatively different levels of both type I and II resistances. So, considering the weak correlation found between the two inoculation methods (Gilbert et al., 1997; Miedaner et al., 2003) (which supports the assumption that types I and II resistances are governed by different *loci*), it would be necessary to apply both inoculation methods to identify highly resistant parents and recombinants of types I and II resistances in segregating populations, since they provide additional information for selection and scientific studies.

5. Conclusion

An accurate characterization of the FHB resistance is essential when QTL mapping studies associated with the trait are carried out. In this work, a methodology for phenotyping of type II resistance to FHB under field conditions in bread wheat was presented, which considers the environmental conditions at flowering. Incorporating the anthesis date of the genotypes in the prediction of FHB response allows to greatly reduce the effects of the environmental conditions occurring during the moment when the host meets with the pathogen. Thus, it is possible to reach a more precise and objective characterization of the level of genetic type II resistance to FHB under field conditions.

Funding

This work was partially supported by INTA (PNCyO 1127044).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank members of the Grupo Trigo Balcarce (EEA Balcarce INTA) for help with the experiments and technical assistance. Scholarships granted to M.F. Franco and M.P. Alonso by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), to J.S. Panelo by the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) and to N.E. Mirabella by Instituto Nacional de Tecnología Agropecuaria (INTA) are acknowledged. This work is part of a thesis by M.F. Franco in partial fulfillment of the requirements for a Doctor's degree (Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina).

Appendices.

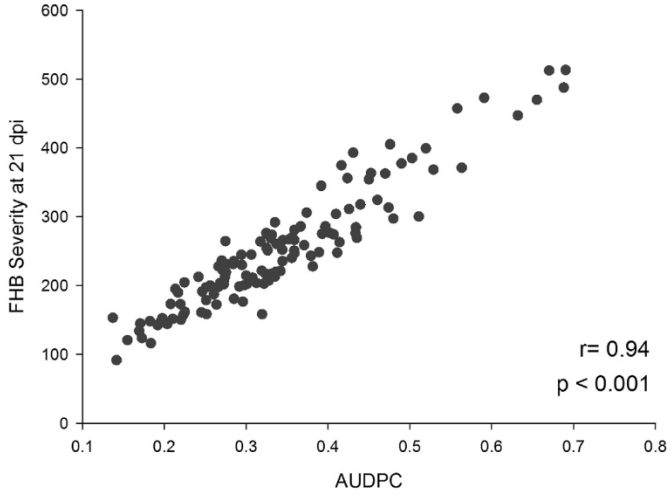

Fig. A1. Correlation between FHB Severity at 21 dpi and the Area under the Disease Progress Curve (AUDPC).

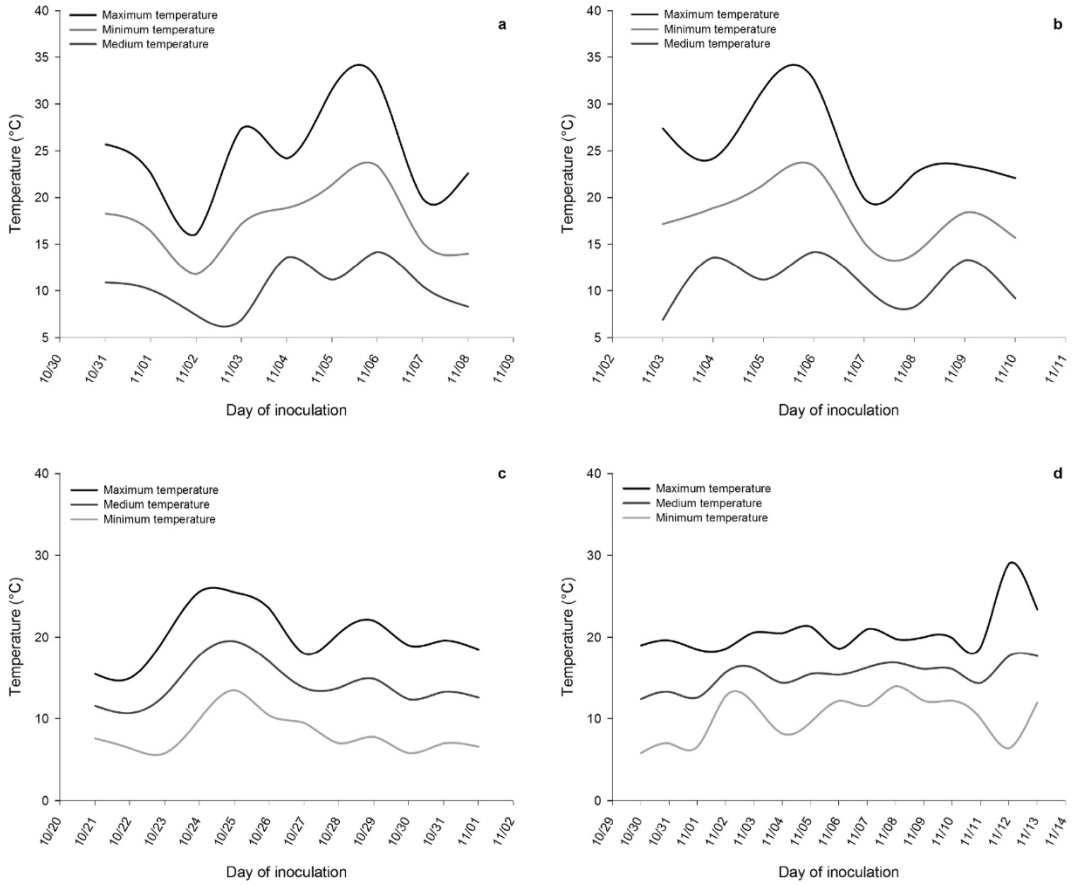

Fig. A2. Maximum, minimum, and medium temperature during the anthesis period in Environment 1 (a), Environment 2 (b), Environment 3 (c), and Environment 4 (d).

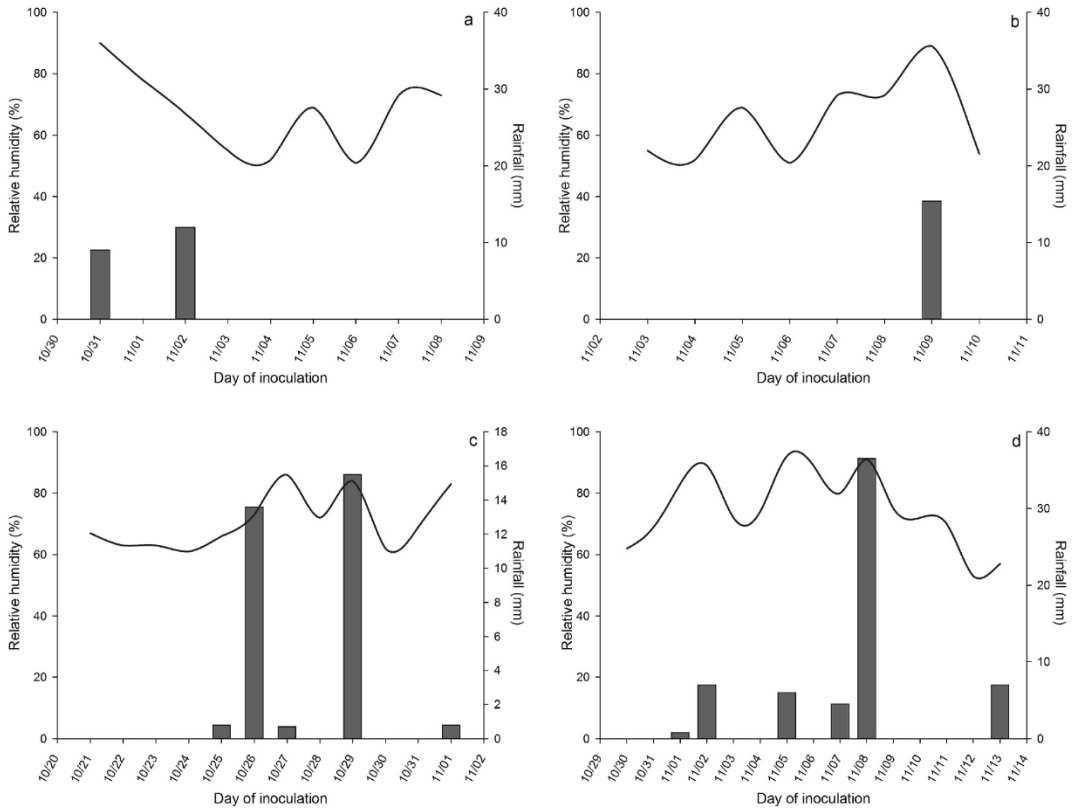

Fig. A3. Relative humidity (black lines) and rainfall (grey bars) during the anthesis period in Environment 1 (a), Environment 2 (b), Environment 3 (c), and Environment 4 (d).

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