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ORIGINAL ARTICLE

Grain discoloration in different genotypes of durum wheat (*Triticum durum* L.) in Argentina: associated mycobiota and peroxidase activity

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Abstract

Wheat grain discoloration, a worldwide disease that lowers grain quality and decreases grain yield, does not have a single etiology. It has been proposed that it is a consequence of an abiotic mechanism, a response to environmental conditions or enzymatic activity. It has also been suggest that it is a biotic mechanism, a fungal infection principally by *Alternaria* spp. and *Bipolaris sorokiniana*. The present work was carried out to analyze the possible etiology of this disease in nine durum wheat genotypes from two localities of southern Buenos Aires province (Argentina) on two sowing dates. Incidence (percentage of grain discoloration) was recorded and mycobiota associated with this pathology was registered following ISTA rules. Peroxidase activity in an extract obtained from grains belonging to genotypes of the locality that showed the highest incidence was measured.

The incidence among genotypes, localities and sowing dates varied, although the genotypes with the higher and lower values of incidence were the same for all the variables tested. The fungus *Alternaria* spp. was isolated the most frequently followed by *Fusarium* spp., while *Bipolaris sorokiniana* was found the least frequently. Peroxidase activity showed that all the treatments had similar levels of enzymatic activity, but there was no clear differentiation between controls either between genotypes with the lowest or the highest incidence values. This suggests that peroxidase activity did not have a clear relationship with grain discoloration. In this research, it is presumed that fungal infection is the main cause of this disease.

Keywords: durum wheat, grain discoloration, mycobiota, peroxidase activity

Introduction

Discoloration of wheat grains and other cereals is characterized by a browning of the embryo (black point) which can extend along the crease and over the shoulders (kernel smudge) (Wiese 1987). In severe infections many of the seeds can be stained and wrinkled. The symptoms are not observed until the plants are harvested and the ears or panicles are threshed (Southwell *et al*. 1980). The seed germination, vigor, seedling growth and grain yield of diseased kernels are decreased (Özer 2005; Toklu *et al*. 2008; Cristea *et al*. 2013; Li *et al*. 2019). Also, this pathology lowers the quality of the grain and interferes with milling and

baking properties (Conner and Davidson 1988). The diseased grains are undesirable for industry because flour, semolina and their products are affected, since they have colored particles that decrease the commercial value (Dexter and Matsuo 1982; Lorenz 1986). These particles can be eliminated, but this would cause a loss of up to 20% of the yield of semolina (Bird 1975).

Generally, durum wheat (*Triticum durum* L.) is more susceptible to discoloration than common wheat (*Triticum aestivum* L.) (Greaney and Wallace 1943; Fernandez *et al*. 2000, 2001). In Argentina, this crop is mainly concentrated in the southern part of Buenos Aires province. In this country, although the first report of grain discoloration was cited by Marchionatto (1934) it was just in the 1990's that the natural incidence of this disease increased and its different aspects were studied (Sisterna and Sarandón 2010). Nevertheless, grain discoloration is not included in the Official Standard for the wheat grain trade (Sisterna and Sarandón 2000). In contrast, in Australia, Canada and the European Union, the commercialization of discolored grains is penalized, being within the parameters that affect grain quality in the Official Trade Standard of each country (Delwiche and Miskelly 2017).

There is not a single etiology related to the symptoms of discoloration. It has been proposed that it is a consequence of an abiotic mechanism (Khani *et al*. 2018), a physiological response to environmental conditions mainly due to high humidity during grain filling (Fernandez and Conner 2011) associated with low temperatures (Walker *et al*. 2008).

These symptoms have also been cited as a consequence of enzymatic activity. It has been suggested that it is the effect of peroxidase activity that generates the formation of colored phenolic products in the grain embryo (Williamson 1997).

However, discoloration symptoms are related also to a fungal complex (biotic mechanism), mainly dematiaceous such as *Alternaria alternata* and *Bipolaris sorokiniana* (Fernandez *et al*. 2011), considered to be the primary causal agents of the disease. Also, *Fusarium* spp., an important toxigenic genus, is associated with this pathology (Conner *et al*. 1996; Desjardins *et al*. 2007). On the other hand, a weak mycobiota, e.g. *Cladosporium*, *Nigrospora*, *Epicoccum*, *Stemphylium* spp., can coparasitize the seeds with the main genera, differing in aggressiveness (Warham *et al*. 1999).

The aim of this research was to analyze the main fungal genera and peroxidase activity associated with grain discoloration of nine durum wheat genotypes obtained from two localities of Buenos Aires province (Argentina) on two sowing dates. This study was designed to provide information about the possible cause of this pathology.

Materials and Methods

Seed samples

The seeds analyzed were obtained from trials carried out in two localities, on two sowing dates. The localities of study correspond to different subregions of durum wheat crops in Buenos Aires Province, Argentina. Miramar (lat 38°10´S, long 58°0´O) is in the southeast while La Dulce (lat 38°20´S, long 59°0´O) is in the central southern subregion. The mean rainfall in Miramar was 106.1 mm, and the sowing dates were: June 23 for the first and July 21 for the second. In La Dulce, the mean rainfall was 86.3 mm, and the sowing dates were July 11 and August 4.

The nine genotypes evaluated were: Bonaerense INTA Facón (BIF), Buck Topacio (BT), Buck Esmeralda (BE), Bonaerense INTA Cariló (BIC), Buck Platino (BP), ACA 1801F, ACA 1901F, Buck Granate (BG), Bonaerense INTA Quillén (BIQ).

Evaluation of natural incidence of grain discoloration

The disease incidence (percentage of discolored grains) was quantified in random samples of 200 grains for each genotype. The absence or presence of discoloration in each grain were observed. Three replications for each sample were done.

Associated mycobiota

Wheat grain samples were analyzed following ISTA rules (International Seed Testing Association 1979). One hundred seeds per treatment were disinfected in 5% sodium hypoclorite for 5 min followed by rinsing twice with sterile distilled water. The disinfected seeds were placed in Petri dishes with Potato Dextrose Agar (broth of 200 g potatoes, 20 g agar, 20 g dextrose and distilled water up to 1 liter) and incubated in a growth chamber at 21 ± 1°C with 12 h of light + UV/12 h of darkness. After 7 days, the plates were evaluated. Through microscopic observation of the micromorphology (vegetative and reproductive structures, conidial ontogeny), the main fungal pathogens associated with the disease were identified (Sivanesan 1987; Nelson *et al*. 1994; Rotem 1994; Manamgoda *et al*. 2014). Three replications for each sample were done.

Peroxidase activity

Guaiacol peroxidase (EC 1.11.17) activity was measured by following the H_2O_2 dependent oxidation of guaiacol at 470 nm, using an extinction coefficient of 26,600 $M^{-1} \cdot cm^{-1}$. All genotypes from the locality and

sowing date that showed the highest incidence were evaluated. Completely healthy grains (C1) and totally diseased grains (C2) were taken as controls. Assays were carried out at 25°C with a Shimadzu 1800 spectrophotometer. According to Dann and Deverall´s (2000) protocol, 25 µl of grain extract, obtained from each genotype, were added to a mixture constituted by 0.15 M sodium phosphate buffer pH 5.8 and 0.38 mM guaiacol.

Peroxidase reaction was started by the addition of 73 mM H_2O_2. One unit of guaicol peroxidase (G.P.) activity was defined as the amount of enzyme that caused the formation of 1 µmol of tetraguaiacol per minute (U) and expressed per unit of grain mass (based on fresh mass).

Statistical analysis

Data of each experiment was analyzed by an analysis of variance (ANOVA) and means were compared by Tukey test (p = 0.05). All statistical analyses were performed using InfoStat version 2017. The analysis of correlation between discoloration incidence and peroxidase activity was made by Statistica (Stat Soft, Inc. 2005).

Results

Evaluation of natural incidence of grain discoloration

Discoloration incidence had comparable values for Miramar and La Dulce for the two sowing dates. The general mean registered was 27.2%. The genotypes showed similar behavior. Buck Granate had the highest incidence while Buck Topacio presented the lowest percentage (Table 1).

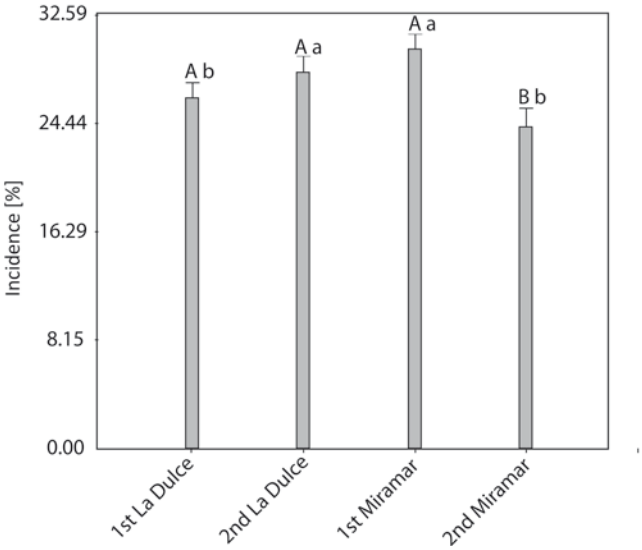

Fig. 1. Comparison of mean discoloration incidence between sowing dates in each locality (capital letters), and between localities on each sowing date (lowercase letters). The same capital letter or lowercase letter on the bars do not differ significantly at p = 0.05 probability level according to Tukey´s test

According to the sowing date, Miramar had significant differences. In this locality, the first sowing date had the highest incidence. In contrast, in La Dulce the second sowing date had major values, though both sowing dates did not show significant differences (Fig. 1).

The correlation coefficient between incidence and sowing date (factor of environmental condition) was analyzed but we decided not to include it in the study because the results were inconclusive.

Associated mycobiota

In all samples analyzed, the fungi isolated were *Alternaria* spp., *Fusarium* spp., *Bipolaris* spp., while genera

Table 1. Discoloration incidence values (%) for the nine genotypes of durum wheat at Miramar and La Dulce in both sowing dates (s.d.)

Genotypes	Miramar		La Dulce	
	1st s.d.	2nd s.d.	1st s.d.	2nd s.d.
Bonaerense INTA Facón	24.17 de	23.3 bcd	26.0 abc	30.5 ab
Buck Topacio	21.5 e	10.16 d	16.7 c	18.0 c
Buck Esmeralda	31.0 bcd	20.7 bcd	22.5 bc	38.0 a
Bonaerense INTA Cariló	35.5 ab	26.5 abc	30.17 ab	18.17 c
Buck Platino	24.8 cde	24.0 abcd	26.5 abc	25.0 bc
ACA 1801F	23.3 de	15.5 cd	22.8 bc	30.8 ab
ACA 1901F	33.3 abc	25.5 abc	26.0 abc	21.5 c
Buck Granate	40.7 a	38.0 a	36.8 a	38.8 a
Bonaerense INTA Quillén	36.7 ab	33.8 ab	30.3 ab	34.0 a
p value	< 0.0001	< 0.0001	0.0001	< 0.0001

Values within each column with a common letter do not differ significantly at p = 0.05 according to Tukey´s test

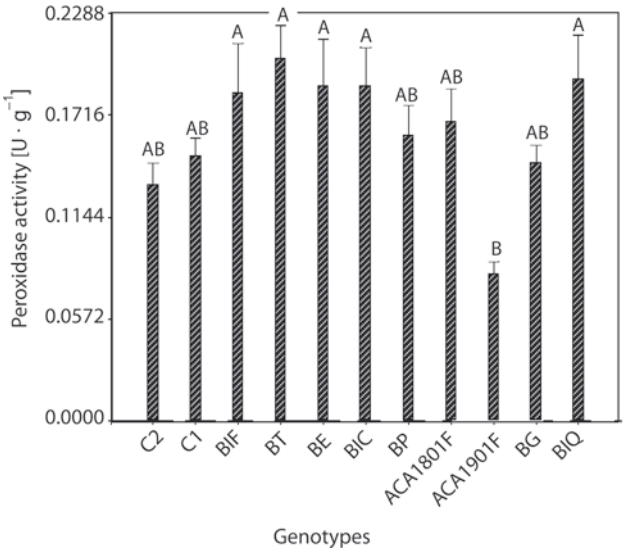

Fig. 2. Peroxidase activity in samples of different genotypes of durum wheat from Miramar with two controls (C1 – completely healthy grains, C2 – totally diseased grains). The same letter on the bars show that there were no significant differences between peroxidase activity at $p = 0.05$ according to Tukey´s test

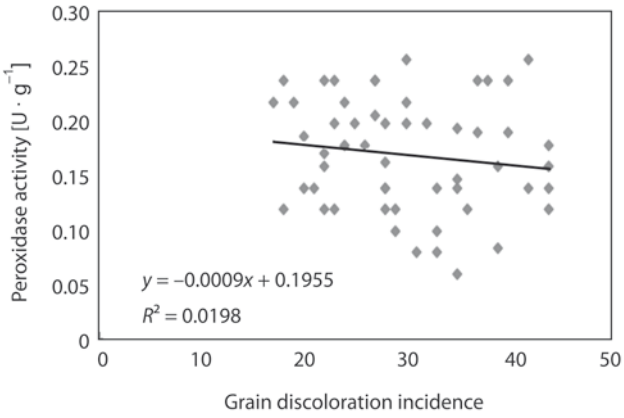

Fig. 3. Correlation analysis between peroxidase activity and grain discoloration incidence of durum wheat

such as *Epicoccum* spp., *Stemphylium* spp., *Cladosporium* spp., *Curvularia* spp. and *Dreschlera* spp. were present in lower values.

Alternaria spp. was the predominant genus isolated in both localities, sowing dates and genotypes. In La Dulce, on the second sowing date there were higher values (85.48%) than on the first (76.62%). On the other hand, in Miramar the results were almost equivalent for both sowing dates (75% for the first and 75.33% for the second).

The other fungus associated with grain discoloration, *Bipolaris sorokiniana*, was isolated less frequently. In La Dulce, on both sowing dates the value of infection was the same (1.11%), while in Miramar on the second sowing date it was 0.82% and on the first, 0.26%.

Fusarium spp. followed *Alternaria* spp. in percentage of isolates in all genotypes. In Miramar, on the first sowing date, the infection was 13.07%, while on the second the percentage was 9.11%. In La Dulce, the values on the first date were 12.44% and on the second, 5.29%. This last one had the lowest values of *Fusarium* contamination.

For the three genera, the total values of fungal infection were in La Dulce 91.03% and in Miramar 86.91%.

Peroxidase activity

To study peroxidase activity, seed samples of the first sowing date from Miramar were analyzed. It was observed that Buck Topacio (BT) showed the highest values while the level of peroxidase activity for ACA 1901F was the lowest (Fig. 2). Although there were significant differences between these treatments, there was not a clear differentiation between the two controls [completely healthy grains (C1) and totally diseased grains (C2)] and between the genotypes Buck Topacio and Buck Granate (BG), which had the lowest and the highest incidence values, respectively.

The correlation coefficient indicated that there was no direct correlation between grain discoloration incidence and peroxidase activity in the genotypes analyzed (Fig. 3).

Discussion

The mean values of discoloration incidence were similar to those found by Sisterna and Sarandón (2000, 2005) for durum wheat. However, for the same crop region Moschini *et al.* (2006) and Miravalles *et al.* (2008) quantified less incidence values.

Worldwide, many researchers have found important differences between genotypes (Conner and Davidson 1988; Madariaga and Mellado 1988; Fernandez *et al.* 1994, 2000; Toklu *et al.* 2008; Li *et al.* 2014). Also, in Argentina the incidence values of discoloration were influenced mainly by the genotypes (Sisterna and Sarandón 2000, 2005).

Grain discoloration incidence in genotypes at different localities could depend on whether the environmental conditions were favorable or unfavorable for disease development (Conner and Davidson 1988). These latter authors found that no genotype was completely free of discoloration. This agrees with our results in which no genetic material was free of disease. The same was found by other researchers (Sisterna and Sarandón 2000; Wang *et al.* 2003; Li *et al.* 2014).

Buck Topacio registered the best behavior under all conditions, having the lowest incidence. This result was similar to that found by Miravalles *et al.*

(2008), making it a suitable resistant genotype for future breeding programs and a good strategy for disease management.

According to the sowing date, the incidence results for both were different. In Miramar the earlier crop had the highest incidence. Solanki *et al.* (2006) observed similar data for wheat in India. Nevertheless, in La Dulce there were no differences in the incidence values. Working at the same locality, Moschini *et al.* (2006) had similar results.

The mycobiota observed in the analyzed samples were similar to those recorded worldwide (Wiese 1987; Mathur and Cunfer 1993). The genera of the fungi isolated less frequently agreed with the results of Cristea *et al.* (2013), Patel and Minipara (2016) and Xu *et al.* (2018).

In accordance with the results found by other researchers (Toklu *et al.* 2008; Cristea *et al.* 2013; Gargouri-Kammoun *et al.* 2014; Draz *et al.* 2016; Patel and Minipara 2016; Xu *et al.* 2018), the genera *Alternaria* spp. was the most frequently isolated.

In our findings, all the treatments had a high percentage of infection, more than 75% for the predominant fungus. *Fusarium* spp. was the second fungus isolated, present in all the genotypes, agreeing with Gargouri-Kammoun *et al.* (2014).

Similar results respecting the order of frequency of *Alternaria* and *Fusarium* were found by Hudec and Muchová (2008) working in the Slovak Republic. Conversely, other researchers observed different frequencies among genera (Toklu *et al.* 2008; Xu *et al.* 2018).

Bipolaris sorokiniana, the other pathogen associated with grain discoloration, was isolated less frequently than the above mentioned genera. These results agree with Sisterna and Sarandón (2000) but were opposite to the findings of other authors (Draz *et al.* 2016; Xu *et al.* 2018). The very small infection percentage could be due to environmental conditions, because this pathogen requires moderate to warm temperatures for growth (Acharya *et al.* 2011). These conditions did not occur in any of the localities where the genotypes were found. In spite of the low infection, *Bipolaris sorokiniana* is an important pathogen because it affects seed germination, and reduces both seedling emergence and yield of the subsequent crop. It is more aggressive than *Alternaria* spp. (Mathur and Cunfer 1993). However, some *Alternaria* species are producers of secondary toxic metabolites, *Alternaria*-mycotoxins (Ostry 2008).

As *Alternaria* spp. was the most frequently isolated genus, it was associated as the main cause of grain discoloration. This result agreed with Sisterna and Sarandón (2000) for Argentina, and with researchers of other countries (Conner and Kuzyk 1988; Fernandez *et al.* 1994). They thought that this pathology is a consequence of *Alternaria* infection instead of *B. sorokiniana,* which had been found in lower values.

The total percentage colonization by *Alternaria* spp., *Fusarium* spp. and *B. sorokiniana*, in all the genotypes per locality, indicated that all the samples had a high degree of infection.

In the present study, the oxidizing activity of guaiacol peroxidase hydrogen dependence was observed in all the treatments. It is known that the presence of peroxidases in all plant tissues, is commonly associated with biotic or abiotic stress (Scandalios 2005), and are related to plant defense responses against pathogens and deterioration (Uarrota *et al.* 2016).

The values of peroxidase activity recorded in all of the treatments and the correlation analysis, suggested that there is no association of enzymatic activity with grain discoloration. These results agree with the findings of Sulman *et al.* (2001) and Hadaway *et al.* (2003).

March *et al.* (2007) did not detect the presence of barley grain peroxidase in healthy or discolored grains, but they confirmed that discoloration was associated with physiological changes within the grain. In contrast, Williamson (1997) established that these enzymes are responsible for discoloration. He disregarded the fungal process and focused on abiotic conditions.

Conclusions

In this research was found that sowing dates and localities were the primary factors which influenced the discoloration incidence. None of the genotypes were free of the disease. Related to the mycobiota present, all samples were infected with the three principal genera associated with discoloration etiology. For both localities and sowing dates these fungi had an important infection percentage, since *Alternaria* genus is closely related with this disease. The peroxidase activity did not show a clear relationship with discoloration, indicating that it probably was not the cause of it.

These results lead to the conclusion that the presence of fungi have a stronger link to this pathology than peroxidase activity.

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