Rev Iberoam Micol. 2013;30(1):9–13

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Original

# Mycotoxin production by *Aspergillus niger* aggregate strains isolated from harvested maize in three Portuguese regions

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ARTICLE INFO

*Article history:*
Received 15 November 2011
Accepted 4 May 2012
Available online 24 May 2012

*Keywords:*
*Aspergillus niger*
Maize
Fumonisin $B_2$
Ochratoxin A

ABSTRACT

*Background:* Maize is considered one of the crops more susceptible to mycotoxins in the world. Two of the mycotoxins commonly associated with maize are fumonisins and ochratoxin A. *Aspergillus niger* is a known producer of ochratoxin A and is easily found in maize. Recently, however, *A. niger* has been reported to produce as well fumonisins, mainly fumonisin $B_2$.
*Aims:* The aim of this study was to isolate *A. niger* strains from maize samples collected in three Portuguese maize growing regions and to detect the production of both fumonisin $B_2$ and ochratoxin A.
*Methods:* Ninety five maize samples were collected, plated, and all observable *Aspergillus* section *Nigri* strains were isolated. Strains were morphologically characterized and mycotoxin production was determined by HPLC-FD.
*Results:* Isolations resulted in a total of 270 strains of black *Aspergillus* from 73 samples (77% of the samples). About 14% of those strains were found to produce ochratoxin A and 39% of the strains were found to produce fumonisin $B_2$.
*Conclusions:* An association between the production of these two mycotoxins could not be found and no conclusions could be taken whether the presence of *A. niger* aggregate strains will increase the risk of maize contamination with fumonisins and more specifically with fumonisin $B_2$.


## Producción de micotoxinas por aislamientos de Aspergillus niger procedentes de muestras de maíz recogido en tres regiones portuguesas

*Palabras clave:*
*Aspergillus niger*
Maíz
Fumonisina $B_2$
Ocratoxina A

RESUMEN

*Antecedentes:* En todo el mundo se considera que el maíz es uno de los cereales más vulnerables a las micotoxinas. Dos de las asociadas con más frecuencia a este cereal son las fumonisinas y la ocratoxina A. Se sabe que *A. niger* produce ocratoxina A, que suele estar presente en el maíz. Sin embargo, recientemente, se ha descrito que *A. niger* puede producir fumonisinas, principalmente fumonisina $B_2$.
*Objetivos:* El objetivo del presente estudio fue aislar las cepas de *Aspergillus niger* a partir de muestras de maíz provenientes de la siega en tres regiones agrícolas portuguesas y detectar la producción de fumonisina $B_2$ y ocratoxina A.
*Métodos:* Se obtuvieron 95 muestras de maíz de la siega en estas regiones, se sembraron en placas y se aislaron todas las cepas de *Aspergillus* sección *Nigri* observadas. Se caracterizaron morfológicamente las cepas y se determinó la producción de micotoxinas mediante cromatografía líquida de alto rendimiento con detección de fluorescencia.
*Resultados:* Se aislaron un total de 270 cepas de *Aspergillus* sección *Nigri* de 73 muestras (77% de las muestras). Se observó que alrededor del 14% de las cepas producían ocratoxina A, mientras que aproximadamente el 39% producían fumonisina $B_2$.
*Conclusiones:* No pudo identificarse una asociación entre la producción de estas dos micotoxinas y no pudimos extraer conclusiones sobre si la presencia de cepas de *A. niger* aumentará el riesgo de contaminación del maíz por fumonisinas, en especial por fumonisina $B_2$.


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1130-1406/$ – see front matter 
http://dx.doi.org/10.1016/j.riam.2012.05.002

Maize (*Zea mays* L.) constitutes one of the most important crops worldwide, being, however, a good substrate for growth, development and activity of filamentous fungi.[13,32] Maize is associated with a large number of fungal species belonging to the genders of *Aspergillus*, *Fusarium*, and *Penicillium* that can cause spoilage and mycotoxin contamination.[17,26] Toxin-producing fungi can invade food at pre-harvest period, harvest-time or post-harvest during storage. Colonization and eventual contamination may occur in the same stage or a later one.[18] Concerns about mycotoxin occurrence in maize are usually associated to *Aspergillus flavus*, for its ability to produce aflatoxins, and to *Fusarium verticillioides* and *Fusarium proliferatum*, for the production of fumonisins.[7] Other fungi associated with this commodity are the ochratoxigenic ones.[41] In a recent survey, ochratoxin A (OTA) was detected in 70% of maize bread samples, from the central zone of Portugal. From these, one sample exceeded the European maximum limit established for OTA in cereal products for human consumption (3 μg $kg^{-1}$).[8,15] The presence of OTA in maize has also been associated with the contamination of beer,[30] belonging the main *Aspergillus* OTA producer species to the *A. niger* aggregate.[33]

*A. niger* has a great economical and biotechnological interest and is extensively used for production of extracellular enzymes and organic acids such as citric acid.[5,37] *A. niger* has even been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government.[35]

The discovery of *A. niger* strains producing OTA by Abarca et al.[1] raised concerns not only for their biotechnological safety but also for their food safety risk, due to their common presence in different commodities.[10,14,20] OTA is a potent nephrotoxin and has teratogenic, immunosuppressive and carcinogenic properties.[11] Cereals and cereal based food and feed are the main contributors to OTA intake in humans and animals, since OTA is stable under normal food processing operation conditions and it is carried-over from raw materials to processed products.[24] Recently, however, these concerns may have widened. There are several reports stating that *A. niger* can also produce fumonisin $B_2$ ($FB_2$) along with OTA.[9,19,27] Fumonisins are suspected to cause human and animal toxicoses and are regarded as carcinogenic.[12] Of the seven fumonisins currently identified, fumonisins $B_1$ ($FB_1$), $B_2$ ($FB_2$) and $B_3$ ($FB_3$) are the most frequently detected in fungal cultures or in naturally contaminated maize.[31] What concerns *Aspergillus niger* aggregate strains, the main fumonisin produced is the $FB_2$, and reports show that $FB_2$ production by these species is higher while growing in low water activity ($a_w$) culture media,[28] when comparing with its production by *Fusarium* species. Therefore, maize and other commodities with low $a_w$ may be more susceptible to $FB_2$ contamination in the presence of *A. niger* aggregate species.

The aim of this study was to evaluate the $FB_2$ and OTA production by *A. niger* aggregate strains isolated from maize grains of three Portuguese regions.

## Materials and methods

### Chemicals

OTA standard was supplied by Biopure (Austria). A standard solution of OTA was prepared in the OTA vial purchased from Biopure. The stock solution was made in 4 mL toluene:acetic acid (99:1) at 250 μg $ml^{-1}$, and stored at −20 °C. An intermediate standard solution was prepared at 250 ng $ml^{-1}$ by dilution with the same solvent, and stored at −20 °C.

Fumonisin $B_2$ standard was supplied by Sigma Chemical Co. (St. Louis, MO, USA) at 5 mg $ml^{-1}$. An intermediate standard solution was prepared at 5 μg $ml^{-1}$ by dilution in acetonitrile:distilled water (50:50), and stored at −20 °C.

All solvents employed were of glass distilled or HPLC grade and all reagents used were of analytical grade.

### Sampling plan

The survey was carried out in three center-south Portuguese regions, from north to south: Beira Litoral, Ribatejo, and Alto Alentejo. Beira Litoral region (center west) climate is transitional between Atlantic and Mediterranean with annual precipitation of 984 mm and the annual average temperatures are very moderate, 14.6 °C. In higher areas the annual precipitation can easily exceed 1000 mm. Ribatejo (central west) climate is temperate south-Mediterranean. The annual average temperatures are very moderate, 15 °C with an annual precipitation of about 500–600 mm. The region of Alentejo (center-southeast) has a Mediterranean climate feature of continental, i.e. hot and dry. The average annual temperature exceeds 17 °C, and in the summer temperatures above 30 °C are recorded throughout the eastern region. Average annual precipitation is less than 600 mm.

Thirty-two samples were taken in Beira Litoral region, forty samples from the Ribatejo and twenty three from Alto Alentejo, making a total of ninety five samples being taken in three distinct stages of the storage chain. Samples were collected into paper bags to prevent water condensation with the indication of the date, water content and transported to the laboratory within 24 hours.

Twenty samples were taken at reception with a water content between 19 and 22%, 42 samples were taken after drying with a water content between 14 and 15% and 33 samples were taken after storage with a water content between 10 and 14%, between the months of November of 2008 and April of 2009 (Table 1).

### Isolation of fungi

Fifteen grains of each maize sample were plated in Petri dishes with MEA10 (containing per liter: malt extract 20 g; peptone 1 g; glucose 20 g; agar 20 g; sodium chloride 100 g) for 7 days at 25 °C. All *Aspergillus* section *Nigri* strains were isolated after observing the plates under a stereomicroscope. The strains were purified by transferring them to Malt Extract agar (MEA; containing per liter: malt extract 20 g, glucose 20 g, peptone 1 g, agar 20 g) and incubating for 7 days at 25 °C. Identification of the isolates was carried out by morphological characterization following the taxonomic keys and guides available for the *Aspergillus* genus.[16,36] The taxonomy of fungi belonging to the section *Nigri* comprises one of the most confusing and complex due to the subtle differences between the species. In this manner, some biseriate species, such as *Aspergillus carbonarius* and *Aspergillus ibericus* can be recognized through the larger conidia size (6–8 μm), for instance, and the uniseriate species (*Aspergillus aculeatus*, *Aspergillus japonicus* and *Aspergillus uvarum*), can be easily recognized through the absence of metullae. On the other hand, colonies representative of *A. niger* aggregate are very difficult to distinguish morphologically. In this study, because molecular analysis was not carried out, all black biseriate aspergilli strains other than *A. carbonarius*, *Aspergillus ellipticus* and *A. ibericus*

**Table 1**
Number of samples taken at reception, dryer and storage.

| Region | Storing stage | | | Total |
|---|---|---|---|---|
| | Reception | Dryer | Storage | |
| Beira Litoral | 0 | 22 | 10 | 32 |
| Ribatejo | 10 | 10 | 20 | 40 |
| Alentejo | 10 | 10 | 3 | 23 |
| Total | 20 | 42 | 33 | 95 |

will be referred to as the *A. niger* aggregate. Fungal frequency at each region was calculated as the ratio between the number of isolates and the number of samples taken. Fungal incidence was determined as the percentage of samples where the fungus was isolated.

### *HPLC-FD*

OTA samples were analysed by HPLC-FD with a Jasco FP-920 fluorescence detector (333 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 mm × 4.6 mm ID, 5 μm) fitted with a precolumn with the same stationary phase. The mobile phase was water:acetonitrile:methanol (99:99:2, v/v) pumped at 0.8 ml $min^{-1}$. The injection volume was 30 μl.

$FB_2$ samples were analysed by HPLC-FD with a Jasco FP-920 fluorescence detector (460 nm excitation wavelength; 500 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 mm × 4.6 mm ID, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase was acetonitrile: water:acetic acid (60:40:1, v/v) pumped at 1.0 ml $min^{-1}$. The injection volume was 30 μl.

### *Mycotoxin production*

#### *OTA production*

Strains were tested for OTA production in Yeast Extract Sucrose (YES) agar medium (containing per liter: yeast extract 20 g, sucrose 150 g, agar 15 g). Strains were inoculated on 6 cm diameter plates and incubated at 25 °C for 7 days in the dark. Extraction methodology consisted in removing 3 agar plugs (mean 3 agar plug weight of 0.424 g) from one colony and placing into a 4 ml vial where 1 ml of methanol was added. After 60 minutes, the extract was filtered through 0.45 μm filters, evaporated and further dissolved in 1 ml of mobile phase as described by Bragulat et al.[6]

Samples were taken as positive for each of the toxins when a peak was obtained at a retention time similar to each standard, with a height five times higher than the baseline noise. Limit of detection (LOD) was of 0.7 ng $ml^{-1}$.

OTA production values were converted to μg $kg^{-1}$ using the mean agar plugs value.

#### *Fumonisin $B_2$ production*

All strains were tested for fumonisin $B_2$ production in Czapek Yeast Autolysate (CYA) agar (containing per liter: sucrose 30 g, Difco yeast extract 5 g, $K_2HPO_4$ 1 g, $NaNO_3$ 3 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, $CuSO_4 \cdot 5H_2O$ 0.005 g, agar 20 g). Strains were inoculated on 6 cm diameter plates and incubated at 25 °C for 7 days in the dark. Extraction and derivatization methodology as described in Abrunhosa et al.[2] was employed: 5 agar plugs (mean 5 agar plug weight of 0.707 g) were removed from one colony, and placed into a 4 ml vial, where 1 ml of methanol:water (75:25) was added. After sonication for 50 minutes, the extract was filtered through 0.45 μm filters, evaporated and further derivatized with 200 μl of methanol, 200 μl of borate buffer, 100 μl of sodium cyanide and 100 μl of NDA.

Samples were taken as positive for each of the toxins when a peak was obtained at a retention time similar to each standard, with a height five times higher than the baseline noise. LOD was of 75 ng $ml^{-1}$.

$FB_2$ production values were converted to μg $kg^{-1}$ using the mean agar plugs value.

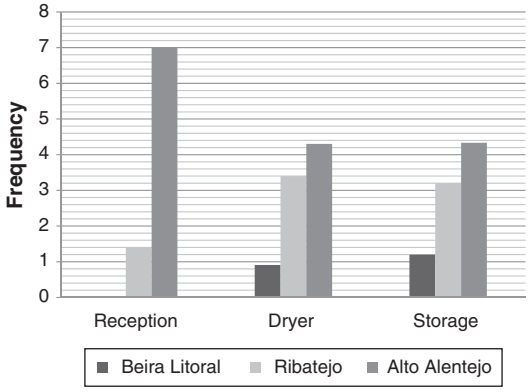


**Fig. 1.** *Aspergillus* section *Nigri* frequency at each region per storing stage.

### *Association between OTA and $FB_2$ production*

To estimate the possible association between OTA and $FB_2$ production, a 2 × 2 contingency table test was used.[4] A low association between variables is indicated when *Phi* is close to 0, and a strong association is indicated when *Phi* is close to 1. Statistical analyses were performed using SPSS Statistics version 19.0.

## Results

### *Isolation of black Aspergillus*

From the ninety five samples of maize grains obtained in the three Portuguese regions, *Aspergillus* section *Nigri* isolates were found in seventy three samples (77%). From these, two hundred and seventy strains were isolated (Table 2). Morphological analysis revealed that all were bisseriate and within the *A. niger* aggregate; uniseriate species and other bisseriate species, such as *A. carbonarius* or *A. ibericus*, were not isolated. The incidence of *Aspergillus* section *Nigri* differed among regions being present in 59%, 80% and 96% of the samples from Beira Litoral, Ribatejo and Alto Alentejo, respectively. Fungal frequency followed the same trend, being higher in Alto Alentejo, in all stages, than in the other regions. Beira Litoral is the region that exhibits the lowest frequency (Fig. 1).

### *Mycotoxin production*

In *Aspergillus* section *Nigri* strains isolated from maize, OTA and $FB_2$ were produced by 14% and 39% of the isolates, respectively (Table 3). Thirty-seven OTA producer strains and 105 $FB_2$ producer strains were isolated. Beira Litoral exhibits the lowest incidence of ochratoxigenic strains (6%) and the highest incidence of $FB_2$ strains (50%); Ribatejo has the highest incidence of ochratoxigenic strains (18%) and the smallest incidence of $FB_2$ producers (34%); while Alto Alentejo has a mean incidence of OTA (12%) and $FB_2$ (38%) producing strains.

To evaluate if there is an association between the production of OTA and $FB_2$, the *Phi* association value was calculated (Table 3). For

**Table 2**
Number of isolates and samples from three Portuguese regions.

| Region | Number of samples with isolations per total of samples (mean frequency) | Number of *Aspergillus niger* aggregate isolates (mean incidence) |
|---|---|---|
| Beira Litoral | 19/32 (59%) | 32 (1.0) |
| Ribatejo | 32/40 (80%) | 111 (2.8) |
| Alto Alentejo | 22/23 (96%) | 127 (5.5) |
| Total | 73/95 (77%) | 270 (2.8) |

**Table 3**
Mycotoxin production of *Aspergillus niger* aggregate isolated in three Portuguese regions.

| Region | OTA | | $FB_2$ | | *Phi* | *p*-Value |
|---|---|---|---|---|---|---|
| | Negative | Positive | Negative | Positive | | |
| Beira Litoral | 30 | 2 | 16 | 16 | 0.000 | 1.000 |
| Ribatejo | 92 | 20 | 71 | 41 | −0.064 | 0.499 |
| Alto Alentejo | 111 | 15 | 78 | 48 | 0.065 | 0.466 |
| Total | 233 | 37 | 165 | 105 | −0.009 | 0.888 |

**Table 4**
Mean values of OTA and $FB_2$ production ($\mu g\,kg^{-1}$) by *Aspergillus niger* aggregate isolated in three Portuguese regions.

| Region | OTA ($\mu g\,kg^{-1}$) | Mean levels[a] ($\mu g\,kg^{-1}$) | $FB_2$ ($\mu g\,kg^{-1}$) | Mean levels[a] ($\mu g\,kg^{-1}$) |
|---|---|---|---|---|
| Beira Litoral | 6–20 | 13 | 1–2323 | 477 |
| Ribatejo | 1–18 | 6 | 1–3536 | 524 |
| Alto Alentejo | 1–18 | 6 | 1–5382 | 366 |

[a] Mean values were determined taking into consideration only the producing strains.

**Table 5**
Number of *A. niger* aggregate strains producing OTA and $FB_2$ and its distribution in three Portuguese regions.

| Region | No. strains tested | No. OTA producing strains (0.8–18 $\mu g\,kg^{-1}$) | No. of strains that produce $FB_2$ | | |
|---|---|---|---|---|---|
| | | | <100 $\mu g\,kg^{-1}$ | 100–1000 $\mu g\,kg^{-1}$ | >1000 $\mu g\,kg^{-1}$ |
| Beira Litoral | 34 | 2 | 5 | 9 | 2 |
| Ribatejo | 121 | 20 | 19 | 15 | 7 |
| Alto Alentejo | 132 | 15 | 28 | 12 | 8 |

the overall results the *Phi* – association value is fair (*Phi* < 0.1), and not significant ($p > 0.05$), meaning that their production is independent from each other.

As expected the OTA production by these strains is relatively low in all regions (Table 4), with production values ranging between 1 $\mu g\,kg^{-1}$ and 20 $\mu g\,kg^{-1}$ (0.4 $ng\,ml^{-1}$ and 8.5 $ng\,ml^{-1}$). The $FB_2$ production is much higher and ranged between 1 $\mu g\,kg^{-1}$ and 5382 $\mu g\,kg^{-1}$. Regarding OTA, even though Beira Litoral exhibits the lowest amount of producing isolates, the production mean for OTA is the highest when comparing with Ribatejo and Alto Alentejo. The same does not apply to $FB_2$, being Ribatejo the region with the highest production mean. The region with higher amount of isolates, Alto Alentejo, is the one with lower production means.

$FB_2$ production varied between strains where almost 50% produce below 100 $\mu g\,kg^{-1}$, 34% produce between 100 and 1000 $\mu g\,kg^{-1}$, and 16% produce at levels above 1000 $\mu g\,kg^{-1}$ (Table 5).

## Discussion

### *Isolation of Aspergillus section Nigri*

Morphological analysis revealed that all isolated strains were bisseriate and within the *Aspergillus niger* aggregate. Uniseriate species were not found in these maize samples, even though it had been previously isolated from Nigerian and Argentinean maize.[1,23] Also in other study, with Portuguese wine grapes, uniseriate strains were rarely isolated (2 isolates in 770 isolated strains),[40] and only in a grape growing area located northern from these maize growing areas.

*Aspergillus* section *Nigri* species are common soil inhabitants contaminating ripening crops in Mediterranean, tropical and subtropical regions[21] being more resistant to higher solar exposure and higher temperatures. Therefore, the higher incidence of isolates in Ribatejo and in Alto Alentejo (Table 2) may be explained by the fact that both these regions have Mediterranean climates, very hot and dry during summer time, frequently achieving temperatures around 40 °C. Beira Litoral, on the other hand, has Atlantic influences with more moderate temperatures and higher precipitation, being more common other fungal species. The black aspergilla presence in Portuguese regions with Mediterranean climate had already been assessed in a survey of ochratoxigenic fungi in Portuguese wine grapes[39,40] where these species have been isolated from 22% of grape samples. Although the sampling sites were not the same, it was also possible to observe a higher incidence in Alto Alentejo (72–100%) than in Ribatejo (18–48%).[39] In these studies, temperature and relative humidity were found to determine the relative incidence of *Aspergillus* section *Nigri* in grapes.

### *Mycotoxin production by fungi*

The production of OTA within *Aspergillus* section *Nigri* varies according to different authors: from 3% to 28%, being extreme value up to 80% reported in the literature, as reviewed by Palumbo et al.[34] In the previous Portuguese study concerning the production of OTA from strains isolated from grapes, percentages of OTA producing strains of *A. niger* aggregate were lower (4%).[40] However, OTA producing strains were not equally distributed among all regions, being the incidence in Southern regions higher.[38] Magnoli et al.[23] reported 6% of ochratoxigenic *A. niger* strains in previously disinfected Argentinean maize kernels, which lies within the value obtained from Portuguese grapes and maize.

The number of fumonigenic *A. niger* aggregate strains is slightly higher (36%) in comparison with similar studies in grapes where 23% and 29% of *Aspergillus niger* aggregate strains produced $FB_2$.[3,42] However, the values are much lower than in other studies in grapes and coffee beans [19,27,29] where 60–70% of the isolates were found to produce $FB_2$ although in these cases a very low number of strains were tested.

It is clear the *A. niger* species can produce both OTA and $FB_2$; however, it was not clear if there is an association between the productions of these two mycotoxins. Although statistically not significant, the distribution of OTA and $FB_2$ producing strains among regions seems to indicate that there is no connection between the production of both these toxins by the same fungus.

OTA production values (0.4 $\mu g\,l^{-1}$ to 8.5 $\mu g\,l^{-1}$) are lower than the ones reported by Magnoli et al. [24] which ranged between 2 $\mu g\,l^{-1}$ and 24.5 $\mu g\,l^{-1}$. The $FB_2$ production is much higher and ranged between 1 $\mu g\,kg^{-1}$ and 5382 $\mu g\,kg^{-1}$, being lower, however, than the ones obtained by Mogensen et al. [27] that ranged between 229 $\mu g\,kg^{-1}$ and 6476 $\mu g\,kg^{-1}$ and by Susca et al. [42] that ranged between 100 $\mu g\,kg^{-1}$ and 293,000 $\mu g\,kg^{-1}$. The requirements for $FB_2$ production by *Aspergillus* and *Fusarium* strains are not the same. *Fusarium* spp. are predominantly considered as field fungi and except under extreme conditions, the concentrations of fumonisins produced by *Fusarium* spp. do not increase during grain storage.[25] However, *Aspergillus* have the ability to grow and produce mycotoxins at lower water availability conditions.[22] Although the optimum temperature and $a_w$ differ, the levels produced under optimum condition are of the same order of magnitude.[28]

The fact that *Aspergillus* section *Nigri* fungi are associated to commodities not previously associated with *Fusarium* and, consequently, with fumonisins, the production of $FB_2$ by *Aspergillus* section *Nigri* strains may extend the concern about fumonisins to a wider range of commodities.

In conclusion, *Aspergillus* section *Nigri* strains isolated from maize can produce both OTA and $FB_2$ but, being maize a well-known natural substrate for *Fusarium* species and their fumonisins, it is still unclear if the presence of black aspergilla will increase the risk of maize contamination with fumonisins.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

Célia Soares and Thalita Calado were supported by grants from Fundação para a Ciência e Tecnologia (references SFRH/BD/37264/2007 and SFRH/BD/79364/2011, respectively).

The authors are grateful for the support and cooperation of the National Producers Association of Maize and Sorghum (ANPROMIS – Associação Nacional de Produtores de Milho e de Sorgo).

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