BÁRBARA ALVES DOS SANTOS-CISCON

MOLECULAR IDENTIFICATION, TOXIGENIC POTENTIAL AND EFFECTS OF *Aspergillus* ASSOCIATED TO BEAN SEEDS AND GRAINS

LAVRAS-MG
2018

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutora.

PhD José da Cruz Machado
Orientador

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BÁRBARA ALVES DOS SANTOS-CISCON

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IDENTIFICAÇÃO MOLECULAR, POTENCIAL TOXIGÊNICO E EFEITOS DE *Aspergillus* ASSOCIADOS A SEMENTES E GRÃOS DE FEIJÃO

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Ao meu esposo, Giancarlo, grande companheiro nesta jornada.

Com amor, dedico.

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RESUMO

O Brasil é um dos líderes mundiais na produção de feijão, além de ser um dos maiores consumidores deste grão. A deterioração das sementes de feijão durante o armazenamento pode ser acelerada devido às condições do ambiente de armazenamento bem como à ação de microrganismos. Os fungos do gênero *Aspergillus* são conhecidos pela sua capacidade de proliferar em condições de baixa humidade, o que favorece sua associação a sementes e grãos armazenados. Além disso, estes fungos são importantes produtores de micotoxinas, constituindo um problema de ordem fitossanitária e de segurança de alimentos. Com presente trabalho, objetivou-se estudar as relações biológicas entre sementes de feijão e espécies de *Aspergillus* em condições de armazenamento natural e controlado, assim como em condições de estresse causado pelo envelhecimento acelerado e restrição hídrica do substrato. Além disso, buscou-se identificar por meio de técnicas moleculares, as principais espécies de *Aspergillus* associadas a sementes de feijão no Brasil e caracterizar seu potencial toxigênico por meio da prospecção de genes envolvidos na biossíntese de micotoxinas. Observou-se que, o armazenamento natural ocasionou maior redução na qualidade fisiológica das sementes de feijão, no entanto, a associação com *A. ohraceus* e *A. parasiticus* mostrou-se capaz de afetar a germinação das sementes mesmo em ambiente controlado. Nas sementes submetidas a condições de estresse, o condicionamento causou menos danos à qualidade fisiológica das sementes que o envelhecimento acelerado, além de evidenciar as diferenças entre os efeitos de *A. ochraceus* e *A. parasiticus*. Em ambas as condições de estresse, observou-se que a associação com A. *parasiticus* foi mais danosa à qualidade das sementes. A identificação molecular de isolados de *Aspergillus* coletados a partir de 35 lotes de sementes de feijão revelou a presença de sete espécies*:* *A. flavus* (n=39), *A. pseudocaelatus* (n=1), *A. westerdijkiae* (n=7), *A. ostianus* (n=3), *A. wentii* (n=2), *A. ochraceus* (n=1), *A. niger* (n=24) e *A. luchuensis* (n=10). Foram detectados genes envolvidos na síntese de aflatoxina somente em isolados de *A. flavus*. Todos os isolados de *A. niger* apresentaram todos os genes da via biossintética de fumonisinas. Em nenhum dos isolados foi detectada a presença de genes envolvidos na biossíntese de ocratoxinas.

Palvras-chave: *Phaseolus vulgaris*. *Vigna unguiculata*. Germinação. Vigor. Armazenamento. Micotoxinas. Calmodulina. β-tubulina.

ABSTRACT

Brazil is one of the world leaders in bean production, besides being one of the biggest consumers of this grain. The deterioration of bean seeds during storage can be enhanced due to the conditions of the storage environment as well as the action of microorganisms. Fungi of the genus *Aspergillus* are known for their ability to proliferate under low humidity conditions, which favors their association with stored seeds and grains. In addition, these are the main producers of mycotoxins, being a problem of seed health and food safety. The objective of this work was to study the biological relationships between bean seeds and *Aspergillus* species under natural and controlled storage conditions, as well as under stress conditions caused by accelerated aging and substrate water restriction. In addition, we aimed to identify, using molecular techniques, the main *Aspergillus* species associated with bean seeds in Brazil, and to characterize their toxigenic potential by prospecting genes involved in mycotoxin biosynthesis. It was observed that the natural storage caused a greater reduction in the physiological quality of bean seeds, however, the association with *A. ochraceus* and *A. parasiticus* affected the seed germination even in the controlled environment. In the seeds subjected to stress conditions, water conditioning caused less damage to the physiological quality of the seeds than accelerated aging, and evidenced the differences between the effects of *A. ochraceus* and *A. parasiticus*. In both stress conditions, it was observed that the association with *A. parasiticus* was more harmful to seed quality than *A. ochraceus*. The molecular identification of *Aspergillus* strains isolated from 35 lots of Brazilian bean seeds revealed the presence of seven species: *A. flavus* (n=39), *A. pseudocaelatus* (n=1), *A. westerdijkiae* (n=7), *A. ostinaus* (n=3), *A. wentii* (n=2), *A. ochraceus* (n=1), *A. niger* (n=24) e *A. luchuensis* (n=10). Genes involved in aflatoxin synthesis were detected only in *A. flavus* strains. All *A. niger* isolates showed the full complement of genes belonging to the fumonisins bissinthetic pathway. In none of the isolates was detected the presence of genes involved in ochratoxin biosynthesis.

Keywords: *Phaseolus vulgaris*. *Vigna unguiculata*. Germination. Vigor. Storage. Mycotoxins. Calmodulin. β-tubulin.

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1 INTRODUCTION

Brazil is one of the largest producers and exporters of agricultural commodities in the world. Nowadays, the participation of agribusiness in the international market has been fundamental for the country's economy, contributing positively to the results of the Brazilian trade balance. However, the export of agricultural products intended for human and animal consumption require special attention to the internationally required quality standards.

Food safety is one of the major concerns regarding international trade in agricultural commodities. Mycotoxin contamination is one of the parameters evaluated to ensure food safety. Some mycotoxins have carcinogenic, teratogenic and immunosuppressive actions and are related to several diseases in humans and animals. Fungi of the genus *Aspergillus* are major contamination of agricultural products by *Aspergillus* characterizes a phytosanitary problem, since the inoculum of these fungi comes from the field and they are capable of affecting seed physiological quality.

Beans are one of the main components of Brazilian's basic dietary. Its production is associated with the most diverse income levels and is distributed throughout the country. The association of *Aspergillus* with bean seeds has been reported frequently, but little is known about the contamination of this product with mycotoxins.

In Brazil, the Brazilian Health Regulatory Agency (Anvisa) and the Ministry of Agriculture Livestock and Food Supply are responsible for determining and monitoring the tolerable limits of mycotoxins in food. Concerning beans, the legislation determines the quantification of aflatoxins and ochratoxin. However, it is known that some *Aspergillus* species can produce fumonisin B_2, which test is not required by the current standards.

Therefore, it is remarkable the great necessity for the correct identification of the *Aspergillus* species frequently associated to bean seeds and grains in Brazil. From the species identification, it is possible to infer about which mycotoxins can be produced, as well as the risk of bean grains contamination. In addition, the identification of the *Aspergillus* species associated to this grain in Brazil makes possible the detailed study of the effects caused by each species on seed physiological quality, demonstrating the importance of sanitary quality of seeds and reinforcing the need for using certified seeds in bean production.

2. LITERATURE REVIEW

2.1 Dry beans: production and seed quality

Historically, agribusiness is one of the main sectors of the Brazilian economy and the main responsible for its participation in the international market. Brazil is one of the world leaders in the production and export of agricultural products, with emphasis on grain production. Currently, the country is the third largest producer of dry beans in the world, with an estimated production of 2.6 million tons (FAOSTAT, 2018). Myanmar is the world's largest producer, followed by India. Besides these countries, China, USA, and Mexico are among the six largest producers, which account for 61% of world beans production.

Due to the great diversity of beans in Brazil and in the world, the dietary habits are very variable and it is possible to detect regional preferences of consumption. In Brazil, dry beans are classified into groups and classes. The groups refer to the botanical species, being: (I) common bean (*Phaseolus vulgaris* L.) and (II) cowpea (*Vigna unguiculata* L. Walp.); while the classes refer to the predominant tegument color: white, black or mixed colors (MAPA, 2008). The Carioca variety (Group I, class colors) is the most consumed in Brazil and represents about 70% of the total beans consumed in the country. According to Ribeiro et al. (2014), Carioca beans and Black beans (Group I, class black) together account for about 85% of the bean market in Brazil. Other types of beans have regional importance, such as Fradinho or Caupi (Group II), which are popular in the Northeast region and represent about 10% of the market for this grain (VOGT et al., 2011). As one of the main constituents of Brazilian basic dietary, beans have their production strongly adjusted to consumption; in addition, this grain is produced under the most diverse levels of technology and stands out for the great socioeconomic importance. Nowadays, beans are harvested all over the country, in three crops during the year. The first crop, or water crop, is planted from September to November; the second one, or drought crop, is planted from January to March; and the third crop, or autumn-winter crop, planted in from May to July, conducted in mild winter regions, with massive inputs and need for irrigation. According to the National Supply Company - CONAB (2017), the production of common beans class colors, is evenly distributed in the three annual crops; differently, about 63.5% of the production of common beans class black comes from the first harvest and is concentrated in the South region.

The cowpea (Group II) is cultivated in the North and Northeast regions (except Bahia) and Mato Grosso and the major part of its production comes from the second harvest. In Brazil,

the main producer and consumer of cowpea is the Northeast region, however, Egypt, India, and China are potential importers of this product (FREIRE FILHO et al., 2012). In order to export Brazilian cowpea, it is necessary a better understanding of these markets, identifying consumer preferences and reaching the international quality standards. According to Souza et al. (2013), the current expansion of cowpea production in the state of Mato Grosso, aiming the large-scale production of this grain requires the development of upright and semi-erect cultivars with modern architecture, suitable for mechanized cultivation. In addition to the modification in the plant architecture, other goals of the cowpea breeding are productivity increase, resistance to pests and diseases, adaptation to different regions of the country and increase of nutritional quality (FREIRE FILHO et al., 2012). In contrast, common bean access to the international market faces other challenges.

Despite being a major producer of this commodity, Brazil is also a major consumer, which implies a small surplus for export. One of the main obstacles in the international trading of beans produced in Brazil is related to consumer's preferences. Approximately 40% of the national production belongs to the Carioca type, which is not well accepted in other countries (CONAB, 2017). Besides that, traditional Carioca-type cultivars darken rapidly, about two months after harvest. This feature prevents the producer from storing these grains for longer periods since the dark grains do not have good acceptance by the consumer (ALMEIDA et al., 2017). For this reason, new common bean cultivars have been developed in order to offer grains that remain lighter in color for about a year (SIQUEIRA et al., 2014), among them, the cultivars TAA Dama, IAC Milênio, BRS Requinte, IAC Alvorada, and BRS Estilo. It is certain that the increase in the storage period of the beans is economically advantageous for the producer. However, it is important to carefully evaluate other aspects of the prolonged storage of bean grains. Considering that approximately 85% of the bean producers use domestic seeds or commercial grains in the planting, these grains must also be evaluated in terms of seed quality. The physiological quality of seeds is one of the factors that are strongly affected by storage conditions and time. In literature, there are numerous reports about the reduction of germination and vigor of bean seeds as a function of storage time (CASSOL et al., 2016; HENDGES et al., 2017; SANTOS; MENEZES; VILLELA, 2005; SILVA; PAZETO; VIEIRA, 2012; ZUCARELI et al., 2015).

Another aspect of fundamental importance is the phytosanitary quality, since the association between microorganisms, mainly fungi, with the seeds is related to the low physiological quality in many crops. Seeds are effective vehicles for the dissemination and transmission of pathogens, capable of introducing them into disease-free areas and,

consequently, capable of reducing the physiological quality of the seed lots produced in those areas (MACHADO, 1988; NEERGAARD, 1979;). Such pathogens can prevent seed germination, cause seedling death, decrease its growth and significant yield losses. In Brazil, the Ministry of Agriculture Livestock and Food Supply establishes and supervises the quality requirements for seed production, certification and commercialization, according to the IN 45/2013 (MAPA, 2013). This regulation determines, among other characteristics, the tolerable incidence of some pathogens in the seed lot. However, when a seed lot does not attend to these requirements, it can be used as grain – i.e. can be sold for human consumption.

2.2 Health quality of stored seeds and grains

The need to store bean seeds is mainly due to market conditions, allowing the producer to achieve greater profitability, besides ensuring viable seeds for the next harvest. However, the first effects of storage on the physiological quality of bean seeds are noted by the loss of vigor (BRAGANTINI, 2005). Therefore, the storage conditions are fundamental to preserve the physiological potential of the seeds and to promote the longevity of the grains until the consumption (GOLDFARB; QUEIROGA, 2013).

The temperature and the relative air humidity are the main environmental factors that affect the quality of the bean seeds during storage (GOLDFARB; QUEIROGA, 2013; HEDGENS et al., 2017). In Brazil, the most used storage condition for bean grains and seeds is the reduction of moisture content. However, when used alone, this condition is not capable of maintaining the quality of the seed at satisfactory levels for long periods. The refrigerated storage is a very efficient method, however, it has a high cost, especially in the warmer regions of the country (BRACKMANN et al., 2002). Preservation of the physiological quality of stored seeds through the control of temperature and humidity conditions is based on the reduction of the metabolic activity of the seeds as well as the microorganisms associated with them.

In the field, various organisms, including insects, nematodes, fungi, bacteria, and viruses can affect bean crops, reducing the production significantly (GRAHAM; RANALLI, 1997). However, during storage, species belonging to the genera *Aspergillus* and *Penicillium* cause considerable losses due to their ability to grow under low humidity conditions. Francisco and Usberti (2008) reported the decrease in the population of "field fungi" and the increase in the population of "storage fungi" after harvest. In addition, these authors highlighted the incidence of storage fungi as the main concern in the sense of preserving the health and viability of stored bean seeds.

The high incidence of *Aspergillus* species associated with bean seeds has been frequently reported in Brazil (ARAÚJO et al., 1980; BENÍCIO et al., 2003; COSTA; SCUSSEL, 2002; FRANCISCO; USBERTI, 2008; SILVA et al., 2008; TORRES; BRINGEL, 2005). This scenario is quite alarming since these populations tend to increase over the storage period and cause significant losses in seed physiological quality (FRANCISCO; USBERTI, 2008; ROCHA et al., 2014). In addition, the use of contaminated grains in the planting of the next crop takes the inoculum back into the field, contaminating the seeds produced and causing economic losses.

2.2 Mycotoxins produced by *Aspergillus* spp.

Another important aspect of the deterioration of food, mainly grains, caused by *Aspergillus* is the formation of mycotoxins (SAMSON et al., 2010; VARGA et al., 2008). Mycotoxins are toxic, low molecular weight secondary metabolites produced mainly by fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium* (BHATNAGAR-MATHUR et al., 2015). Among those produced by the genus *Aspergillus* are the aflatoxins (AF) B1, B2, G1 and G2, ochratoxin A (OTA) and, more recently, fumonisin B2 (FB2). Such toxins exhibit carcinogenic, teratogenic mutagenic effects in humans and animals, and are thermostable, remaining in the food indefinitely (PITTET, 1998, WILLIAMS et al., 2004).

Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins (YU et al., 2004). There are four major aflatoxins: B1, B2, G1, and G2; aflatoxin B1 is the most toxic and prevalent and is classified as a Group 1a carcinogen by the International

Agency for Research on Cancer (BANDYOPADHYAY et al., 2016; IARC, 2002;). *Aspergillus flavus* produces AFB_1 and AFB_2 and *Aspergillus parasiticus* produces AFB_1, AFB_2, AFG_1 and AFG_2, but other species like *Aspergillus nomius, Aspergillus pseudotamarii, Aspergillus ochraceoroseus, Aspergillus pseudocaelatus* and *Aspergillus ostianus* have also been reported to produce aflatoxin as reviewed by Bezerra da Rocha et al. (2014). In humans, aflatoxin contamination is associated with several diseases such as hepatitis, liver cirrhosis, liver cancer, and gallbladder cancer (KOSHIOL et al., 2017; MCKEAN et al., 2006).

Crop aflatoxin contamination starts in the field and varies according to environmental and biological factors such as host susceptibility, heat and high-temperature stress, insect attack, and aflatoxin-producing potentials (MEHL et al., 2012; WILLIAMS, 2006). Aflatoxin contamination can start or continue after harvesting weather the storage environment is favorable for fungal proliferation and aflatoxin formation (COTTY; MELLON, 2006). As

reviewed by Bandyopadhyay et al., (2016), the only commercially effective, environmentally friendly technology to reduce aflatoxin accumulation of crops is the use of atoxigenic strains as biocontrol agents to displace aflatoxigenic fungi. The biocontrol formulation provides atoxigenic strains with both reproductive and dispersal advantages over resident aflatoxin-producers. According to Bandyopadhyay and Cotty (2013), biocontrol is a simple intervention in the field that reduces aflatoxin contamination in crops from pre-harvest until consumption. However, the use of non-toxigenic strains requires the selection of local strains that occur endemically on target crops in target regions (MEHL et al., 2012).

Ochratoxin A (OTA) is known as the most toxic member of the ochratoxin family of mycotoxins, displaying nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic effects (JECFA, 2001). It naturally occurs in a wide variety of agricultural commodities worldwide. *Aspergillus ochraceus* and *Penicillium verrucosum* were considered for a long time the main OTA producers (PITT, 2000). *A. ochraceus* strains have been shown to be capable of producing high amounts of OTA under certain circumstances, while eight other species within the section *Circumdati* were described as robust OTA producers: *Aspergillus cretensis*, *Aspergillus flocculosus*, *Aspergillus pseudoelegans*, *Aspergillus roseoglobulosus*, *Aspergillus westerdijkiae*, *Aspergillus sulphurous*, and *Neopetromyces muricatus* (FRISVAD; FRANK; HOUBRAKEN, 2004).

As reviewed by Amézqueta et al., (2009), the critical factors that affect fungal growth and OTA production are temperature, moisture content and the time a product remains under adverse conditions, besides mechanical damages and insect attack. It is recommended to store products completely dried and to maintain them at a water activity under 0.70 and at temperature under 20ºC (BUCHELI; TANIWAKI, 2002)

The exposure to fumonisins can lead to carcinogenic, nephrotoxic and hepatotoxic effects in humans and animals (JECFA, 2001). Fumonisins were considered to be produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum* (GELDERBLOM et al., 1988), therefore, regulated mainly in maize-based products. Recently, fumonisin B_2 production was detected in *Aspergillus niger* and *Aspergillus welwitschiae* (FRISVAD et al., 2007; HONG et al., 2013; PERRONE et al., 2011) highlighting the importance of testing *Aspergillus* infected commodities.

Regarding beans, the Brazilian mycotoxin regulation establishes the bearable limts of aflatoxins and ochratoxins. Fumonisin testing is required only on maize and its derivatives. However the literature already reports the occurrence of *Aspergillus* fumonisin producing strains affecting onions, nuts, cocoa, coffee and dried fruits in Brazil (MASSI et al., 2016).

Based on reports of bean contamination by ochratoxin A and aflatoxin (SILVA et al., 2002; SOARES; RODRIGUES-AMAYA, 1989), Costa and Scussel (2002) alerted to the low amount of information on the presence of mycotoxins and the toxigenic behavior of fungi associated with bean grains in Brazil, a scenario that persists today. Therefore, it is clear the need for new research on the risk of mycotoxin contamination on beans, the toxigenic potential of the fungi associated with them and the prospection of isolates that to be used as biological control agents in mycotoxins management.

2.4 Identification of *Aspergillus* spp.

Nowadays, it is accepted that the genus *Aspergillus* comprises 339 formally described species, grouped into four subgenera and 19 sections (HOUBRAKEN; VRIES; SAMSON, 2014; SAMSON et al., 2014). Given the great importance of these fungi and their wide geographical distribution, it is essential to identify them correctly, regardless of the area of interest for study or practical use.

Morphology is an important part of the concept of *Aspergillus*, whose name was given by Antonio Micheli in 1729 due to the similarity of the conidiophore with an aspergillum (GIBBONS; ROKAS, 2012). The classification of *Aspergillus* is traditionally based on morphological characters, such as size and arrangement of conidial heads, color, the growth rate in solid media and physiological features. However, classification and identification based only on phenotypic characters is difficult and requires experienced staff (HOUBRAKEN; VRIES; SAMSON, 2014). In addition, an evaluation of macro and micromorphological characters requires good growth, which takes around five days; besides, it is strongly influenced by the composition of the culture medium, inoculation technique and incubation conditions (OKUDA; KLICH; SEIFERT, 2000)

Currently, molecular techniques, based especially on the DNA sequence have often been used to identify fungi. The ITS region is like the primary barcode in the identification of these microorganisms (SCHOCH et al., 2102). However, regarding the genus *Aspergillus*, this locus offers a low level of discrimination and cannot be used for species identification (HUBKA; KOLARIK, 2012; SAMSON et al., 2014). Thus, protein-encoding genes are quite promising, since they contain a high content of functional information and have, in general, greater variability than the ITS region (HOUBRAKEN; VRIES; SAMSON, 2014; LIU; WHELEN; HALL, 1999).

According to Samson et al. (2014), an ideal marker, must be detected by using universal primers, be easily amplified and distinguish between all species. Another important consideration is the amount of information currently available, for each gene, in the databases; that is, how many species have been actually sequenced. Based on these criteria, these authors proposed the use of the calmodulin (*CaM*), β-tubulin (*BenA*) or the second major subunit of RNA polymerase II (*RPB2*) as ideal barcodes for the identification of *Aspergillus* species. However, the precise species identification should consider morphological and phylogenetic data, as well as the metabolic profile, which is called polyphasic approach (SAMSON; VARGA, 2009).

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SEGUNDA PARTE – ARTIGOS

(Formatação baseada nas exigências do *Journal of Seed Sciences*)

ARTIGO 1

EFFECTS OF *Aspergillus ochraceus* AND *Aspergillus parasiticus* ON QUALITY OF STORED DRY BEAN SEEDS.

EFEITOS DE *Aspergillus ochraceus* E *Aspergillus parasiticus* NA QUALIDADE DE SEMENTES DE FEIJÃO ARMAZENADAS

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1 **Effects of *Aspergillus ochraceus* and *Aspergillus parasiticus* on quality of**
2 **stored dry bean seeds**

3 Bárbara Alves dos Santos-Ciscon[1], José da Cruz Machado[1*], Iara Eleutério Dias[1], Poliana
4 Patrícia Lima[1], Luís Roberto Batista[2]

5 ABSTRACT – Currently, Brazil is the third largest producer of beans in the world. Among the
6 phytosanitary problems associated with this crop, the association of seeds with *Aspergillus*
7 species is frequently reported during storage. Thus, the objective of this work was to study the
8 effect of *A. ochraceus* and *A. parasiticus* on the quality of bean seeds of the cultivar TAA Dama
9 under different storage conditions. Healthy and contaminated seeds with *A. ochraceus* and *A.*
10 *parasiticus* were submitted to natural storage (uncontrolled conditions) and controlled storage
11 (cold and dry chamber) for six months. During this period, germination, electrical conductivity,
12 and water activity tests were carried out to evaluate seed quality. Controlled storage proved to
13 be more efficient in preserving the physiological quality of bean seeds. However, in the seeds
14 associated with *A. ochraceus* under controlled storage, the percentage of normal seedlings was
15 similar to that shown by healthy seeds under natural storage. The electrical conductivity and
16 water activity varied according to the time and storage method but were not influenced by the
17 association of the seeds with the fungi under study.

18

19 Index terms: Germination; Vigor; Dama cultivar; Storage; Water activity.

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20 # Efeitos de *Aspergillus ochraceus* e *Aspergillus parasiticus* na qualidade de
21 # sementes de feijão armazenadas.

22 Bárbara Alves dos Santos-Ciscon[1], José da Cruz Machado[1*], Iara Eleutério Dias[1], Poliana
23 Patrícia Lima[1], Luís Roberto Batista[2]

24 RESUMO – Atualmente, o Brasil é o terceiro maior produtor mundial de feijão. Dentre os
25 problemas fitossanitários associados à essa cultura, a associação de sementes com espécies de
26 *Aspergillus* é frequentemente relatada durante o armazenamento. Sendo assim, objetivou-se
27 com o presente trabalho estudar o efeito de *A. parasiticus* e *A. ochraceus* na qualidade de
28 sementes de feijão da cultivar TAA Dama sob diferentes condições de armazenamento.
29 Sementes sadias e contaminadas com *A. ochraceus* e *A. parasiticus* foram submetidas a
30 armazenamento natural (condições não controladas) e controlado (câmara fria e seca) durante
31 seis meses. Neste período, foram realizados testes de germinação, condutividade elétrica e
32 atividade de água para avaliar a qualidade das sementes. O armazenamento controlado mostrou-
33 se mais eficiente na preservação da qualidade fisiológica das sementes de feijão. No entanto,
34 nas sementes associadas à *A. ochraceus* a porcentagem de germinação foi semelhante à das
35 sementes sadias em armazenamento natural. A condutividade elétrica e a atividade de água
36 variaram de acordo com o tempo e método de armazenamento, porém não foram influenciadas
37 pela associação das sementes com os fungos em estudo.

38

39 Termos para indexação: Germinação; vigor; cultivar dama; armazenamento; atividade de água.

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1. Introduction

Brazil is one of the world's leading grain producers and is currently the third largest producer of dry beans, with around 2.6 million tons. (FAOSTAT, 2018). *Phaseolus vulgaris* is the main cultivated species, with the Carioca and Preto varieties representing 85% of the dry bean consumption in the country (Ribeiro et al. 2014).

Although it is produced all over the year, the demands of the regional market and price speculation imply the need for bean grains storage during long periods. The Carioca variety presents beige grains with brown streaks and is the most appreciated by Brazilian consumers (Ribeiro et al., 2014). However, the darkening of the tegument and its consequent depreciation in the market is observed about 60 days after harvest, what limits the storage time of these grains. For this reason, new common bean cultivars have been developed in order to provide grains that remain lighter in color for about a year, such as the cultivars TAA Dama, IAC Milênio, BRS Requinte, IAC Alvorada and BRS Estilo (CONAB, 2017).

Another factor that requires the storage of bean grains is its use as seeds. According to the National Supply Company (2017), approximately 85% of Brazilian bean producers use domestic seeds or commercial grains in planting this crop, which causes loss of vigor, varietal degeneration in addition to pathogen spreading.

During storage, the incidence of fungi is one of the main concerns regarding the preservation of seed physiological quality. Due to the ability to grow in low humidity environments, fungi belonging to the genera *Aspergillus* and *Penicillium* are the most frequently reported in association with stored seeds and grains. Besides the sanitary quality, the conditions of temperature and relative humidity affect directly the physiological quality of bean seeds (Goldfarb and Queiroga, 2013; Hedgens et al., 2017), and the control of such environmental conditions are the basis of bean storage methodologies used in Brazil.

65 The association between *Aspergillus* and bean seeds has been frequently reported in the
66 literature (Araújo et al., 1980; Benício et al., 2003; Costa and Scussel, 2002; Francisco and
67 Usberti, 2008; Silva et al., 2008; Torres and Bringel, 2005). However, little is known about the
68 species that occur associated with this grain, as well as if there are differences in the effects
69 caused by each of them on bean seeds. Thus, in order to elucidate the relationships between
70 different species of *Aspergillus* and bean seeds, the objective of this work was to study the
71 effects caused by *A. ochraceus* and *A. parasiticus* on the physiological quality of bean seeds
72 submitted to different conditions of storage.
73

74 ## 2. Material and methods

75 Bean seeds of TAA Dama cultivar were disinfected with 1% sodium hypochlorite
76 (NaClO) for 30 seconds, washed with distilled water and dried on filter paper for 48h. After de
77 disinfestation, the seed lot profile was determined according to the Rules for Seed Testing
78 (BRASIL, 2009). The germination rate of the studied bean seeds was 91%. In the seed health
79 test were detected *Aspergillus* sp. section *Nigri* (5%), *Penicillium* sp. (3%) and *Cladosporium*
80 sp. (0.5%).

81 Seed contamination was performed, according to Coutinho et al., (2011), using a powder
82 formulation containing, separately, conidia of isolates of *A. ochraceus* (CCDCA 1034) and *A.*
83 *parasiticus* (CCDCA 1059) at the concentration of $1x10^6$ spores g^{-1} kaolin. The contamination
84 was performed at the ratio of 200g of the formulation per 100kg of bean seeds. Contaminated
85 and uncontaminated seeds were packaged in trifoliate paper bags and submitted to two storage
86 conditions: natural storage, with uncontrolled conditions of temperature and relative humidity;
87 and cold room temperature of 10°C and 50% relative humidity (RH). The seeds were stored for
88 180 days and their percentage of germination, electrical conductivity and water activity were
89 evaluated at intervals of 45 days. Healthy and contaminated seeds not submitted to storage were

90 also evaluated, which were used as controls. Therefore, a total of five evaluations were carried
91 out: 0, 45, 90, 135 and 180 days of storage.

92 The germination test was performed by using the paper roll method based in the Rules
93 for Seed Testing – Brazilian Ministry of Agriculture, Livestock and Supply (BRASIL, 2009). The
94 sterilized paper was moistened with autoclaved distilled water in a volume equivalent to 2,5x
95 the mass of the paper and incubated at 25ºC. For each evaluation were used 200 seeds
96 distributed in eight replicates of 25. Normal seedlings were counted five and nine days after
97 sowing. The results were expressed as percentages of normal seedlings.

98 In the electrical conductivity test, which indicates seed vigor, four replicates of 50 seeds
99 were used. The seeds were weighed on a precision scale and transferred to a plastic container
100 containing 75ml of deionized water for imbibition. The seeds were incubated for 24h at 25°C
101 in the dark. After the incubation, the electrical conductivity of the soaking solution was read
102 using the MS TECNOPON® conductivity meter. The data obtained were analyzed as described
103 by Krzyzanowski et al. (1999).

104 For the water activity evaluation, the seeds were removed from storage and sent
105 immediately to perform the test. The readings were performed directly using the Aqualab®
106 CX2 (Decagon Devices Inc.) apparatus by determining the dew point. Samples were placed in
107 plastic containers and the readings were performed at 25°C ± 0,3°C using three replicates.

108 The seed health was assessed at each evaluation time, in order to confirm the presence
109 of the studied fungi during the whole storage period. The seed health test was performed
110 according to Brasil (2009) by using the method of incubation on paper disc (blotter test).

111 The conditions of temperature and relative humidity during the period of natural storage
112 were collected by the station of the National Institute of Meteorology (INMET) located on the
113 campus of the Federal University of Lavras.

114

115 Statistical analysis were carried out by using Sisvar® software version 5.3 (Ferreira,
116 2011) employing a completely randomized design. The experiment was conducted in a 2 x 3 x
117 5 factorial scheme, with two storage conditions: natural and controlled; three treatments:
118 healthy seeds, *A. ochraceus* seeds and *A. parasiticus* seeds; five times: 0, 45, 90, 135 and 180
119 days). The data were submited to Shapiro-Wilk test and then the analysis of variance was
120 performed by the F-test and regression ($p \leq 0.05$) with the adjustment to the linear and quadratic
121 models. The model presenting the highest R^2, significant equation parameters and non-
122 significant regression deviation was chosen to represent the data.

123 **3. Results**

124 The results observed by the tests of germination and electrical conductivity
125 demonstrated the process of natural deterioration of the seeds during storage. The reduction of
126 vigor and germination occurred more intensely in seeds subjected to natural storage than in
127 those stored in a controlled environment. This effect was observed in both healthy seeds and
128 those contaminated with the *Aspergillus* isolates under study. The seed health test confirmed
129 the presence of the studied isolates in 100% of the contaminated seeds at all evaluation periods.

130 The effect of the studied fungi was also observed in both storage conditions, causing
131 greater loss of germination potential, especially after prolonged periods of storage. Under
132 controlled conditions (10°C, 50% RH) a reduction of 37% of the germination was observed in
133 healthy seeds at 180 days of storage. In the contaminated seeds, the observed reduction was 52
134 and 39% for *A. ochraceus* and *A. parasiticus*, respectively (Figure 1A and B). Another
135 important result concerns the behavior of the fungi used in this study: under controlled
136 conditions, the reduction of germination caused by *A. ochraceus* was remarkable at 90 days of
137 storage (dropping from 76 to 53%), whereas in contaminated seeds with *A. parasiticus*, this
138 phenomenon occurred at 135 days, dropping from 73 to 45%. Under uncontrolled conditions,
139 the percentage of germination was even more affected by the association with the studied fungi,

140 being more pronounced at the end of the storage period, at 180 days (Figure 1C and D). At this
141 time, the seeds contaminated with *A. ochraceus* presented a germination percentage 47% lower
142 than the uncontaminated control, whereas germination percentage of the seeds contaminated
143 with *A. parasiticus* was 36% lower.

144 The results revealed by the electrical conductivity test did not indicate differences in
145 seed vigor due to the association with *A. parasiticus* and *A. ochraceus*. However, the electrical
146 conductivity increased over the storage period, this effect was more intense in the seeds
147 submitted to natural storage (Figure 2).

148 The water activity displayed variations in the range of 0.585 to 0.754 in seeds submitted
149 to controlled storage, and from 0.585 to 0.749 in the seeds submitted to natural storage. Under
150 uncontrolled conditions, seed water activity reached its peak at 90 days of storage, followed by
151 reduction at 135 and 180 days (Figure 3A and B). Differently, in the seeds stored under
152 controlled conditions, water activity increased over time reaching higher values than those
153 observed in natural storage at 135 and 180 days (Figure 3C and D). No differences were
154 observed in the water activity of *A. ochraceus* and *A. parasiticus* contaminated seeds compared
155 to the healthy ones.

156 **4. Discussion**

157 The observed differences in the physiological quality of bean seeds submitted to natural
158 and controlled storage conditions demonstrate the efficiency of the last one in preventing seed
159 deterioration. The control of temperature and humidity conditions aims to reduce the speed of
160 biochemical reactions that lead to seed deterioration, such as increased respiration and activity
161 of microorganisms. Several authors have reported the effectiveness of temperature and
162 humidity reduction in preserving the physiological quality of different bean cultivars (Francisco
163 and Usberti, 2008; Hendges et al., 2017; Zucareli et al., 2015), however only a few address to
164 the effect of sanitary quality during storage.

165 Despite the proven superiority of the controlled storage in relation to the natural storage,
166 it was observed that after 180 days of storage, the seeds contaminated with *A. ochraceus* and
167 *A. parasiticus* presented germination percentages very close to that observed in healthy seeds
168 under natural storage. These results demonstrate the great importance of seed health since the
169 intensity of fungal damage can mitigate the benefits of the controlled environment.

170 According to Bragantini (2005), the first effects of storage on the physiological quality
171 of bean seeds are noted by the loss of vigor. The electrical conductivity test is one of the most
172 used methodologies in the evaluation of seed vigor. The test itself measures the amount of
173 leachate from the seed soak solution, whose value is directly related to the integrity of the cell
174 membranes (Krzyzanowski et al., 1999). In the present work, the results obtained by the
175 electrical conductivity test indicated that the association with *A. ochraceus* and *A. parasiticus*
176 did not affect seed vigor, whereas germination was strongly reduced by these microorganisms.
177 On the other hand, the electrical conductivity test demonstrated the loss of vigor over the storage
178 time, distinguishing between the two storage conditions studied. This test showed to be unable
179 to detect slight differences on seed vigor, like those caused by fungal activity; while it was
180 capable to detect only the stronger effects, like the ones caused by the storage time and
181 environmental conditions. Similar results were found by Soares et al. (2010) studying vigor
182 tests in sorghum seeds. These authors reported that the electrical conductivity was not efficient
183 in classifying seed lots in levels of vigor, just identifying lots with low vigor.

184 The values of water activity did not present significant differences between healthy and
185 contaminated seeds. Despite the increase observed over the storage time, the values did not
186 exceed 0.754. According to Beuchat (1983), the minimum values of water activity for the
187 growth of *A. ochraceus* and *A. parasiticus* are 0.77 and 0.82 respectively, what suggests that
188 the values observed during the storage period were insufficient for their growth and

189 proliferation. In this case, it might be that the effects caused by these fungi under uncontrolled
190 storage conditions can be even stronger if the water activity reaches favorable values.

191 Water activity is defined as the ratio of equilibrium vapor pressure in the seed to the
192 vapor pressure of pure water at the same temperature; being, therefore, affected mainly by the
193 temperature and relative air humidity (Troller and Christian, 1978). Hence, the observed
194 variation in the water activity of seeds stored under uncontrolled conditions was probably due
195 to the variations of temperature and humidity occurred during the storage period. In addition,
196 the peak of water activity observed in bean seeds occurred during the same period (45 to 90
197 days) as the highest mean relative air humidity recorded (Figure 4B). Differently, the increase
198 in water activity of the seeds under controlled storage was not expected, since the conditions of
199 temperature and relative humidity are constant.

200 Even though the results the vigor tests did not indicate the exclusive effect of the
201 association of *A. ochraceus* and *A. parasiticus* with the bean seeds, it was demonstrated that the
202 prolonged storage of seeds reduces their physiological quality. The grains of the Dama cultivar
203 are characterized by the potential of prolonged storage without the darkening of the tegument.
204 However, the present work demonstrated that even if the technological characteristics of the
205 grains are maintained, they should not be used as seeds, given the reduction of vigor and
206 germination potential after storage. These results reinforce the need for the use of certified seeds
207 by bean producers in Brazil, which guarantees a material with high physiological and sanitary
208 quality. In addition, it should be noted that differences in the behavior of the studied isolates
209 demonstrate the importance of the correct species identification, determining the ones with a
210 higher incidence during storage, as well as the study of their biological relationships with seeds
211 of other crops.

5. FIGURES

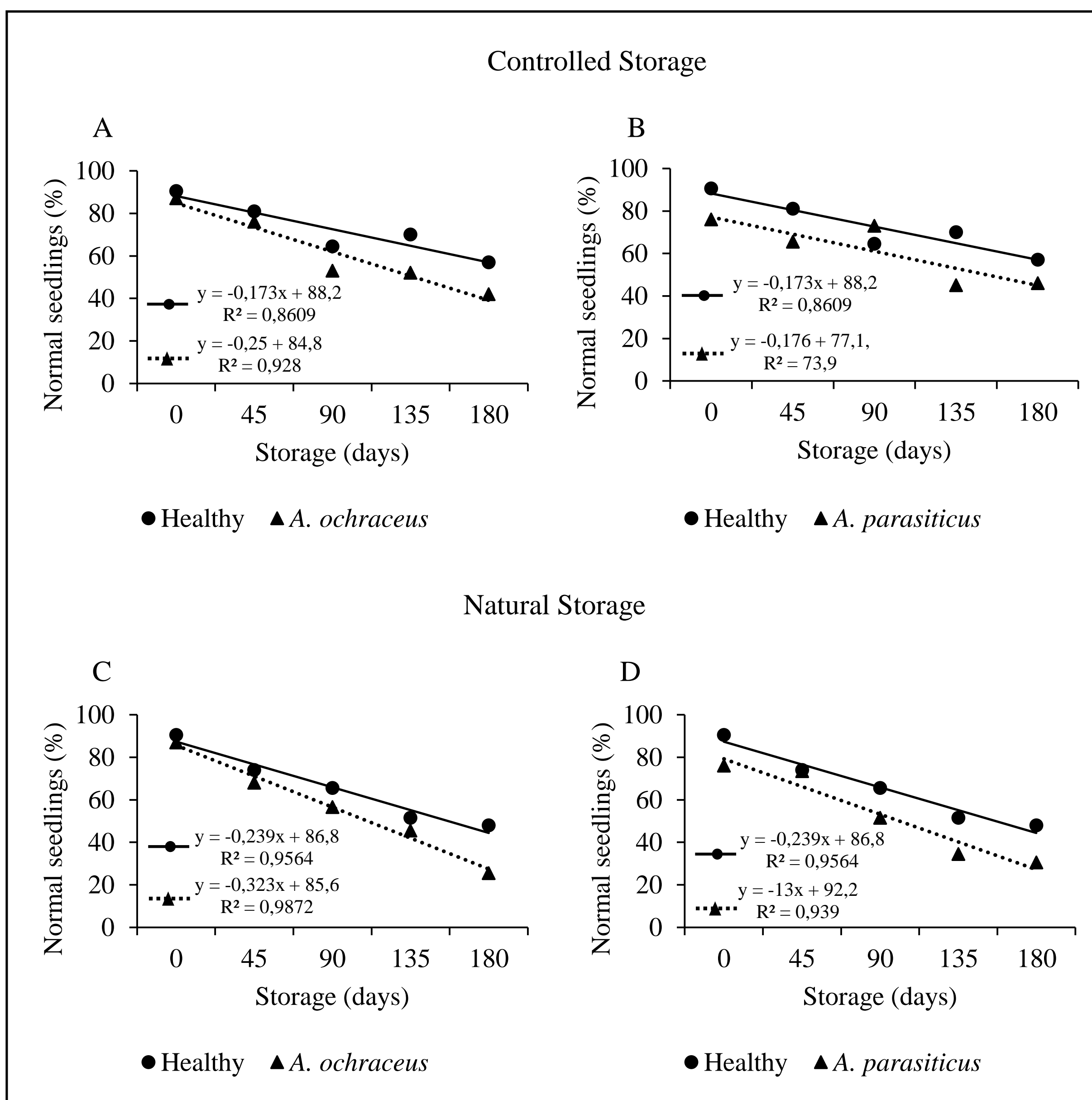

Figure1- Percentage of normal seedlings over the storage time.

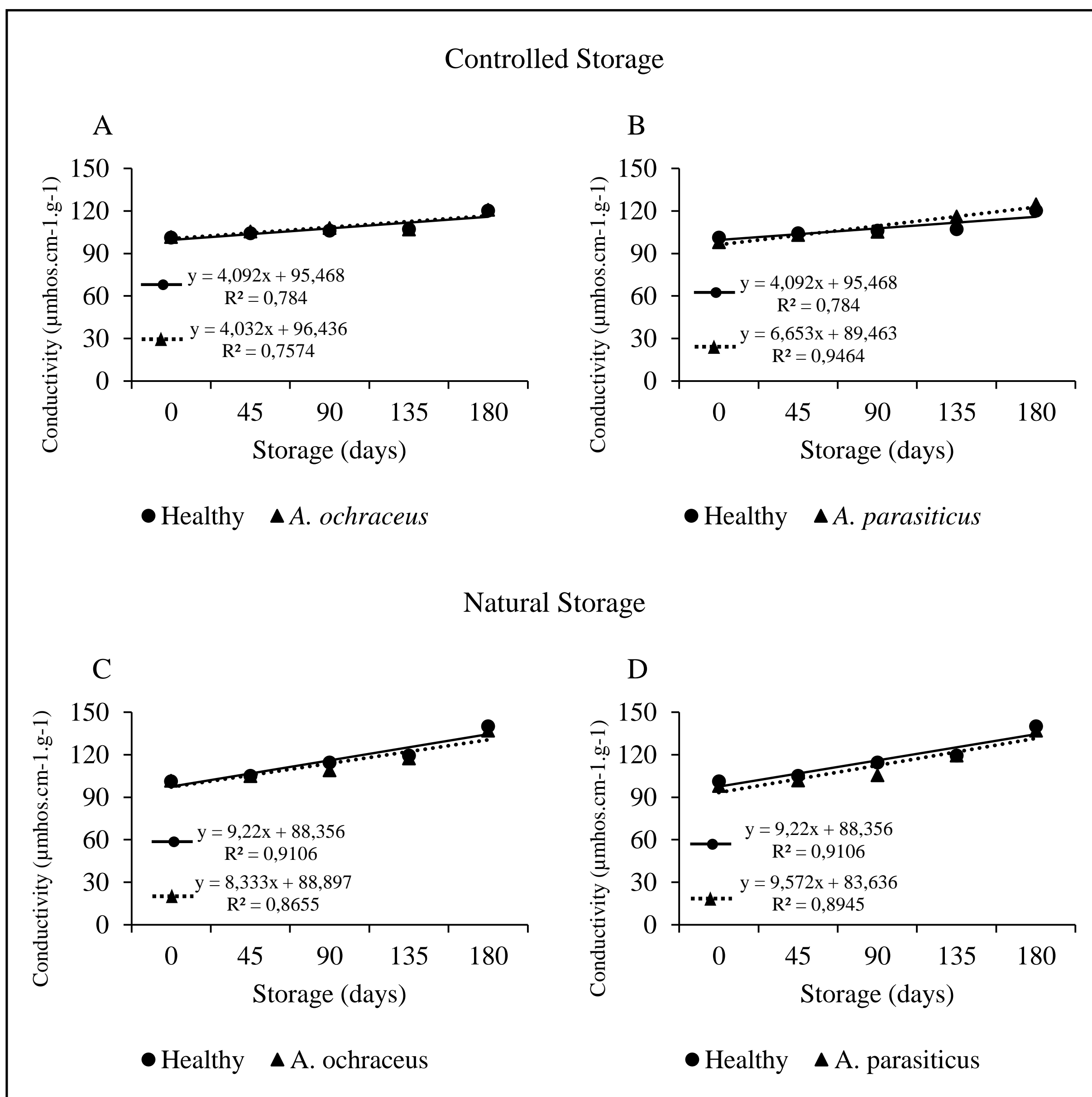

Figure 2 – Electrical conductivity over the storage time.

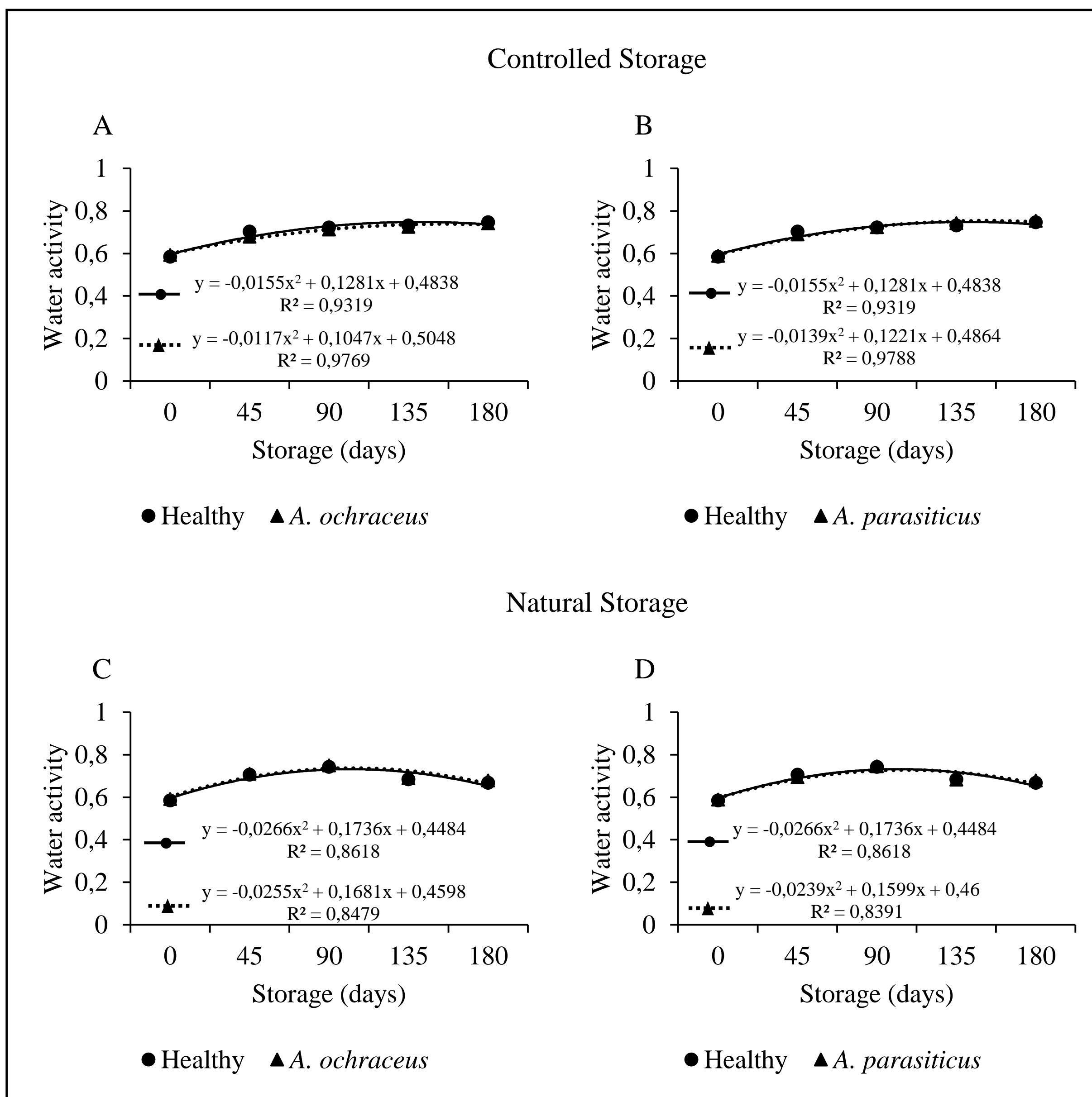

Figure 3- Water activity over the storage time.

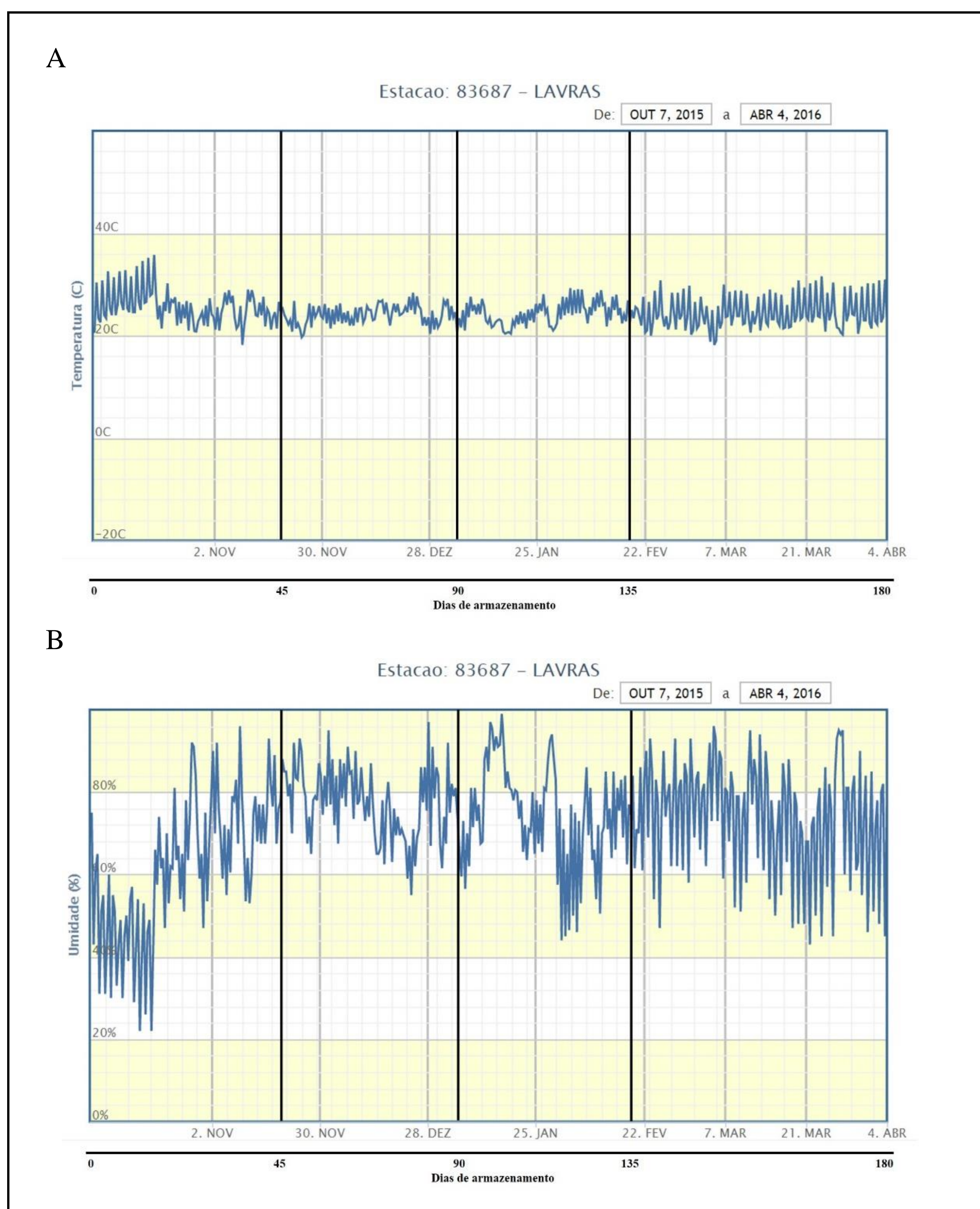

Figure 4 – Meteorological data during the natural storage period. Source: INMET. (A) Temperature; (B) Relative humidity.

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ARTIGO 2

(Formatação baseada nas exigências do *Journal of Seed Sciences*)

EFFECTS OF *Aspergillus ochraceus* AND *Aspergillus parasiticus* ON QUALITY OF DRY BEAN SEEDS UNDER STRESS CONDITIONS.

EFEITOS DE *Aspergillus ochraceus* E *Aspergillus parasiticus* NA QUALIDADE DE SEMENTES DE FEIJÃO SOB CONDIÇÕES DE ESTRESSE

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1 **Effects of *Aspergillus ochraceus* and *Aspergillus parasiticus* on quality of dry**
2 **bean seeds under stress conditions.**

3 Bárbara Alves dos Santos-Ciscon[1], José da Cruz Machado[1*], Iara Eleutério Dias[1], Poliana
4 Patrícia Lima[1], Luís Roberto Batista[2]

5 ABSTRACT – *Aspergillus* is one of the fungal genera most commonly associated with seeds
6 and grains in storage. The objective of this work was to evaluate the effects of toxigenic isolates
7 of *Aspergillus ochraceus* and *Aspergillus parasiticus* in the physiological quality of bean seeds
8 under stress conditions induced by accelerated aging and water conditioning. Healthy and
9 contaminated seeds with *A. ochraceus* and *A. parasiticus* were subjected to accelerated aging
10 (42ºC) and water conditioning induced by mannitol during 24, 48, 72 and 96 hours.
11 Contaminated and healthy seeds not submitted to the referred stress conditions were used as
12 controls (0h). Then, germination percentage, emergence speed index (ESI), initial and final
13 stand and fresh and dry weight of shoot and root, were evaluated. In all tests, accelerated aging
14 caused more severe damage to seed quality than observed in water conditioning. In the seeds
15 under accelerated aging, *A. ochraceus* affected only the germination percentage, while *A.*
16 *parasiticus* reduced germination, ESI, initial and final stands and fresh and dry weight of shoot.
17 In the seeds under water conditioning, *A. ochraceus* affected the germination and caused a slight
18 reduction in the initial and final stands, while *A. parasiticus* affected more strongly the
19 germination percentage, ESI, final and initial stands and the fresh weight of shoot especially
20 after 72 hours and 96 hours. The fresh and dry weight of root were not influenced by the
21 association with the isolates tested.

22 Keywords: Accelerated aging; Water restriction; *Phaseolus vulgaris*; Germination; Vigor

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23 # **Efeitos de *Aspergillus ochraceus* e *Aspergillus parasiticus* na qualidade de**
24 # **sementes de feijão sob condições de estresse.**

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27 RESUMO – *Aspergillus* são fungos comumente associados a sementes e grãos no
28 armazenamento. Objetivou-se com este trabalho avaliar os efeitos de isolados toxigênicos de
29 *A. ochraceus* e *A. parasiticus* na qualidade de sementes de feijão sob condições estresse causado
30 pelo envelhecimento acelerado e condicionamento hídrico. Sementes sadias e contaminadas
31 com os fungos estudados foram submetidas a envelhecimento acelerado (42ºC) e
32 condicionamento hídrico induzido por manitol durante 24, 48, 72 e 96 h. Sementes não
33 submetidas às referidas condições foram utilizadas como controle (0h). Em seguida, foram
34 avaliados: germinação, índice de velocidade de emergência (IVE), estande inicial e final e peso
35 de parte aérea e raiz frescas e secas. De modo geral, o envelhecimento acelerado causou maiores
36 danos à qualidade das sementes que o condicionamento hídrico. Nas sementes sob
37 envelhecimento acelerado, *A. ochraceus* afetou apenas a germinação, enquanto *A. parasiticus*
38 reduziu a germinação, o IVE, o estande inicial e final e o peso de parte aérea fresca e seca. Nas
39 sementes sob condicionamento hídrico, *A. ochraceus* afetou a germinação e reduziu sutilmente
40 o estande inicial e final, enquanto que *A. parasiticus* afetou intensamente a germinação, o IVE,
41 o estande final e inicial e o peso de parte aérea fresca e seca. O peso da raiz fresca e seca não
42 foi influenciado pelos isolados das espécies testadas.

43 Termos para indexação: Envelhecimento acelerado; Restrição hídrica; Germinação; Vigor.

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44 # 1. Introduction

45 Brazil is one of the world leaders in the production of beans (*Phaseolus vulgaris*),
46 besides being one of the main consuming markets of this grain (CONAB, 2017). Several studies
47 have demonstrated the high incidence of fungi belonging to the genus *Aspergillus* associated
48 with bean seeds and grains (Araújo et al., 1980; Benício et al., 2003; Costa and Scussel, 2002;
49 Francisco and Usberti, 2008; Silva et al., 2008), what represents a phytosanitary issue as well
50 as food safety, since these fungi are important mycotoxin producers.

51 The association between *Aspergillus* species and seeds occurs especially during the
52 storage stage, although it is a consensus that the inoculum of these fungi comes mainly from
53 the field. This type of interaction is little studied regarding the common bean. In addition, little
54 is known about the effect of these fungi on seed vigor, as well as on possible differences in the
55 levels of damage caused by different species of this genus. Storage is a crucial step in seed
56 production and it is subject to the interference of several factors. The presence of
57 microorganisms, for example, can accelerate the deterioration of the seeds and, ultimately,
58 compromise seed quality. The association with *Aspergillus* and *Penicillium* is one of the factors
59 responsible for large losses of seed quality, due to cell modifications, biochemical changes or
60 toxin production, what can result in loss of vigor and germination capacity or even interfere
61 with seed quality testing (Borém et al., 2006; Machado, 1988;).

62 Thus, many vigor tests are based on the deterioration rate of seeds exposed to stress
63 conditions in order to estimate their storage potential as well as the seedlings vigor in the field.
64 In addition, they may be an effective tool in the study of the biological relationships between
65 fungi and seeds or grains. The accelerated aging test is one of the most used tools for evaluating
66 seed vigor. Developed by Delouche and Baskin (1973), accelerated aging is based on seed
67 exposure to high temperature and relative humidity conditions (Marcos Filho, 1999).
68 Differently, the stress caused by water conditioning or water restriction is due to the reduced

69 availability of water to the seed, as well as to the associated microorganisms, by the addition of
70 a restrictor (e.g., mannitol) to the substrate. Therefore, water stress can also be used to estimate
71 seed storage potential, since the storage environment also offers low availability of water to the
72 seed and its associated microflora.

73 Hence, the objective of this work was to evaluate the effect of toxigenic isolates of *A.*
74 *ochraceus* and *A. parasiticus* on the quality of bean seeds subjected to stress conditions due to
75 accelerated aging and substrate water restriction.

76

77 2. Material and Methods

78 Bean seeds of the TAA Dama cultivar were previously disinfected with 1% sodium
79 hypochlorite (NaClO) for 30 seconds, washed with distilled water and dried on filter paper for
80 48h. After de disinfestation, the seed lot profile was determined according to the Rules for Seed
81 Testing (BRASIL, 2009). The germination rate of the studied bean seeds was 91%. In the seed
82 health test were detected *Aspergillus* sp. section *Nigri* (5%), *Penicillium* sp. (3%) and
83 *Cladosporium* sp. (0.5%).

84 The seeds were contaminated with a powder formulation containing, separately, conidia
85 of *A. parasiticus* (CCDCA 1059) and *A. ochraceus* (CCDCA 1034), according to the
86 methodology described by Coutinho et al. (2011).

87 Accelerated aging was performed according to the methodology described by Marcos
88 Filho (1999), by placing a single layer of bean seeds on the surface of a metallic screen
89 suspended inside plastic boxes with lid (Gerbox; 11 x 11 x 3.5 cm) containing 40ml of distilled
90 water. The boxes containing healthy and contaminated bean seeds were transferred to an
91 incubator at 42°C, where they remained for 24, 48, 72 and 96 hours. Contaminated and
92 uncontaminated seeds not subjected to accelerated aging (0 hours) were used as controls. In
93 water conditioning, healthy and contaminated seeds were placed in 15 cm diameter Petri dishes

94 containing PDA medium plus mannitol (Machado et al., 2012), with water potential adjusted
95 to -3MPa calculated by the SPPM Software (Michel and Radcliffe, 1995), based on previous
96 works of our research group. Plates were incubated at 25°C for 24, 48, 72 and 96 hours.
97 Contaminated and uncontaminated seeds not subjected to water conditioning were used as
98 controls. After each stress period, the seeds were disinfected with 1% sodium hypochlorite for
99 30 seconds, followed by washing with distilled water. The seeds were forwarded to the
100 germination test immediately after washing. For the emergence test, the seeds were previously
101 dried on filter paper during 48 hours and then, sent to planting.

102 The germination test was performed on a sterile paper roll, moistened with autoclaved
103 distilled water in a volume equivalent to 2.5x the paper mass, and then incubated at 25°C, as
104 described in the Rules for Seed Testing (BRASIL, 2009). In this test were used 200 seeds
105 distributed in eight replicates of 25. The counting was performed on the fifth and ninth days
106 after sowing. The results were expressed as percentages of normal seedlings.

107 The emergence test was carried out in a plant growth chamber at 25±2°C, with a
108 photoperiod of 12h (daylight NSK T10 40W 6500K FL40T10-6 60Hz) / 12h dark. One hundred
109 seeds were individually sown in 200 ml plastic vessels containing commercial substrate
110 Topstrato Hortaliças® and sand (2: 1), which were irrigated daily. The vessels were arranged
111 in four trays (25x4), each tray corresponding to one replicate. The number of emerged seedlings
112 was accessed daily, from the first emerged seedling until the stand stabilization. The seedlings
113 were kept in a growth chamber up to 30 days after sowing (d.a.s). The Emergency Speed Index
114 (ESI) was calculated according to Maguire (1962).

115 The initial and final stands were recorded at 10 and 30 days after sowing, respectively.
116 The results were expressed as percentage of plants. In order to evaluate the fresh and dry weight
117 of shoot, plants emerged until 30 days were cut in the neck region, weighed and submitted to
118 oven drying a 70 °C for five days, when they were weighed again. The same procedure was

119 carried out to evaluate the fresh and dry weight of roots, which were extracted from the
120 substrate, removing to the maximum the residues adhered to it. The roots were weighed after
121 extraction, oven dried and reweighed as performed for the shoot.

122 The seed health was assessed at each evaluation time, in order to confirm the presence
123 of the studied fungi during the whole storage period. The seed health test was performed
124 according to Brasil (2009) by using the method of incubation on paper disc (blotter test).

125 Statistical analysis were carried out by using Sisvar® software version 5.3 (Ferreira,
126 2011) employing a completely randomized design. The experiment was performed in a 2x3x5
127 factorial scheme, with two stress conditions (accelerated aging and water restriction), three
128 treatments (healthy seeds, *A. ochraceus* contaminated seeds and *A. parasiticus* contaminated
129 seeds) and five times (0, 24, 48, 72 and 96 hours). The data were submited to Shapiro-Wilk test
130 and then the analysis of variance was performed by the F-test and regression ($p \leq 0.05$) with the
131 adjustment to the linear and quadratic models. The model presenting the highest R^2, significant
132 equation parameters and non-significant regression deviation was chosen to represent the data.
133

134 **3. Results**

135 In general, the effects observed by *A. ochraceus* and *A. parasiticus* were more subtle in
136 seeds submitted to water restriction than in those submitted to accelerated aging. Therefore, the
137 water restriction methodology revealed the differences in the effects caused by each of the
138 studied species. The germination percentage was strongly affected by the stress caused by
139 accelerated aging. After 96h, healthy seed presented a reduction of 83% in the normal seedlings,
140 whereas the seeds contaminated with *A. ochraceus* e *A. parasiticus*, presented reduction of 91
141 and 97%, respectively (Figure 1 C and D). The intense decrease observed in the contaminated
142 seeds demonstrates the effect of these fungi reducing seed germination. Regardless the fungal
143 presence, the stress caused by high temperature and relative humidity strongly affected seed

144 germination, notably after 72h-exposure. Using the water restriction methodology, the
145 differences on the germination of healthy and contaminated seeds were clearly observed. In
146 uncontaminated seeds, the percentage of normal seedlings dropped from 86 (0h) to 75% (96h);
147 while in the contaminated seeds, germination fell from 78 (0h) to 58% (96h) for *A. ochraceus*
148 and from 75.5 (0h) to 19.5% (96h) for *A. parasiticus* (Figure 1A and B). Up to 48h of water
149 restriction, the percentage of normal seedlings was similar in healthy seeds and contaminated
150 seeds containing *A. ochraceus*. The effect caused by this fungus became noticed from 72h of
151 water restriction, when the contaminated seeds presented germination percentage 46% (72h)
152 and 55% (96h) lower than the healthy ones at the same times. Differently, the reduction in the
153 germination percentage due to the association with *A. parasiticus* was observed since the initial
154 periods and enhanced from 72h.

155 The emergence speed index (ESI) also revealed different behaviors according to the
156 stress conditions employed. Similar to that observed in the germination test, the accelerated
157 aging caused a more intense reduction in ESI, compared to water restriction. Contaminated and
158 uncontaminated seeds submitted to accelerated aging presented similar behavior, showing
159 marked reduction in ESI at 72 and 96 hours (Figure 2 C and D). No significant differences were
160 observed between ESI of healthy and *A. ochraceus* contaminated seeds. In contrast, ESI was
161 significantly lower in seeds contaminated with *A. parasiticus* under 96h of accelerated aging,
162 showing a reduction of 64% against healthy seeds in the same period. Regarding the seeds
163 submitted to the water restriction, the ESI of healthy seeds was reduced in approximately 9%
164 throughout the incubation time (0-96h). In seeds contaminated with *A. ochraceus* and *A.*
165 *parasiticus*, this reduction was around 15 and 38% respectively (Figure 2 A and B). The
166 association of seeds with *A. ochraceus* caused a slight and progressive reduction on ESI, quite
167 similar to the observed in healthy seeds. On the other hand, the association with *A. parasiticus*
168 caused a marked reduction in the ESI, especially in the last periods, dropping from 3.0 in 72

169 hours of water stress to 2.1 in 96 hours. Such results mean a reduction of 6 and 38% in the ESI
170 of seeds contaminated with *A. ochraceus* and *A. parasiticus* respectively, compared to healthy
171 seeds at the same period.

172 The initial and final stands obtained from healthy and contaminated seeds were not
173 affected by the accelerated aging up to 48h (Figure 3C and D). At 72 and 96h was possible to
174 observe a reduction in the percentage of seedlings in the initial stand. However, only the seeds
175 associated with *A. parasiticus* differed from the control, presenting an initial stand 36% lower
176 than that observed from healthy seeds. The initial stand of healthy seeds under water restriction
177 showed a slight reduction, approximately 10% over the incubation time (0-96h). Similar
178 behavior was observed in *A. ochraceus* contaminated seeds submitted to water stress. The initial
179 stand obtained from these seeds decreased in 9% (0-96h), presenting lower means than the ones
180 observed in healthy seeds over the incubation period (Figure 3A). Differently, the initial stand
181 obtained from seeds contaminated with *A. parasiticus* presented remarkable decrease at 72 and
182 96h, with a reduction of 12 and 38%, respectively, compared to healthy seeds (Figure 3B). The
183 final stand was also more affected by the accelerated aging than the water restriction. The final
184 stand of *A. ochraceus* contaminated seeds was similar to that observed in healthy seeds in all
185 evaluated times (Figure 4C). In contrast, the final stand of *A. parasiticus* contaminated seeds
186 displayed a progressive reduction after 72h under accelerated aging, with averages 21 (72h) and
187 58% (96h) lower than that observed in uncontaminated seeds (Figure 4D). Regarding the seeds
188 submitted to water restriction, a slight decrease in the final stand was observed due to the
189 association with *A. ochraceus* in all times tested; while the association with *A. parasiticus*
190 caused a decrease of 28% in the final stand only at 96h (Figure 4 A and B). In addition, on
191 seedlings obtained from contaminated seeds submitted to long period of stress was observed a
192 massive colonization of the cotyledons by the inoculated fungi, causing seedling death or
193 reducing its growth.

194 The fresh and dry weight of shoot was slightly reduced over the exposure time to the
195 used stress conditions. However, in both accelerated aging and water restriction the association
196 of *A. ochraceus* with seeds did not affect this feature (Figure 5 A and C; Figure 6 A and C). In
197 the seeds containing *A. parasiticus* submitted to accelerated aging, the effect of the fungal
198 association was observed in almost all times tested (0, 24, 72 and 96h), showing a sharp
199 reduction of fresh and dry weight of the shoot at 96h (Figure 5D). In the seeds associated with
200 *A. parasiticus* submitted to water restriction, the main effects of the fungi were observed at 72
201 and 96h of incubation. The fresh weight of shoot was 25 (72h) and 48% (96h) lower than that
202 observed in the uncontaminated seeds, whereas this difference was about 34 (72h) and 46%
203 (96h) regarding the dry weight of shoot (Figure 5B). The fresh and dry weight of root was
204 affected neither by the association with the studied fungi nor by the stress conditions employed
205 (data not shown).

206 The seed health test confirmed the presence of the studied isolates in 100% of the
207 contaminated seeds at all evaluation periods.

208 4. Discussion

209 In general, the results suggest that water restriction has proved to be a very useful tool
210 in studies of the relationship between seeds and species of *Aspergillus*. Since the effects of this
211 stress condition were not as drastic as those observed in the seeds subjected to accelerated aging,
212 it was possible to clearly observe the effects caused by the fungi under study.

213 According to Marcos Filho (1999), a good vigor test is the one that distances itself from
214 seed death, that is, its methodology should not cause serious damage to the seed. This
215 characteristic allows discriminating seed lots in a detailed manner, estimating their potential in
216 the field. The conditions of high temperature and relative humidity to which the seeds are
217 exposed during the accelerated aging promote the protein coagulation, which accelerates the
218 process of natural deterioration of the seed. The accelerated aging test developed by Delouche

219 and Baskin (1973) is currently used to evaluate the vigor of seed lots and recommends the
220 incubation of bean seeds at 42°C for 72 hours. In the present work, it was observed that some
221 effects caused by the fungi under study could only be observed at 96h of accelerated aging, for
222 example, the effect of *A. parasiticus* on ESI, final stand and dry shoot weight. It is likely that
223 the use of the conventional aging test would not indicate differences in the vigor of seed lots as
224 a function of association with *A. parasiticus*, estimating that healthy seeds contaminated with
225 this fungus would have similar storage potentials. Differently, in the seeds subjected to water
226 restriction, the damage caused to the physiological quality of the seeds was less drastic,
227 allowing the identification of the effects resulting from the association with the fungus and
228 evidencing the differences in the effects caused by each species.

229 The methodology of water restriction developed by Machado et al. (2001) has the initial
230 objective of obtaining seeds infected by pathogens. However, besides the possibility of
231 controlling inoculum potential, this methodology proved to be very useful in other areas of seed
232 pathology, such as the control of seed germination in health tests, the study of pathogen/seed
233 relationships and in the reproduction of water stress conditions (Machado et al., 2012). In
234 addition, water stress caused by low water availability may better represent the stress conditions
235 to which seeds and fungi are subjected during storage. At this stage, the environment presents
236 reduced relative humidity in order to prevent the activity of microorganisms. However, the so-
237 called storage fungi - including those belonging to the genus *Aspergillus* - are capable of acting
238 in reduced moisture conditions. This characteristic can be the key to explain the reason why the
239 water restriction methodology was efficient on distinguishing the effects caused by *A.*
240 *ochraceus* and *A. parasiticus* on the physiological quality of seeds.

241 A comparison between the results obtained by the germination test and those obtained
242 by the emergency test shows that effects of the fungi, as well as the employed stress conditions,
243 were much more intense in the germination than in the initial stand. Possibly, this discrepancy

244 was due to the different conditions of temperature and humidity of said tests. The prolonged
245 contact of the tegument with the seeds during the germination test provides favors fungal
246 activity reducing seed quality. A similar situation was reported in other pathosystems such as
247 *Phomopsis phaseolorum* in soybean seeds (Henning, 2005) and *Aspergillus flavus* in peanuts
248 (Santos et al., 2016). These observations suggest that the germination test using paper roll is
249 likely to underestimate the germination percentage of seed lots with high incidence of
250 *Aspergillus.* Therefore, it is of great importance to carry out additional studies to demonstrate
251 the need for specific recommendations of using alternative methodologies, such as the
252 germination test in sand, for the analysis of bean seeds with low phytosanitary quality.
253 According to our results, *A. ochraceus* and *A. parasiticus* affected differently the quality of
254 bean seeds under stress conditions. In general, it was observed that *A. parasiticus* caused a
255 greater reduction in seed quality, however, this effect was observed after longer periods (72 and
256 96h) of exposure to stress conditions. Differently, *A. ochraceus* caused subtle effects on seed
257 quality, which could be observed in all periods tested, mainly in seeds subject to water
258 restriction. The reductions observed in the percentage of seedlings of initial and final stands, as
259 well as the reductions in fresh and dry weight of shoot, may be related to the intense
260 colonization of the cotyledons by the fungus observed after the seedling emergence. Due to the
261 epigeal emergence of bean seedlings, much of the fungal inoculum present on the seeds is
262 retained in the soil, associated with seed tegument, which is detached from the cotyledons
263 during emergency. However, in seeds with high inoculum concentration and/or mechanical
264 damage, conidia can colonize the cotyledons, reducing the nutritional sources that supply the
265 seedlings in the early stages of development. Thus, early decay of cotyledons can lead to
266 seedling death or reduced development (Oliveira and Morais, 1999).

267 Although they are not considered pathogens sensu stricto, fungi of the *Aspergillus* genus
268 have demonstrated their ability to reduce the physiological quality of bean seeds, as well as

269 seeds of other crops, such as rice, soybean and peanuts (Begum et al, 2013; Monajjem et al.,
270 2014; Rocha et al., 2014). The reduction of seed physiological quality implies loss of vigor and
271 consequent yield losses at the end of the growing cycle. In addition, these fungi are important
272 producers of mycotoxin, which have demonstrated the ability to affect seed quality and seedling
273 growth in other crops as reviewed by Ismaiel and Papenbrock (2015). Therefore, it is extremely
274 important to monitor the incidence of *Aspergillus* on bean seeds and encourage the use of
275 certified seeds. Besides affecting seed physiological quality, the capacity of these fungi to
276 proliferate in the storage environment allows the inoculum increase. This inoculum is taken to
277 the field in the subsequent crop cycle, contaminating the seeds produced in the following
278 harvest. In addition, the presence of these fungi represents a high risk of intoxication of the
279 population by mycotoxins, through the ingestion of the bean grains contaminated with these
280 toxic metabolites.

5. FIGURES

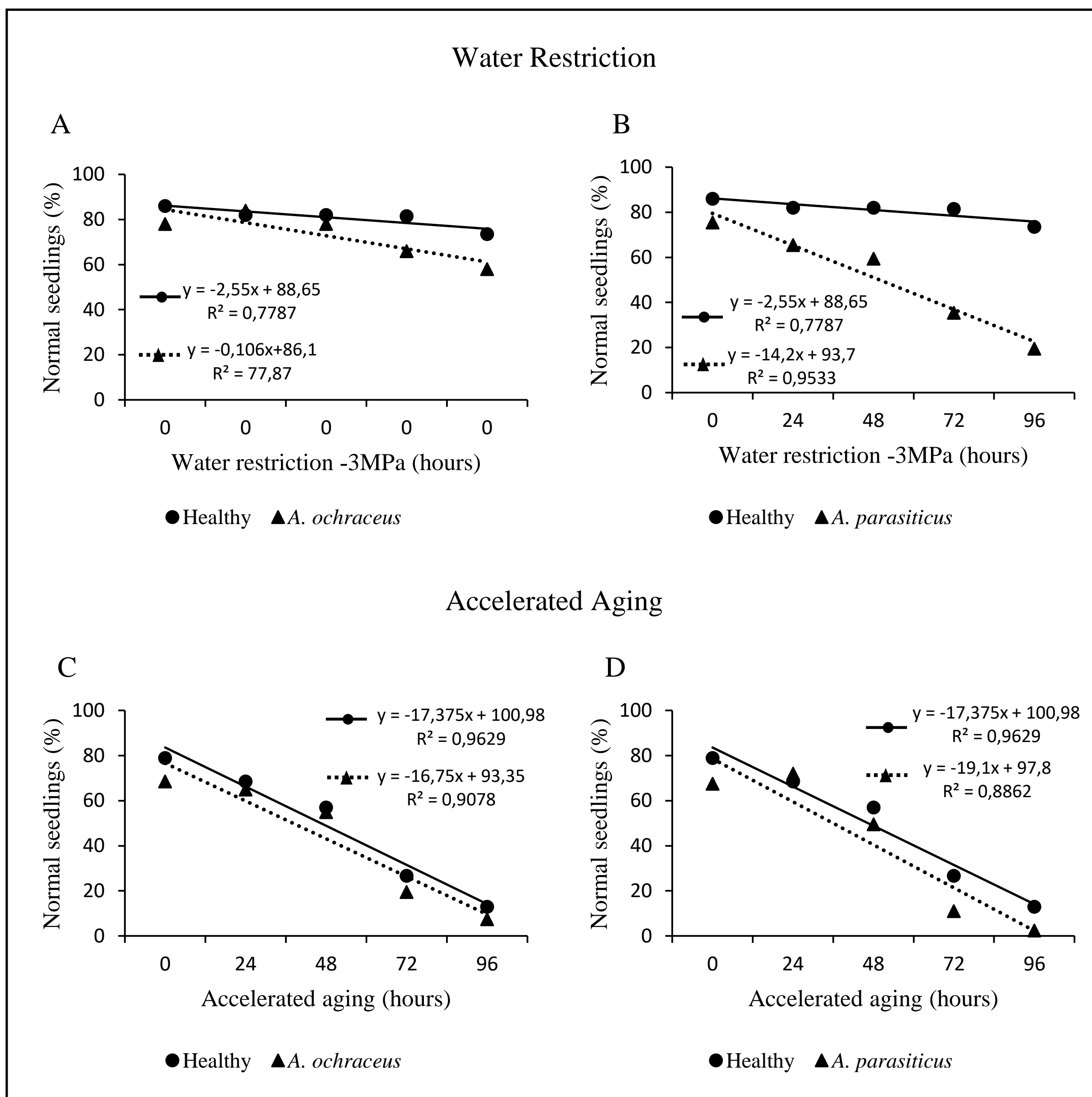

Figure 1- Normal seedlings percentage over time of stress conditions.

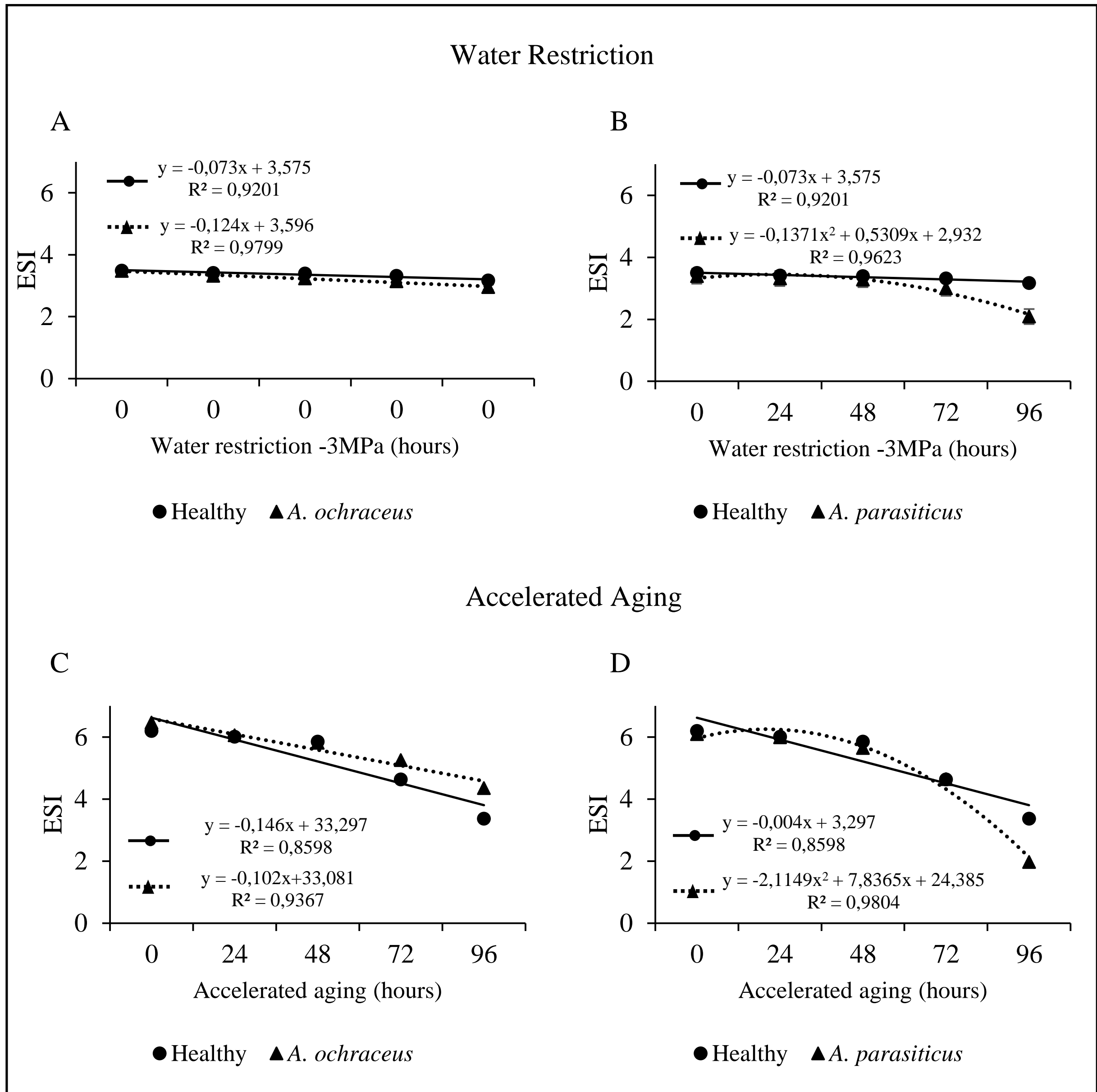

Figure 2- - Emergence speed index (ESI) over time of stress conditions.

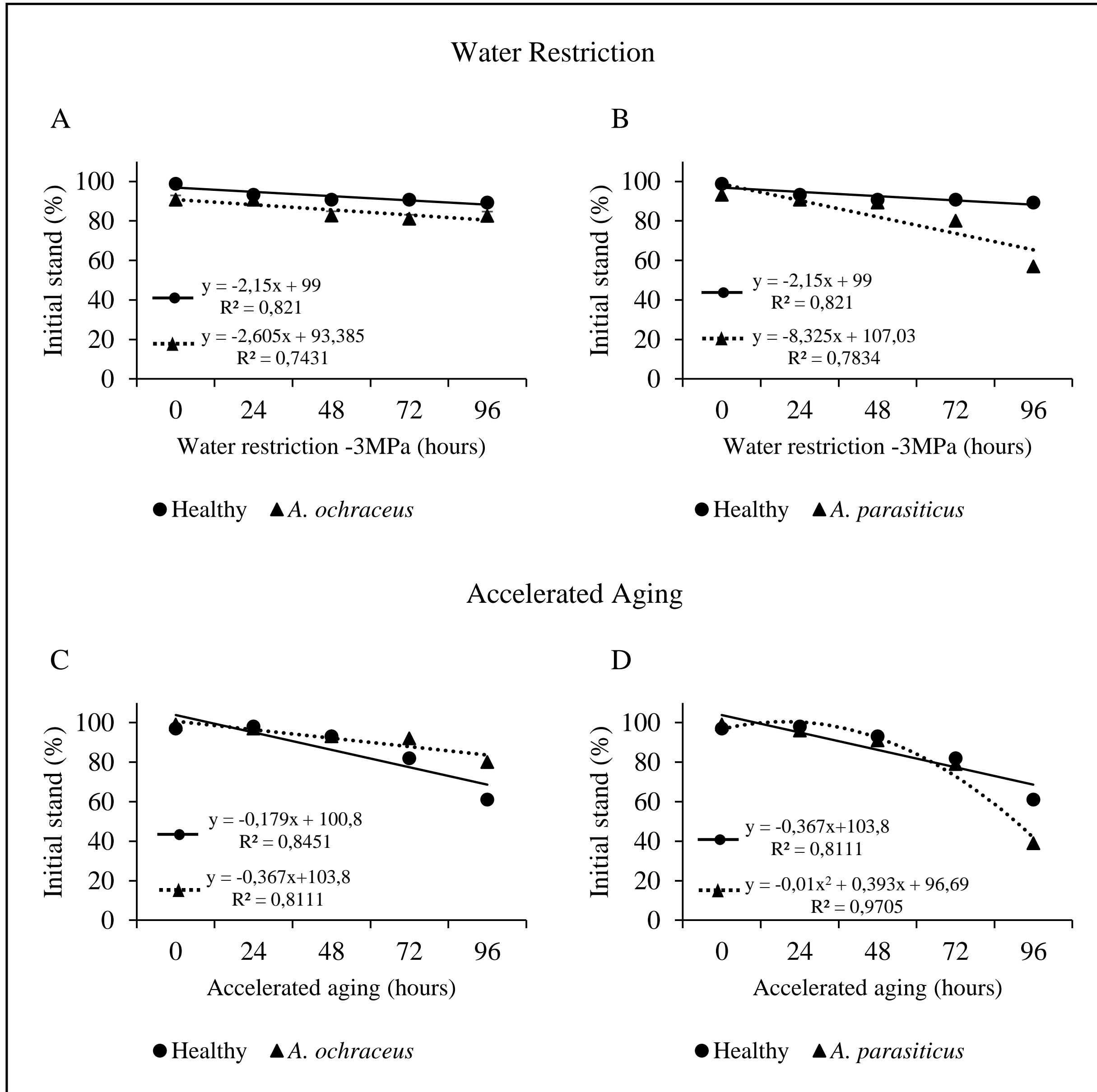

Figure 3 – Initial stand of seedlings over time of stress conditions.

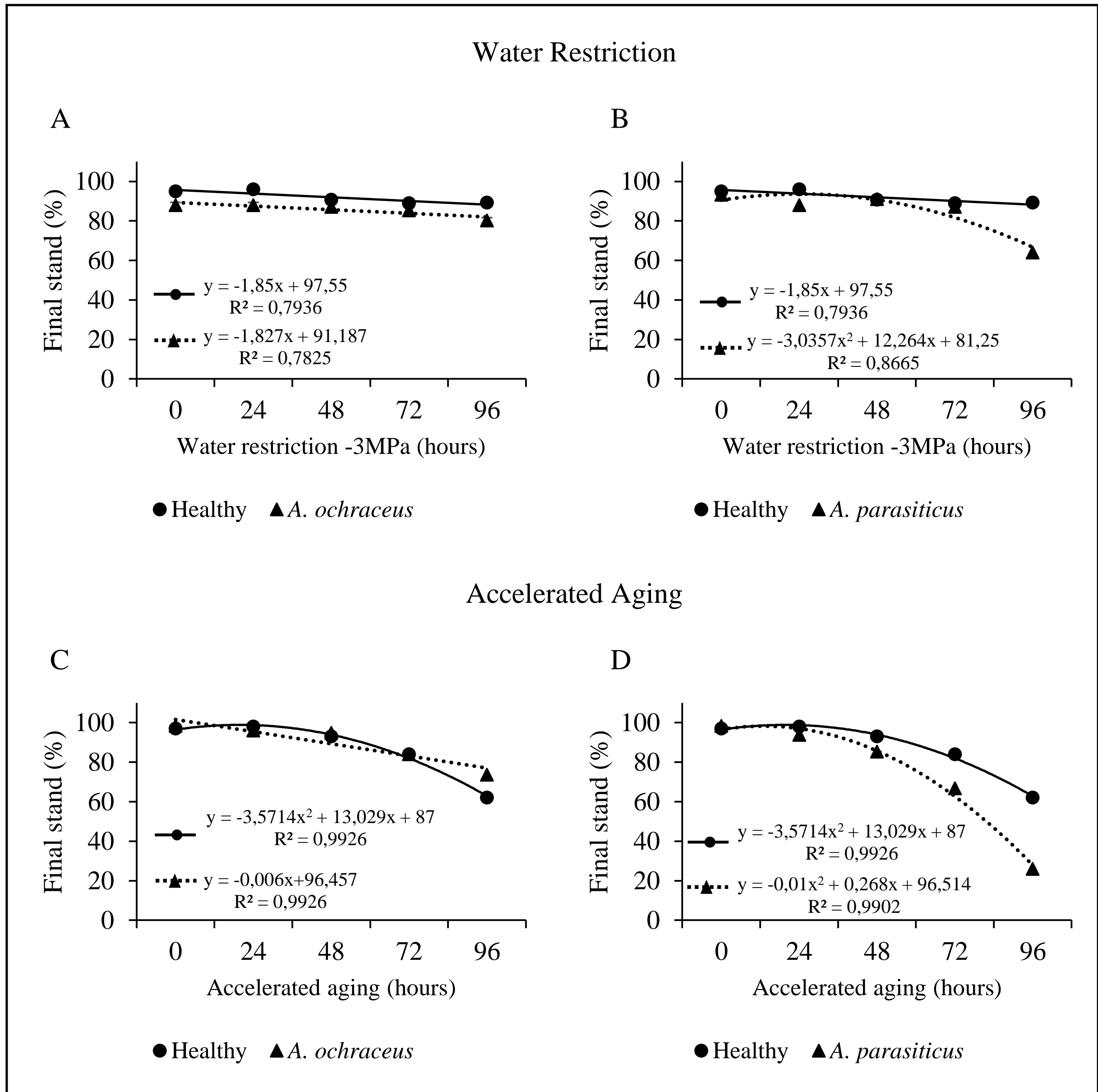

Figure 4- Final stand of seedlings over time of stress conditions.

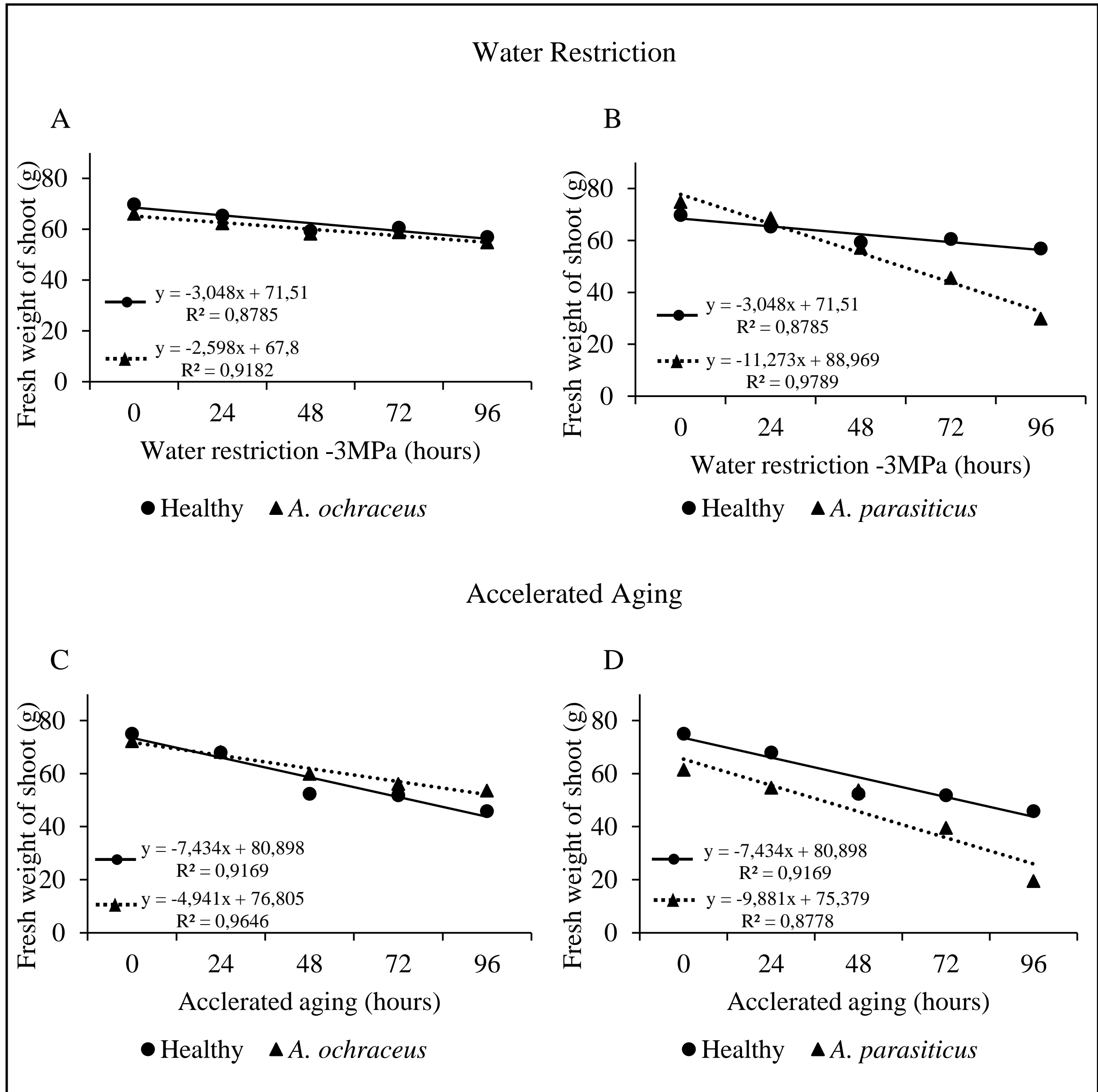

Figure 5 – Fresh weigh of shoot over time of stress conditions.

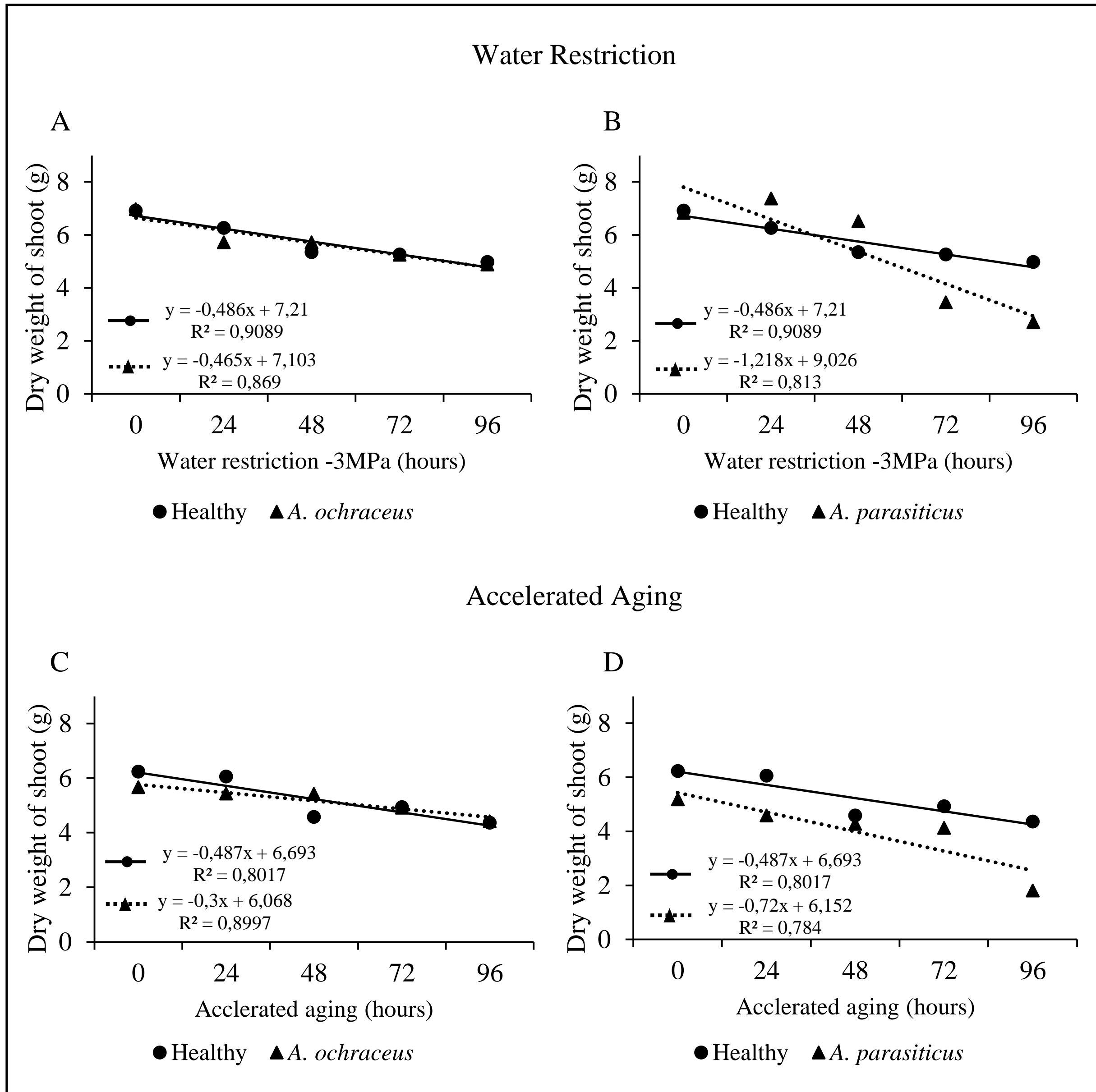

Figure 6 – Dry weigh of shoot over time of stress conditions.

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72

ARTIGO 3

(Formatação baseada nas exigências do *International Journal of Food microbiology*)

Aspergillus SPECIES FROM BRAZILIAN DRY BEANS AND THEIR TOXIGENIC POTENTIAL

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1 *Aspergillus* species from Brazilian dry beans and their toxigenic potential

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9 ABSTRACT – Aspergilli are common contaminants of food and feed and a major source of mycotoxins.
10 In this study, 87 *Aspergillus* strains were isolated from beans from 14 different cities in Brazil and
11 identified to the species level based on partial calmodulin and β-tubulin sequence data. The results
12 revealed that green, black and yellow aspergilli were found in 97, 77 and 29% of the seed lots tested.
13 All green spored isolates belonged to section *Flavi* and were identified as *A. flavus* (n=39) or *A.*
14 *pseudocaelatus* (n=1). All black spored isolates belonged to section *Nigri* and were identified as *A. niger*
15 (n=24) or *A. luchuensis* (n=10). While the yellow spored strains were identified as *A. westerdijkiae*
16 (n=7), *A. ostianus* (n=3) or *A. ochraceus* (n=1), belonging to section *Circumdati,* and *A. wentii* (n=2)
17 belonging to section *Wentii*). The toxigenic potential of these *Aspergillus* strains from beans was studied
18 by the prospection of genes in three of the major mycotoxin clusters: aflatoxin (7 genes checked),
19 ochratoxin A (four genes) and fumonisin (ten genes and two intergenic regions). Genes involved in the
20 biosynthesis of aflatoxin were only detected in *A. flavus* isolates: about half of the *A. flavus* isolates
21 proved to contain all the aflatoxin genes tested, the others missed one or more genes. The full
22 complement of fumonisin biosynthesis genes was identified in all *A. niger* isolates. Finally, no genes for
23 ochratoxin A were detected in any of the isolates. Our work suggests that aflatoxin production by some
24 *A. flavus* strains and fumonisin production by *A. niger* isolates form the largest mycotoxin risks in
25 Brazilian beans.

26 *Keywords: A. flavus*; *A. niger*; *A. luchuensis*; *Aflatoxin*; Fumonisin; Ochratoxin A.
27

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28 **1. Introduction**

29 Dry beans are a traditional staple food of great economic, social and nutrition importance in
30 Brazil. Seven out of ten Brazilians consume beans daily, irrespective of their income level. Brazil is one
31 of the major global producers of dry beans, with a total of 5.9 million tons harvested in 2016 growing
32 seasons (CONAB – National Supply Company, 2017). A variety of beans from different groups and
33 market classes are grown in Brazil: Groups being the botanical species *Phaseolus vulgaris* (I) and *Vigna*
34 *unguiculata* (II), while the class identifies the beans according to their skin colours (black, white or
35 mixed colors). The cream seeded variety *Carioca* belongs to group I and is most widely consumed,
36 accounting for approximately 70% of total beans consumed, followed by black beans (several varieties
37 grouped as *Preto*). *Carioca* and *Preto* beans correspond to around 85% of the Brazilian bean market
38 (Ribeiro et al., 2014). Other types of beans are important regional foods, including the *Fradinho* bean
39 (cowpea), which is popular in Northeastern Brazil, representing approximately 10% of the total
40 Brazilian dry bean market (Vogt et al., 2011).

41 In the field, bean crops can be affected by a diverse range of organisms, including insects,
42 nematodes, fungi, bacteria and viruses, which can reduce yield significantly (Graham and Ranalli, 1997).
43 However, especially during the storage period, fungal species belonging to *Aspergillus* and *Penicillium*
44 genera cause considerable loss due to their ability to grow under low humidity conditions. These fungi
45 not only contaminate the seeds by fungal growth, but also affect the quality by the production of toxic
46 secondary metabolites. The presence of *Aspergillus* on bean seeds has been reported before (Costa and
47 Scussel, 2002; Domijan et al., 2005; Silva et al., 2008; Tseng et al., 1995), but in most reports, the
48 molecular identification to species level or the capacity to produce mycotoxins was not performed. This
49 lack of information may lead to a serious risk of food contamination, once these fungi produce toxins
50 that are detrimental to humans and animals.

51 Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins (Yu
52 et al., 2004). In humans, they are capable of causing diseases such as hepatitis, liver cirrhosis, liver
53 cancer, and gallbladder cancer (Koshiol et al., 2017; McKean et al., 2006). The four major types of
54 aflatoxins are AFB_1, AFB_2, AFG_1 and AFG_2, which can be present on a wide range of commodities.
55 *Aspergillus flavus* produces AFB_1 and AFB_2 and *A. parasiticus* produces AFB_1, AFB_2, AFG_1 and AFG_2,

56 but other species like *A. nomius, A. pseudotamarii, A. ochraceoroseus, A. pseudocaelatus* and *A.*
57 *ostianus* have also been reported to produce aflatoxin as reviewed by Bezerra da Rocha et al. (2014).
58 The aflatoxin biosynthesis is regulated by an intricate group of genes clustered on a 70kb DNA segment.
59 This cluster contains 25 genes involved in the complex reactions in the aflatoxin pathway (Yu, et al.,
60 2004).

61 Ochratoxin A (OTA) is known as the most toxic member of the ochratoxin family of
62 mycotoxins, displaying nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic
63 effects (JECFA, 2001). It is produced by certain *Aspergillus* and *Penicillium* species and it is commonly
64 found as a contaminant in a wide variety of food commodities (Wang et al., 2016). *A. ochraceus* and *P.*
65 *verrucosum* were considered for a long time the main OTA producers (Pitt, 2000). *A. ochraceus* strains
66 have been shown to be capable of producing high amounts of OTA under certain circumstances, while
67 eight other species within the section *Circumdati* - including *A. westerdijkiae* -were described as robust
68 OTA producers (Frisvad et al., 2004). OTA-producing black aspergilli, such as *A. niger*, *A. welwitschiae*
69 and *A. carbonarius* are important contaminants of grape and wine (Einloft et al., 2017; Susca et al.,
70 2016). The gene cluster involved in the production of ochratoxin A was identified for the first time in
71 *Penicillium verrucosum* by Geisen et al. (2006). In *Aspergillus*, OTA biosynthetic cluster is not
72 completely elucidated, but at least a polyketide synthetase gene (PKS) and a non-ribosomal peptide
73 synthase (NRPS) have been demonstrated to be involved in the pathway of OTA biosynthesis (Gallo et
74 al., 2012a). In addition, three other genes are hypothesized to be part of the cluster (Ferracin et al., 2012;
75 Susca et al., 2016).

76 Fumonisins were considered to be produced mainly by *Fusarium verticillioides* and *F.*
77 *proliferatum* (Gelderblom et al., 1988), but recently, fumonisin B_2 production was detected in *A. niger*
78 and *A. welwitschiae* (Frisvad et al., 2007; Hong et al., 2013; Perrone et al., 2011). The exposure to
79 fumonisins can lead to carcinogenic, nephrotoxic and hepatotoxic effects in humans and animals
80 (JECFA, 2001). The fumonisin biosynthetic gene cluster in *Aspergillus* consists of eleven homologues
81 to *Fusarium* genes, and one additional gene (*sdr1*), a short-chain dehydrogenase gene not present in the
82 *Fusarium* cluster (Pel et al., 2007; Susca et al., 2014).

83 The objective of the current study was to provide information about the occurrence of *Aspergillus*
84 species – grouped according to the different sections they belong to with green, black or yellow spores
85 respectively -in association with the most popular dry bean types in Brazil and to identify these isolates
86 to species level using barcode sequences. Furthermore, we characterized their toxigenic potential by
87 detecting the presence of genes involved in toxin biosynthesis. Hence, this paper gives an inventory of
88 potential mycotoxins produced by *Aspergillus* species on dry beans.
89

90 ## 2. Material and methods

91 ### *2.1. Fungal isolates*

92 Fungal strains were obtained from 35 seed lots originated from 14 different cities in Brazil
93 (Figure 1; Table 2). The seed surface was disinfected by soaking seeds in sodium hypochlorite solution
94 (NaClO 1%) during 1 minute and immediately rinsing them twice with sterile distilled water. After
95 drying for 72h under aseptic conditions, 100 seeds were placed in four Petri dishes of 15cm diameter
96 containing a sterile filter paper disc immersed in 0.5% water agar medium with 6% sodium chloride
97 (NaCl) to reduce seed germination and favour *Aspergillus* growth in detriment of other fungi (Protocol
98 from Brazilian Ministry of Agriculture, Livestock and Food Supply, 2009). After 7 days at 25°C
99 *Aspergillus* colonies were selected based on morphological characteristics and representative strains of
100 the different groups based on spore colour (green, black and yellow) were selected for isolation. Strains
101 were subcultured on PDA and grown during 5-7 days at 25°C in a 12/12 hour photoperiod regime. From
102 pure cultures, spore suspensions were prepared in sterile water, and 50µL were spread on water agar
103 medium. After 24-36h of incubation at 25°C, the plates were checked under a stereomicroscope. Single
104 germinating spores were collected and transferred to a PDA plate to obtain single spore cultures.

105

106 **Figure 1**
107 Geographical origin of *Aspergillus* strains of this study. The letters indicate the name of
108 Brazilian provinces: CE (Ceará), BA (Bahia), MG (Minas Gerais), SP (São Paulo), RS (Rio
109 Grande do Sul).

110 *2.2.DNA isolation, amplification and sequencing*

111 Conidia from single spore cultures were inoculated on 2mL of Wickerham's medium
112 and incubated at 25°C for DNA extraction. After 48 hours, mycelia were harvested and
113 transferred to 1.5mL microtubes. DNA was isolated by using the Wizard® Genomic DNA
114 Purification Kit according to the manufacturer's instructions. Amplification of part of the
115 calmodulin gene (*caM*) and the β-tubulin gene (β-tub), was performed using the primers
116 CMD5/CMD6 (Hong et al., 2005) and Bt2a/Bt2b (Glass and Donaldson, 1995) (Table 1). PCR
117 reactions were performed in a 12.5µL-volume reaction, containing 0.5U Roche Taq DNA
118 Polymerase, 1.25x Roche Taq DNA Polymerase buffer, 2mM $MgCl_2$, 200nM of each primer
119 and 200µM dNTPs. The cycling protocol consisted of an initial denaturation at 95°C for 10min,
120 35 cycles of denaturation at 95°C for 50s, annealing for 30s at 55°C for *caM*, or at 58°C for β-
121 tub and extension at 72°C for 40s, followed by a final extension at 72°C for 7min. Alternatively
122 for those strains that did not amplify using CMD5/CMD6, primers CL1/CL2 (O'Donnell et al.,

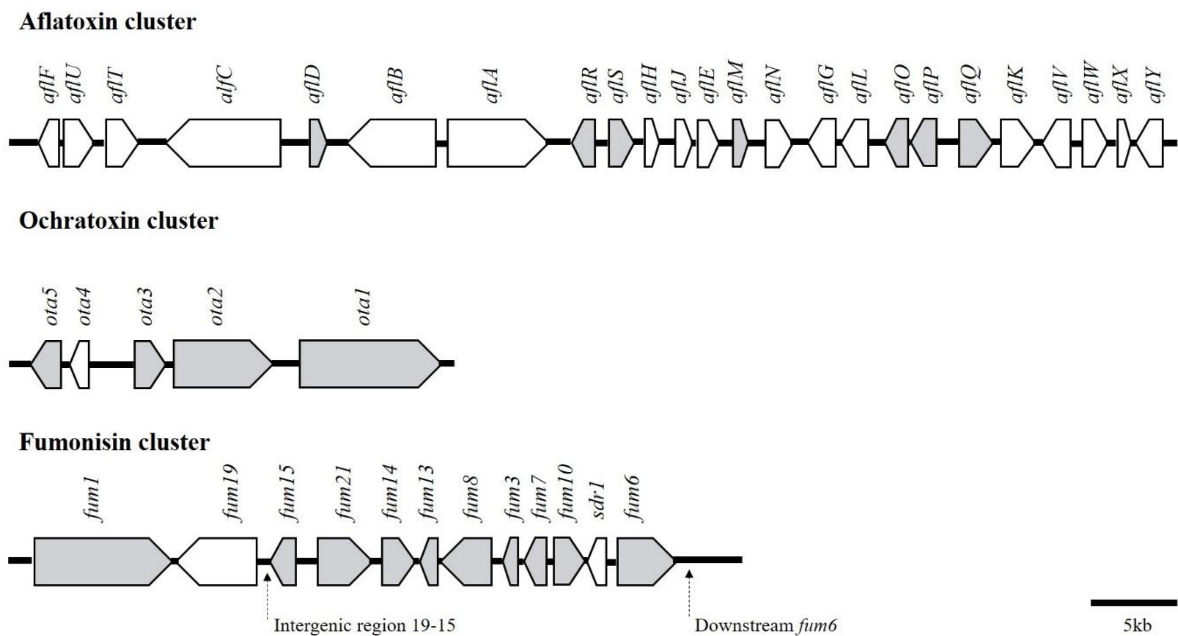
123 2000) were used to obtain their calmodulin amplicons using the same PCR conditions as
124 described above. PCR products were sent to Macrogen Europe for purification and sequencing.

125 *2.3 Sequence analysis*

126 DNA sequences were trimmed, assembled and aligned with CLC Genomic Workbench
127 9.5.1. Phylogenetic trees were obtained using MEGA 7.0.21 (Kumar et al., 2016), by the
128 Maximum Likelihood construction method, using Tamura-Nei model with bootstrap support
129 with 1,000 replicates.

130 *2.4 Toxigenic potential*

131 The presence of genes involved in aflatoxin (*afl*), ochratoxin (*ota*) or fumonisin (*fum*)
132 biosynthesis was assessed by PCR using specific primers for genes and intergenic regions
133 within the respective clusters (Figure 2; Table 1).



134

135 **Figure 2**

136 Graphical representation of the putative aflatoxin, ochratoxin and fumonisin biosynthetic gene
137 clusters in *Aspergillus* (based on Yu et al., 2004, Susca et al., 2016 and Pel et al., 2007,
138 respectively). Arrows represent genes and indicate transcription direction. Genes targeted by
139 PCR in this study are shaded in grey. In the fumonisin gene cluster also the intergenic region
140 between *fum19* and *fum15* was targeted as well as the region downstream of *fum6* (dotted
141 arrows).

142

143

144 The amplifications for all *afl, ota* and *fum* genes were performed in a final volume of
145 12.5µL, containing 0.5U Roche Taq DNA Polymerase, 1x Roche Taq DNA Polymerase buffer,
146 2.25mM $MgCl_2$, 300nM of both forward and reverse primer and 200µM dNTPs. The cycling
147 conditions targeting the *afl* genes were described by Gallo et al. (2012b) consisting of an initial
148 denaturation at 95°C for 10min, 30 cycles of denaturation at 95°C for 50s, annealing at 58°C
149 for 50s and extension at 72°C for 2min, followed by a final extension at 72°C for 5min. In case
150 the *afl* amplifications did not give products or products of unexpected sizes, the reactions were
151 repeated using Premix Ex Taq™ Hot Start Version (Takara) with 300nM of both forward and
152 reverse primer using the same cycling conditions described above. The cycling conditions for
153 *fum* and *ota* amplicons were the same as described by Susca et al. (2016), consisting of an initial
154 denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 94°C, annealing at 58
155 and 60°C, respectively, and extension at 72°C - each step performed for 50s for *fum* primers or
156 30s for *ota* primers and a final extension for 7min at 72°C.

157 **Table 1**
158 Primers used in this study.

Target genes/ cluster	Gene / Intergenic region	Primer sequence (5'-3')	Annealing temperature	Amplicon size	References
Barcodes	*caM* (CMD5/CMD6)	F: CCGAGTACAAGGARGCCTTC R: CCGATRGAGGTCATRACGTGG	55°C	600	Hong et al., 2005
	caM (CL1/CL2)	F: GARTWCAAGGAGGCCTTCTC R: TTTTGCATCATGAGTTGGAC	55°C	750	O'Donnell et al., 2000
	β-tub (Bt2a/Bt2b)	F: GGTAACCAAATCGGTGCTGCTTTC R: ACCCTCAGTGTAGTGACCCTTGGC	58°C	555	Glass and Donaldson, 1995
Aflatoxin	*aflD*	F: CACTTAGCCATCACGGTCA R: GAGTTGAGATCCATCCGTG	58°C	852	Gallo et al., 2012b
	aflR	F: AAGCTCCGGGATAGCTGTA R: AGGCCACTAAACCCGAGTA	58°C	1079	Gallo et al., 2012b
	aflS	F: TGAATCCGTACCCTTTGAGG R: GGAATGGGATGGAGATGAGA	58°C	684	Gallo et al., 2012b
	aflM	F: AAGTTAATGGCGGAGACG R: TCTACCTGCTCATCGGTGA	58°C	470	Gallo et al., 2012b
	aflO	F: TCCAGAACAGACGATGTGG R: CGTTGGCTAGAGTTTGAGG	58°C	790	Gallo et al., 2012b
	aflP	F: AGCCCCGAAGACCATAAAC R: CCGAATGTCATGCTCCATC	58°C	870	Gallo et al., 2012b
	aflQ	F: TCGTCCTTCCATCCTCTTG F: ATGTGAGTAGCATCGGCATTC	58°C	757	Gallo et al., 2012b
Ochratoxin	*ota5*	F: TCCCTCGGTAAGAGTATCCTCGT R: GCGAGTTCTTGGTTCATGACG	60°C	845	Susca et al., 2016
	ota3	F: TTAGACAAACTGCGCGAGGA R: GCGTCGCTATGCCCAGATA	60°C	613	Susca et al., 2016
	ota2	F: GGGAAYRCTGAYGTCGTGTTT R: TCCCACGAGCAWACAGCCTC	60°C	644	Susca et al., 2016
	ota1	F: CAATGCCGTCCAACCGTATG R: CCTTCGCCTCGCCCCGTAG	60°C	776	Ferracin et al.,2012
Fumonisin	*fum1*	F: GGGTTCCAGGCAGAATCGTAC R: GAACTCACATCCTTTTGGGCC	58°C	701	Susca et al., 2014
	fum19-15 IGR	F: ACACCGCGAGAATTCCATG R: GCAGGCTGGTAGTAGCGACAT	58°C	868	Susca et al., 2014
	fum15	F: CGATTGGTAGCCCGAGGAA R: CTTGATATTGCGGAGTGGTCC	58°C	701	Susca et al., 2014
	fum21 region I	F: CATTTCATGGGACCTCAGCC R: AAGCACAGGTTCCGAATTTGA	58°C	703	Susca et al., 2014

fum21 region II	F: GGGTCCCATTGCCTCAATT R: CAATGGAGTCGACGGTGTCAC	58°C	705	Susca et al., 2014
fum14	F: TTGGGCTGATGTGCTCTGTC R: CCTCGTAGACGTAATTGAGTAGTCCT	58°C	730	Susca et al., 2014
fum13	F: ATGCTCTTCACCTCCTCCGG R: CACTCAACGAGGAGCCTTCG	58°C	651	Susca et al., 2014
fum8	F: TTCGTTTGAGTGGTGGCA R: CAACTCCATASTTCWWGRRAGCCT	58°C	862	Susca et al., 2010
fum3	F: TACCATGGACCACTTTCCCG R: AAGTTCCTCAAGCGGCAGTC	58°C	651	Susca et al., 2014
fum7	F: CAACAGCCCGAATCCCAGTA R: GCTCAGTCTTGCCCATCGTG	58°C	681	Susca et al., 2014
fum10	F: GTCATTATTCCTCCGGCCCT R: TGGGATTCGAAAGCATACCG	58°C	651	Susca et al., 2014
fum6	F: CTGTGAGGCCCTGGCACTT R: TCTGCCGGAGCTCAACGTA	58°C	849	Susca et al., 2014
downstream fum6	F: CAAAAGACACCGCCCGTCT R: TTGACGCCCTGTACAAGGC	58°C	667	Susca et al., 2014

159 IGR: intergenic region between *fum19* and *fum15*
160

161 ## 3. Results

162 After fungal isolation, we obtained 87 strains, of which 40 belong to the green group,
163 34 to the black group and 13 to the yellow group (Table 2). All strains were deposited in the
164 Culture Collection of the Food Sciences Department (CCDCA) at Federal University of Lavras,
165 Brazil. Green strains were found in 34 seed lots tested (97%), while black and yellow strains
166 were found in 27 (77%) and 10 (29%) seed lots, respectively.

167 ### *3.1 Species identification*

168 Partial calmodulin and β-tubulin gene sequences were used to determine species identity
169 of all *Aspergillus* strains collected from bean seeds (MG746413 to MG746586). Sequences
170 from both genes gave the same identification. Our work shows that 97.5% of the green strains
171 are *A. flavus* (n=39) and 2.5% are *A. pseudocaelatus* (n=1). Within the black aspergilli, 70.6%
172 of the strains are *A. niger* (n=24) and 29.4% were identified as *A. luchuensis* (n=10). Regarding
173 the yellow group, 53.8% are *A. westerdijkiae* (n=7), 23.1% are *A. ostianus* (n=3), 15.4% are *A.*
174 *wentii* (n=2), and 7.7% are *A. ochraceus* (n=1).

175 **Table 2**
176 Information on the analysed seed lots and molecular identification of the obtained strains.

Strain code	City	Province	Seed lot	Bean Group	Bean Class	Sowing/harvesting year	Molecular identification (*caM* and β-tub)
CCDCA11411	Campo Belo	MG	20	I	Mixed colors	2013/2013	*A. niger*
CCDCA11412	Passos	MG	14	I	Mixed colors	2014/2014	*A. niger*
CCDCA11413	Madre de Deus de Minas	MG	35	I	Mixed colors	2016/2016	*A. flavus*

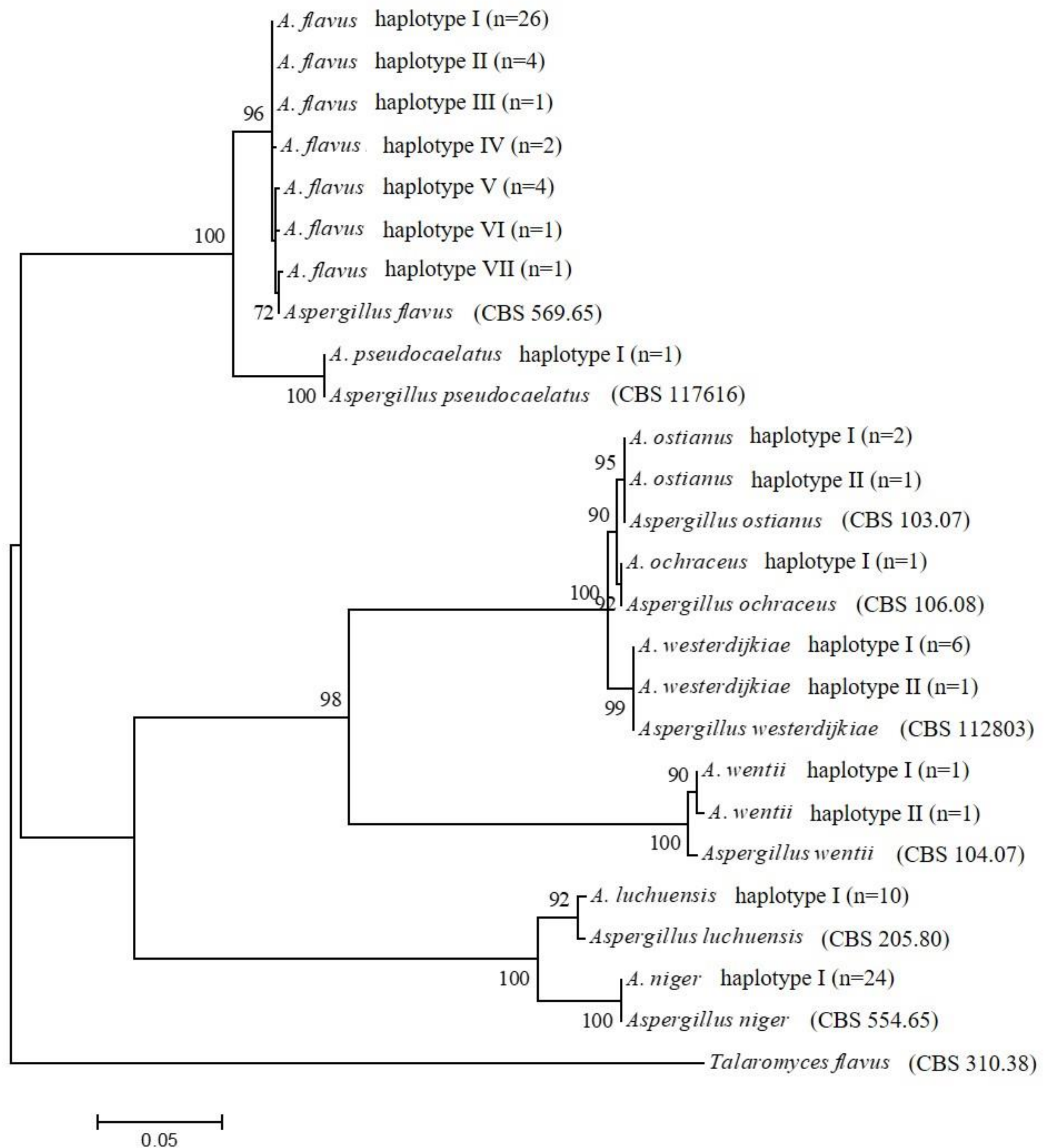
CCDCA11414	Sete Lagoas	MG	30	I	Mixed colors	2015/2015	*A. niger*
CCDCA11415	Ribeirão Vermelho	MG	23	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11416	Ribeirão Vermelho	MG	23	I	Mixed colors	2013/2013	*A. niger*
CCDCA11417	Ribeirão Preto	SP	02	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11418	Ribeirão Preto	SP	05	I	Mixed colors	2012/2013	*A. ostianus*
CCDCA11419	Ribeirão Preto	SP	06	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11420	Ribeirão Preto	SP	32	I	Mixed colors	2016/2016	*A. niger*
CCDCA11421	Ribeirão Preto	SP	16	I	Mixed colors	2013/2014	*A. westerdijkiae*
CCDCA11422	Santo Anastácio	SP	15	I	Mixed colors	-	*A. niger*
CCDCA11423	Cruz das Almas	BA	27	II	White	2015/2015	*A. flavus*
CCDCA11424	Sete Lagoas	MG	10	I	Mixed colors	2013/2013	*A. westerdijkiae*
CCDCA11425	Cruz das Almas	BA	29	I	Black	2015/2015	*A. pseudocaelatus*
CCDCA11426	Ribeirão Preto	SP	08	I	Mixed colors	2012/2013	*A. niger*
CCDCA11427	Ribeirão Preto	SP	04	I	Mixed colors	2013/2014	*A. niger*
CCDCA11428	Ribeirão Preto	SP	03	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11429	Sete Lagoas	MG	31	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11430	Sete Lagoas	MG	10	I	Mixed colors	2013/2013	*A. niger*
CCDCA11431	Sete Lagoas	MG	01	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11432	Ribeirão Preto	SP	12	I	Mixed colors	2013/2014	*A. niger*
CCDCA11433	Cruz das Almas	BA	28	I	Mixed colors	2015/2015	*A. luchuensis*
CCDCA11434	Ribeirão Preto	SP	07	I	Black	2013/2014	*A. niger*
CCDCA11435	Ribeirão Preto	SP	08	I	Mixed colors	2012/2013	*A. flavus*
CCDCA11436	Santo Anastácio	SP	15	I	Mixed colors	-	*A. flavus*
CCDCA11437	Ribeirão Preto	SP	33	I	Mixed colors	2016/2016	*A. flavus*
CCDCA11438	Sete Lagoas	MG	09	I	Mixed colors	2013/2013	*A. luchuensis*
CCDCA11439	Ribeirão Preto	SP	13	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11440	Sete Lagoas	MG	31	I	Mixed colors	2015/2015	*A. luchuensis*
CCDCA11441	Patos de Minas	MG	25	I	Mixed colors	2015/2015	*A. niger*
CCDCA11442	Ribeirão Preto	SP	04	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11443	Campo Belo	MG	20	I	Mixed colors	2013/2013	*A. luchuensis*
CCDCA11444	Ribeirão Preto	SP	16	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11445	Ribeirão Preto	SP	07	I	Black	2013/2014	*A. flavus*
CCDCA11446	Cruz das Almas	BA	29	I	Black	2015/2015	*A. flavus*
CCDCA11447	Itutinga	MG	22	I	Black	2012/2013	*A. westerdijkiae*
CCDCA11448	Ribeirão Preto	SP	12	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11449	Sete Lagoas	MG	10	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11450	Ribeirão Preto	SP	11	I	Black	2013/2014	*A. flavus*
CCDCA11451	-	CE	18	II	White	2015/2015	*A. flavus*
CCDCA11452	Ribeirão Vermelho	MG	23	I	Mixed colors	2013/2013	*A. niger*
CCDCA11453	Cana Verde	MG	24	I	Mixed colors	2012/2012	*A. westerdijkiae*
CCDCA11454	Cruz das Almas	BA	27	II	White	2015/2015	*A. ostianus*
CCDCA11455	Camaquã	RS	17	II	White	-	*A. flavus*
CCDCA11456	Cruz das Almas	BA	26	I	Mixed colors	2015/2015	*A. niger*
CCDCA11457	Cana Verde	MG	24	I	Mixed colors	2012/2012	*A. flavus*
CCDCA11458	Ribeirão Preto	SP	11	I	Black	2013/2014	*A. niger*
CCDCA11459	Ribeirão Preto	SP	05	I	Mixed colors	2012/2013	*A. flavus*

CCDCA11460	Patos de Minas	MG	25	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11461	Sete Lagoas	MG	09	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11462	Ribeirão Vermelho	MG	19	I	Mixed colors	2014/2015	*A. flavus*
CCDCA11463	Sete Lagoas	MG	09	I	Mixed colors	2013/2013	*A. westerdijkiae*
CCDCA11464	Sete Lagoas	MG	09	I	Mixed colors	2013/2013	*A. ochraceus*
CCDCA11465	Ribeirão Preto	SP	03	I	Mixed colors	2013/2014	*A. niger*
CCDCA11466	Campo Belo	MG	20	I	Mixed colors	2013/2013	*A. luchuensis*
CCDCA11467	Perdões	MG	21	I	Mixed colors	2013/2014	*A. niger*
CCDCA11468	Ribeirão Preto	SP	06	I	Mixed colors	2013/2014	*A. niger*
CCDCA11469	Passos	MG	14	I	Mixed colors	2014/2014	*A. westerdijkiae*
CCDCA11470	Ribeirão Preto	SP	34	I	Mixed colors	2016/2016	*A. luchuensis*
CCDCA11471	Sete Lagoas	MG	01	I	Mixed colors	2013/2013	*A. niger*
CCDCA11472	Cruz das Almas	BA	28	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11473	Ribeirão Preto	SP	34	I	Mixed colors	2016/2016	*A. flavus*
CCDCA11474	Sete Lagoas	MG	30	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11475	Camaquã	RS	17	II	White	-	*A. niger*
CCDCA11476	Passos	MG	14	I	Mixed colors	2014/2014	*A. flavus*
CCDCA11477	Ribeirão Vermelho	MG	23	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11478	Ribeirão Preto	SP	16	I	Mixed colors	2013/2014	*A. niger*
CCDCA11479	Passos	MG	14	I	Mixed colors	2014/2014	*A. flavus*
CCDCA11480	Itutinga	MG	22	I	Black	2012/2013	*A. niger*
CCDCA11481	Cana Verde	MG	24	I	Mixed colors	2012/2012	*A. niger*
CCDCA11482	Itutinga	MG	22	I	Black	2012/2013	*A. flavus*
CCDCA11483	Perdões	MG	21	I	Mixed colors	2013/2014	*A. flavus*
CCDCA11484	Ribeirão Vermelho	MG	23	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11485	Cruz das Almas	BA	26	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11486	Campo Belo	MG	20	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11487	Itutinga	MG	22	I	Black	2012/2013	*A. westerdijkiae*
CCDCA11488	Campo Belo	MG	20	I	Mixed colors	2013/2013	*A. flavus*
CCDCA11489	Patos de Minas	MG	25	I	Mixed colors	2015/2015	*A. luchuensis*
CCDCA11490	Cana Verde	MG	24	I	Mixed colors	2012/2012	*A. luchuensis*
CCDCA11491	Itutinga	MG	22	I	Black	2012/2013	*A. niger*
CCDCA11492	Patos de Minas	MG	25	I	Mixed colors	2015/2015	*A. flavus*
CCDCA11493	Patos de Minas	MG	25	I	Mixed colors	2015/2015	*A. ostianus*
CCDCA11494	Itutinga	MG	22	I	Black	2012/2013	*A. luchuensis*
CCDCA11495	Ribeirão Preto	SP	33	I	Mixed colors	2016/2016	*A. luchuensis*
CCDCA11496	Ribeirão Preto	SP	33	I	Mixed colors	2016/2016	*A. wentii*
CCDCA11497	Ribeirão Preto	SP	33	I	Mixed colors	2016/2016	*A. wentii*

177 -: not specified
178 Province: CE (Ceará), BA (Bahia), MG (Minas Gerais), RS (Rio Grande do Sul), SP (São
179 Paulo)
180 Group: I (*Phaseolus vulgaris*), II (*Vigna unguiculata*)
181

182 The sequences obtained from the 87 strains were compared to the reference sequences
183 available at GenBank and shown to be 98-100% similarity to the type strain of each species

184 (Figure 3). The beta-tubulin (*β-tub*) sequences presented a single haplotype identical to the
185 reference strain for each species, except for the two *A. wentii* isolates that both showed 1%
186 variation with the type strain CBS 104.07 (EF652106). Differently, the calmodulin (*caM*)
187 sequences, revealed variations up to 2% compared to the reference sequence, and up to 1%
188 comparing among the isolates from this study (Table 3). The strains identified as *A. luchuensis*
189 were the most divergent from the reference CBS 205.80 (2%), even though they all represented
190 a single haplotype, with no internal variation. The *A. flavus* clade showed the highest internal
191 variation, presenting seven haplotypes different from the reference CBS 569.65, most of them
192 presenting SNPs in the noncoding region. Only the haplotypes IV and V presented SNPs in the
193 coding sequence (Figure 4).



194
195 **Figure 3**
196 Maximum likelihood tree obtained from partial calmodulin and β-tub concatenated sequences.
197 Bootstrap values over 70 are shown.
198

Table 3
Information on the internal variation among the studied strains, number of haplotypes found and similarity to the reference strain, ordered by abundance.

Species	n	β-tub				*caM*			
		Similarity to reference strain (%)	Reference accession[a]	Internal Variation[b] (%)	Haplotypes[c]	Similarity to reference strain (%)	Reference accession[a]	Internal Variation[b] (%)	Haplotypes[c]
A. flavus	39	100	EF661485	0	1	99	EF661508	1	7
A. niger	24	100	EF661089	0	1	100	EF661154	0	1
A. luchuensis	10	100	JX500062	0	1	98	JX500071	0	1
A. westerdijkiae	7	100	EF661329	0	1	99	EF661360	1	2
A. ostianus	3	100	EF661324	0	1	99-100	EF661385	1	2
A. wentii	2	99	EF652106	0	1	99	EF652131	1	2
A. ochraceus	1	100	EF661322	-	1	99	EF661381	-	1
A. pseudocaelatus	1	100	EF203128	-	1	99	EF202037	-	1

[a]Accession numbers of *caM* and β-tub sequences from reference strains (Samson et al., 2014)
[b]Variation observed between the strains analysed in this study
[c]Number of haplotypes found among strains from this study
-: not applicable since only one strain of the species was obtained in this study.

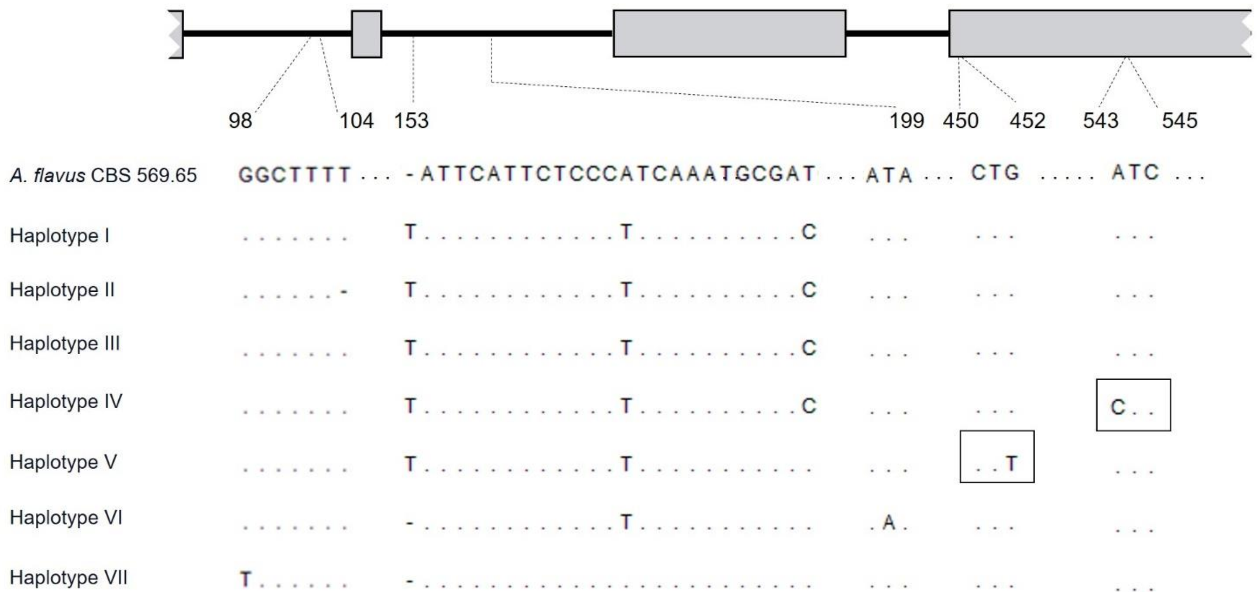


Figure 4
Alignment of partial calmodulin sequences presenting the SNPs observed in seven *A. flavus* haplotypes. Numbers indicate the position on calmodulin sequence of the reference strain *A. flavus* CBS 569.65 (EF661508) starting at the first nucleotide of calmodulin gene. Matching residues are show as dots. SNPs on the coding region in the haplotypes IV and V are boxed.

215 *3.2 Toxigenic characterization*

216 The presence of genes involved in mycotoxin biosynthesis was assessed by PCR,
217 targeting seven, four and ten genes in the aflatoxin, ochratoxin and fumonisin biosynthetic
218 clusters, respectively. In the fumonisin biosynthetic cluster, the presence of one intergenic
219 region (*fum 19*-15) and a region downstream the gene *fum 6* was also determined (Figure 2)

220 Within the studied species, *A. flavus*, *A. ostianus* and *A. pseudocaelatus* are known as
221 potential aflatoxin producers. None of the *A. ostianus* and *A. pseudocaelatus* strains gave the
222 expected amplification products, except one *A. wentii* strain (#89) that amplified a fragment of
223 approximately 650bp with primers *aflO*. This amplicon was sequenced and compared within
224 NCBI databases using BLAST. The fragment had no similarity with the *aflO* gene, but its
225 translation and comparison to protein sequences (blastx) gave 100% identity to a hypothetical
226 protein in *A. wentii* (OJJ31152). The 39 *A. flavus* strains in this study represented 13 different
227 amplification patterns (Table 4), varying from the presence of all tested genes (n=17) to the
228 absence of all of them (n=2), indicating that a large part of the *A. flavus* population on beans in
229 Brazil (43%) is capable of producing aflatoxin.

230

Table 4
Amplification patterns of aflatoxin genes observed within the studied strains. Positive results are shaded in grey.

Species	*aflD*	*aflR*	*aflS*	*aflM*	*aflO*	*aflP*	*aflQ*
A. flavus (n=17)	+	+	+	+	+	+	+
A. flavus (n=5)	-	+	+	+	+	+	+
A. flavus (n=1)	+	-	+	+	+	+	+
A. flavus (n=3)	-	-	+	+	+	+	+
A. flavus (n=2)	+	+	+	-	+	+	+
A. flavus (n=1)	-	+	+	+	+	-	-
A. flavus (n=2)	-	-	+	+	-	-	+
A. flavus (n=1)	-	-	+	-	+	-	+
A. flavus (n=1)	-	-	-	+	+	+	-
A. flavus (n=1)	-	-	-	+	+	-	+
A. flavus (n=1)	-	-	-	+	-	+	-
A. flavus (n=2)	-	-	-	+	-	-	-
A. flavus (n=2)	-	-	-	-	-	-	-

+: amplicon with the expected size
-: no amplicon detected

Regarding fumonisin genes, amplicons were only observed in *A. niger* strains. In all 24 *A. niger* isolates from this work the expected amplicons were obtained for all 13 primer sets, indicating that all strains harbour the 10 genes checked in this pathway, as well as the two intergenic regions (Table 5). On the other hand, none of the ochratoxin genes was detected in any of the 87 studied strains (data not shown).

Table 5
Amplification patterns of fumonisin genes and intergenic regions observed within the studied strains.

Species	*fum1*	*fum19-15*	*fum15*	*fum21 I*	*fum21 II*	*fum14*	*fum13*	*fum8*	*fum3*	*fum7*	*fum10*	*fum6*	*downstream fum6*
A. flavus (n=39)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. niger (n=24)	+	+	+	+	+	+	+	+	+	+	+	+	+
A. luchuensis (n=10)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. westerdijkiae (n=7)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. ostianus (n=3)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. wentii (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. ochraceus (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. pseudocaelatus (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	-

+: amplicon with the expected size
-: no amplicon detected

4. Discussion

The presence of *Aspergillus* strains on beans is the first indication of a potential risk of mycotoxin contamination. Especially in large parts of Brazil, this commodity constitutes the basic diet of the population, increasing the chances of mycotoxin intake leading to a serious public health issue. This is the first study to perform molecular identification of Brazilian strains of *Aspergillus* associated with beans as well as a prospection of genes involved on aflatoxin, fumonisin and ochratoxin biosynthesis. The presence of *A. flavus* in 100% of the seed lots tested and the presence of all the scanned aflatoxin genes in 43% of them reinforces the necessity of legislation on acceptable mycotoxin limits and trading conditions. In addition the occurrence of a single *A. niger* lineage harbouring the whole fumonisin cluster (n=24) in all regions surveyed, alerts to the possibility of fumonisin contamination in many regions of Brazil. Furthermore, our findings indicate the necessity of further studies on *Aspergillus* populations on Brazilian commodities, mainly concerning their toxigenic potentials and the environmental conditions triggering toxin production.

Species identification was based on two barcoding genes, calmodulin and β-tubulin: *A. flavus* and *A. niger* strains which are the most common reported food-borne *Aspergillus* species (Dijksterhuis et al., 2013) were found in 100% and 71% respectively of the seed lots tested. In Brazil, *A. flavus* was also shown to be the prevalent species within the section *Flavi* in peanuts and Brazil nuts (Martins et al., 2017; Reis et al., 2014).

In addition to *A. niger*, another species was found among the black aspergilli from this study: *A. luchuensis*. This species was recently reported as atoxigenic and hence considered as safe for food and beverage fermentation purposes (Hong et al. 2013). In Korea, *A. luchuensis* is commonly isolated from meju, a product based on dried fermented soybeans (Hong et al., 2013). In crops such as grapes, onions, Brazil nuts and coffee *A. niger* along with *A.*

272 *welwitschiae* are most frequently isolated species among the black aspergilli (Ferranti et al.,
273 2017; Massi et al., 2016). However, no *A. welwitschiae* was found in the studied bean samples.
274 Regarding the yellow spored isolates, *A. westerdijkiae*, *A. ochraceus*, *A. ostianus* and *A.*
275 *wentii* were identified in this work. In Brazil, *A. westerdijkiae* and *A. ochraceus* have been
276 frequently reported on coffee and grapevine crops as the main agents of OTA contamination in
277 associated beverages (Morello et al., 2007; Taniwaki et al., 2003). In contrast, only one *A.*
278 *ochraceus* strain was found in this study, while *A. westerdijkiae* represents 54% of the yellow
279 spored strains. In our samples, *A. ostianus* and *A. wentii* were found in lower frequencies,
280 similarly to frequencies reported in other Brazilian food products (Abe et al., 2015; Batista et
281 al., 2003).
282 Sequence variation observed in the calmodulin gene and in the aflatoxin cluster,
283 suggests variability within the species, though no variation was observed in the β-tubulin gene.
284 In *A. flavus*, calmodulin sequences revealed seven haplotypes, including two SNPs in the coding
285 part of the gene. At position 452 of the calmodulin gene there is synonymous SNP in the
286 haplotype V, while there is a nonsynonymous SNP at position 543 of haplotype IV. However,
287 the substitution of an isoleucine by a leucine residue is unlikely to cause significant changes in
288 the structure of the protein.
289 Most of the *A. flavus* strains of this study (n=17) harbour all aflatoxin genes examined,
290 suggesting that these strains possibly contain the entire aflatoxin gene cluster. In addition to the
291 presumable toxigenic strains were found in 44% of the seed lots tested, what may indicate a
292 potential aflatoxin contamination level higher than that observed by Lutfallah and Hussain
293 (2012) in beans from Pakistan. Our data reveal a high risk of aflatoxin contamination on beans
294 in Brazil that can be exacerbated by the low level of technology employed by small farmers and
295 the uncontrolled humidity and temperature conditions during storage.

296 Previous studies reported the incidence of aflatoxin on beans (Silva et al., 2002; Tseng
297 et al., 1995). Hence, Telles et al. (2017) suggested that phenolic compounds found on beans
298 constitute a defence mechanism against fungal attack and aflatoxin production. Nevertheless,
299 no microbiological or molecular tests were performed to confirm the presence or absence of
300 aflatoxigenic strains in the analysed samples, or the proposed antifungal action of such
301 compounds. In addition, *A. flavus* is frequently reported on this substrate (Costa and Scussel,
302 2002; Francisco and Usberti, 2008) and it is the major species present in Brazilian beans tested
303 in this work. Our findings reinforce the need to monitor *A. flavus* populations on beans to
304 support either the defence mechanism proposed by Telles et al. (2017) or actions to reduce
305 aflatoxin intake by the population.

306 Excluding isolates that appear to contain all the genes of the *afl*-cluster (n=17), the most
307 frequent amplification patterns comprise strains lacking the gene *aflD* (n=5) or both *aflD* and
308 *aflR* (n=3). These patterns are in accordance with large (>1kb) deletions at the left end of the
309 cluster observed in several genotypes of non-aflatoxigenic strains used as biocontrol agents
310 (Adhikari et al., 2016). The complete absence of amplicons for all the tested genes as observed
311 in two strains also indicates large deletions, possibly comprising the entire cluster. It has been
312 suggested that the most frequent deletions in the aflatoxin cluster occur at end of the gene cluster
313 closest to the telomeric end of the chromosome (Adhikari et al., 2016). Nonetheless, we
314 observed nine amplification patterns that lack genes in the middle of the cluster, resembling the
315 results found by Fakruddin et al. (2015) using the same sets of primers. These unexpected
316 patterns can be explained by the occurrence of small (<1kb) deletions, which were also reported
317 by Adhikari et al. (2016). Alternatively, sequence variability in the aflatoxin gene cluster, as
318 observed by these authors may prevent amplification by the primers employed. The occurrence
319 of strains lacking some or most of all tested genes strongly suggests the presence of local non-
320 aflatoxigenic strains on beans, which must be further studied in order to confirm them as

321 candidate biocontrol agents in Brazil. Reduction of aflatoxins using non-toxigenic *A. flavus*
322 strains requires the selection of local strains that occur endemically on target crops in target
323 regions (Mehl et al., 2012).

324 Within the black group, calmodulin and β-tubulin sequences revealed a single *A. niger*
325 haplotype 100% identical to the reference strain, and a single *A. luchuensis* haplotype 98%
326 identical to the reference strain. The lack of variation is also observed in the fumonisin cluster:
327 our results showed that all 24 *A. niger* strains tested, harbour the whole biosynthetic cluster,
328 while the 10 *A. luchuensis* strains lack all the genes we screened for. Our observation of
329 complete absence of the fumonisin cluster in *A. luchuensis* is in contrast to the results obtained
330 by Susca et al. (2014) who reported the presence of the genes *fum1* and *fum15*. These results
331 described here suggest the occurrence of specific lineages of *A. niger* and *A. luchuensis*
332 affecting beans, which may not be different in other regions in Brazil.

333 Although the presence of the complete fumonisin cluster was also reported in non-
334 producing *A. niger* strains (Susca et al., 2014), it is necessary to highlight the high risk of
335 fumonisin contamination on beans. This toxin was known to be produced by *Fusarium*
336 *verticillioides* and other Fusaria. Therefore, the current legislation in Brazil regulates the
337 tolerable limits of fumonisins only on corn as the main ecological niche for *F. verticillioides*
338 and corn-based products (Anvisa - The Brazilian Health Regulatory Agency, 2011).
339 Considering the present and previous studies, it is clear that the laws concerning fumonisin
340 obligatory assessments must be extended to products highly affected by *A. niger*, including
341 beans.

342 Among the strains analyzed in this study, three species were reported to be able to
343 produce ochratoxin: *A. niger, A. westerdijkiae* and *A. ochraceus*, however none of the four
344 genes prospected in this work was found among the strains tested in the present study. This
345 result confirms previous studies reporting that only a minority of *A. niger* strains can produce

346 both fumonisin and ochratoxin (Massi et al., 2016). Susca et al. (2014) reported 100% of the
347 Brazilian strains analysed were OTA non-producers with two possible *ota* amplicon patterns:
348 either presence or absence of all four putative genes. These authors also suggested that the
349 deletion of the *ota* cluster occurred in an *A. niger* ancestor and resulted in the formation of two
350 alleles: an intact and a deleted ota-cluster allele. On the other hand, the reasons why no
351 amplification was observed in *A. westerdijkiae* and *A. ochraceus* strains can be a result of
352 specificity problems of the primers employed, since they were designed based on the *A. niger*
353 ochratoxin gene cluster and may be unable to generate amplicons in these species. As the
354 ochratoxin biosynthetic cluster is not completely elucidated in *Aspergillus*, we cannot assume
355 that the same deletion pattern occurred in *A. niger*, *A.ochraceus* and *A. westerdijkiae*.
356 Other *Aspergillus* species were observed more rarely but none gave amplification
357 products with the tested primers, indicating the absence of any of the mycotoxin genes screened
358 for or too much diverging sequences to give the expected products.
359 In conclusion, the current study revealed that *Aspergillus* species containing toxigenic
360 clusters are frequently found on beans in Brazil what suggests a potentially high risk of daily
361 intake of mycotoxins by the population. Therefore, we emphasize the need for further studies
362 in an effort to elucidate the *Aspergillus* diversity in Brazil and contribute to strategies for
363 preventing toxin contamination in food in the world. In addition, we demonstrated the
364 requirement of reliable tests and a very strict regulation on the tolerable mycotoxin levels
365 especially in staple foods.
366

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