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Tese de Doutoramento
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ABSTRACT

The reduction of yield, quality, and nutritional value of grain cereals by filamentous fungi and subsequent contamination with mycotoxins is of great concern around the world. Mycotoxins are known to cause serious health problems in animals and some mycotoxins such as aflatoxins, fumonisins and ochratoxins, in particular, have also been associated with human health problems.

Aflatoxins, fumonisins and ochratoxin A produced by several *Aspergillus* species are prominent among the mycotoxins associated to maize economic losses (*Zea mays* L.). However, the presence of a given fungus does not mean that the mycotoxin(s) associated with that fungus is (are) also present. There are many factors, especially environmental conditions and agricultural practices, involved in the production of mycotoxins.

The aim of this thesis was to contribute to the risk assessment of post-harvested contamination of maize kernels with aflatoxins (AFs), cyclopiazonic acid (CPA), fumonisins B1 and B2 (FB1 and FB2, respectively) and ochratoxin A (OTA) in three Portuguese regions (Beira Litoral, Ribatejo e Alto Alentejo). To this end, studies were made, first to determine maize *Aspergillus* mycoflora and assess the mycotoxigenic potential of the isolated strains, and secondly to determine the incidence of AFs, CPA, OTA and FB in milled maize.

Ninety five maize samples were collected between November 2008 and April 2009 in maize association of producer's facilities in three different agroclimatic regions of Portugal. These samples were taken at reception, after drying and after storage, and moisture content was measured immediately after sampling. Strains of *Aspergillus* were isolated and identified through phenotypic characterization and mycotoxin production. Molecular work was also done in eight isolates that could not be fully identified, up to species level, and a new species belonging to *Aspergillus* section *Flavi* was described and named as *Aspergillus mottae*.

Around ninety one percent of the maize samples were contaminated with strains of *Aspergillus*. These were subsequently grouped into five sections: *Flavi* (423 isolates) followed by *Nigri* (270 isolates), *Wentii* (214 isolates), *Circumdati* (15 isolates) and *Candidus* (2 isolates). The fungal frequency of the isolations obtained from the samples belonging to the three steps of the maize storage chain differs. The highest frequency occurs after drying, being the lowest frequency at reception followed by storage. What concerns *Aspergillus* species isolations at

each region, the regions with Mediterranean climates had a higher percentage of *Aspergillus* section *Nigri* isolates in comparison with the region with transitional between Atlantic and Mediterranean climate.

Maize samples were analyzed by HPLC for mycotoxin contamination. Mycotoxins were detected in 83% of the samples, with prevalence for FB1. Hence, 83% percent of the samples were positive for FB1, 46% were positive for FB2 and only 5% were positive for AFs. None of the samples were positive for OTA and CPA.

In conclusion, the two Producer's Society and the Agricultural Cooperative showed to have good practices, minimizing the occurrence of mycotoxins. Even though *Aspergillus* section *Flavi*, especially of *A. flavus*, and *Aspergillus* section *Nigri* isolates were present in high numbers in the maize kernels, very few samples were contaminated with aflatoxins (5%), and none were positive for either CPA and OTA. The presence of fumonisins (FB1 and FB2) in the majority of the samples (83 and 46%, respectively) suggests that there was pre-harvest contamination with *Fusarium* species. There is no evidence that the presence of FB2 is due to the presence of *Aspergillus niger* aggregate isolates.

Key-Words:

Maize; mycotoxigenic fungi; aflatoxins, cyclopiazonic acid, fumonisins, ochratoxin A; *Aspergillus* section *Flavi*; *Aspergillus* section *Nigri*

SUMÁRIO

A redução do rendimento, qualidade e valor nutricional dos cereais por fungos filamentosos e consequente contaminação com micotoxinas é de grande preocupação em todo o mundo. As micotoxinas são conhecidos por causar sérios problemas de saúde nos animais e algumas micotoxinas em particular as aflatoxinas, fumonisinas e ocratoxinas, também têm sido associadas a problemas de saúde nos seres humanos.

As aflatoxinas, fumonisinas e ocratoxina A, produzidas por várias espécies de *Aspergillus*, estão entre as micotoxinas associadas a perdas económicas no milho (*Zea mays* L.). No entanto, a presença de um dado fungo não significa que a(s) micotoxina(s) associada também esteja(m) presente. Há muitos factores envolvidos na produção de micotoxinas, tendo especial relevância as condições ambientais e as práticas agrícolas.

O objectivo desta tese foi contribuir para a avaliação do risco de contaminação pós-colheita de grãos de milho com aflatoxinas (AFs), ácido ciclopiazónico (CPA), fumonisinas B1 e B2 (FB1 e FB2, respectivamente) e de ocratoxina A (OTA), em três regiões portuguesas (Beira Litoral, Ribatejo e Alto Alentejo). Para este fim, foram realizados estudos com o intuito de determinar, em primeiro lugar, a variedade de *Aspergillus* associada ao milho e o potencial micotoxigénico das estirpes isoladas, e, em segundo lugar, determinar a incidência de AFs, CPA, OTA e FB em farinha de milho.

De modo a atingir os objectivos, foram recolhidas noventa e cinco amostras de milho entre Novembro de 2008 e Abril de 2009 em Associações de Produtores de milho situadas em três regiões de Portugal com diferenças agroclimáticas. Estas amostras foram retiradas na recepção, após a secagem e após o armazenamento, tendo sido medido o teor de humidade logo após a amostragem. As estirpes de *Aspergillus* foram isoladas e identificadas através da caracterização fenotípica e da produção de micotoxinas. Foram realizados estudos moleculares em oito isolamentos, uma vez que não puderam ser identificados até ao nível de espécie. O resultado deste trabalho permitiu descrever uma nova espécie pertencente aos *Aspergillus* secção *Flavi*, cuja designação passou a ser *Aspergillus mottae*.

À volta de de 91% das amostras de milho estavam contaminadas com estirpes de *Aspergillus*. As estirpes foram identificadas como pertencentes a cinco diferentes secções: *Flav*i (423 isolamentos), seguida por *Nigri* (270 isolamentos), *Wentii* (214 isolamentos), *Circumdati* (15 isolamentos) e *Candidus* (2 isolamentos). A frequência fúngica entre os

isolamentos obtidos a partir das amostras pertencentes às três etapas da cadeia de armazenamento de milho difere. A maior frequência ocorre após a secagem, sendo a menor frequência na recepção seguida do armazenamento. No que refere a isolamentos de espécies de *Aspergillus* em cada região, as regiões com climas Mediterrânicos apresentaram maior percentagem de isolamentos de *Aspergillus* secção *Nigri* em comparação com a região de clima de transição entre Atlântico e Mediterrânico.

As amostras de milho recolhidas foram analisadas por HPLC para determinar se estariam contaminadas com micotoxinas. As micotoxinas foram detectadas em 83% das amostras, com prevalência para FB1. Assim, 83% por cento das amostras foram positivas para FB1, 46% foram positivas para FB2 e apenas 5% foram positivas para AFs. Nenhuma das amostras acusou a presença de OTA e CPA.

Em conclusão, as Associações de Produtores mostraram ter boas práticas, minimizando a ocorrência de micotoxinas. Mesmo que a presença de estirpes de *Aspergillus* secção *Flavi*, principalmente de *A. flavus*, e de estirpes de *Aspergillus* secção *Nigri* estivessem presentes em números elevados nos grãos de milho, poucas amostras mostraram estar contaminadas com aflatoxinas (5%), e nenhum delas foi positiva quer para CPA quer para OTA. A presença de fumonisinas (FB1 e FB2) na maioria das amostras (83 e 46%, respectivamente) sugere que houve uma contaminação pré-colheita com espécies de *Fusarium*. Não há nenhuma prova que indique que a presença de FB2 é devido à presença de estirpes pertencentes ao agregado *Aspergillus niger*.

Palavras-Chave:

Milho; fungos micotoxigénicos; aflatoxinas, ácido ciclopiazónico, fumonisinas, ocratoxina A; *Aspergillus* secção *Flavi*; *Aspergillus* secção *Nigri*

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NOMENCLATURE

AFs - aflatoxins

AFB1 - aflatoxin B1

AFB2 - aflatoxin B2

AFG1 - aflatoxin G1

AFG2 - aflatoxin G2

AFLP - Amplified Fragment Length Polymorphism

*a*W - water activity

BT2 - beta tubulin gene

CAST - Council for Agricultural Science and Technology

CF - calmodulin gene

CPA - cyclopiazonic acid

CYA - Czapek Yeast Autolysate agar

CZ20S - Czapek Yeast Autolysate agar with 20% of sucrose

DNA - deoxyribonucleic acid

EFSA - European Food Safety Authority

FAO - Food and Agricultural Organization

FDA - Food and Drug Administration

FBs - fumonisins

FB1 - fumonisin B1

FB2 - fumonisin B2

FL - fluorescence

GC - gas chromatography

HPLC - high-performance liquid chromatography

IARC - International Agency for Research on Cancer

ITS – internal transcribed spacer

MA - modified atmosphere

MAS - modified atmosphere storage systems

MALDI-TOF ICMS - Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Intact-Cell Mass Spectrometry

MAT1-1 - Mating type 1-1

MAT1-2 - Mating type 1-2

Mcm7 - minichromosome maintenance complex component 7

MEA - malt extract agar

MEA10 - malt extract agar with 10% of sodium chloride

NDA - naphthalene-2, 3-dicarboxialdehyde

OPA - ortho-Phthalaldehyde

OTA - ochratoxin A

PBS - phosphate buffered saline

ppm – part per million

RAPD - Random Amplification of Polymorphic DNA

RFLP - Restriction Fragment Length Polymorphisms

RPB2 - RNA polymerase

TLC - thin-layer chromatography

TFA - trifluoroacetic acid

UV - Ultraviolet

WHO - World Health Organization

YES - Yeast extract sucrose agar

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LIST OF ORIGINAL PAPERS

I. Soares, C., Rodrigues, P., Freitas-Silva, O., Abrunhosa, L., Venâncio, A., 2010. HPLC method for simultaneous detection of aflatoxins and cyclopiazonic acid. World Mycotoxin Journal, 3: 225-231.

II. Soares, C., Rodrigues, P., Peterson, S.W., Lima, N., Venâncio, A., 2012. Three new species of *Aspergillus* section *Flavi* isolated from almonds and maize in Portugal. Mycologia, DOI: 10.3852, in press.

III. Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R.R.M., Lima, N., Venâncio, A., 2007. Identification and characterization of *Aspergillus flavus* and aflatoxins. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Formatex, pp 527-534.

Some of the information used in this thesis was taken in from the published articles.

DISSERTATION STRUCTURE

This dissertation was divided into five chapters.

Chapter 1, entitled "Introduction", introduces the background and motivation of this work, and integrates the different aspects developed throughout the dissertation. The aim of the work is also presented in this chapter.

Chapter two, it is the state of the art based on research findings related to the topics covered during the present study. The goals of the dissertation are exposed according to the approached themes, which are:

i) The fungal and mycotoxin contamination of food commodities, with special emphasis on cereals;

ii) Post-harvest contamination of maize and the presence of aflatoxins (AFs), cyclopiazonic acid (CPA), fumonisins B1 and B2 (FB1, FB2) and ochratoxin A (OTA) in this commodity;

The subsequent structure follows the established working plan by dividing the work in additional three chapters. The three chapters have a similar structure where a small introduction, the materials and the methods used in the experimental phase, as well as the results and their discussion are developed.

In Chapter 3, “Sample Collection”, regional choice and climate are described as well as the materials and methodologies used during sampling.

Chapter 4, “Isolation and identification of *Aspergillus* species” focuses in the isolation, preservation and identification techniques used at this stage of the working plan.

In Chapter 5, entitled “Mycotoxin in maize samples”, includes the description of the different trials to develop a simultaneous mycotoxin extraction protocol for AFs, FBs and OTA from grounded maize.

As a supplement to the body of the work, 3 appendices give additional information.

Appendix 1 lists all *Aspergillus* section *Flavi* isolates obtained and used in this study with some of their main characteristics. **Appendix 2** lists all *Aspergillus* section *Nigri* isolates obtained and

used in this study with some of their main characteristics. **Appendix 3** includes several figures and tables that complement the data shown during the thesis.

CHAPTER ONE: INTRODUCTION

1.1 Background

Consumers make choices about the food products they acquire based on a number of factors, which the consumer perceives as quality. In addition to the price of the product, factors such as appearance, convenience, texture and smell influence their choices. More recently food safety has been increasingly attracting consumer's attention, such as the risk of food contamination by mycotoxins.

Mycotoxins are toxic compounds with known activity in animals, produced by some species of filamentous fungi, which may be present in food. Being natural contaminants, it is not possible to eliminate their presence completely, but their levels can and should be reduced so that they do not represent a risk to populations.

Over the last two decades various international inquiries on worldwide limits and regulations for mycotoxins were published. Although regulations over food production, processing, distribution and marketing may create benefits by increasing the safety level, these regulations can also increase producers costs and potentially raise food prices. There is, therefore, a dichotomy in the world with demands from the First World for uncontaminated quality food and the need from developing countries populations for food security (i.e., availability of food and one's access to it) and for the protection of their agricultural products from insect pests and fungal contamination, to get a minimum level of food safety.

Maize (*Zea mays* L) is one of the most important cereals of the world and provides more human food than any other cereal. Maize provides nutrients for humans and animals and serves as a base raw material for the production of several products such as starch, oil, alcoholic beverages, and, more recently, fuel. It is also a versatile crop, allowing it to grow across a range of agroecological zones. In Portugal, maize is one of the most important field crops of the country. In 2010, maize occupied 95 656 ha at national level, with a production of 657 960 tons (INE, 2010). However, it is also a commodity considered to be one of the most susceptible to mycotoxins world-wide.

Producing species have different ecological requirements, which will affect its distribution in the world and the occurrence of contaminations. With the clarification of the existing mycoflora, mycotoxins contamination can be prevented and appropriate control actions can be taken. A systematic approach to understand the problem is, therefore, required in order to combat the adverse effects of these mycotoxins. It should involve strategies for

prevention, control and quality control at all stages of production, from the field to the final consumer. Knowing the key critical points during harvest, drying and storage stages in maize production chain is essential to develop effective post-harvest prevention strategies and assure the protection of consumers and animal health.

1.2 Aim

The aim of this thesis is to contribute to the risk assessment of post-harvested contamination of maize kernels with aflatoxins (AFs), cyclopiazonic acid (CPA), Fumonisins B1 and B2 (FB1 and FB2, respectively) and ochratoxin A (OTA) in the studied regions. To this end, studies were made at 3 stages:

• Determination of maize mycoflora. The mycoflora of maize was studied in three stages of the storage chain in three distinct regions: Alto Alentejo, Beira Litoral and Ribatejo. The used method for assessing the total mycoflora was by direct plating. Thus, we sought to:

i) Identify the species of *Aspergillus* present in maize capable of producing relevant mycotoxins in maize, especially for OTA, AFs, CPA and FBs;

ii) Determine the variation with the geographic region of origin.

• Assessment of the mycotoxigenic potential of the isolated strains. The ability of strains to produce mycotoxins has been evaluated in laboratory facilities to see the potential toxigenic strains of potential producing species.

• Determination of AFs, CPA, OTA and FBs in milled maize. It was intended to develop a method for simultaneous extraction of AFs, CPA, FBs and OTA in maize. Since this was not achieved, it was developed a method for the simultaneous extraction of AFs, FBs and OTA, being CPA analysed in parallel.

CHAPTER TWO: BIBLIOGRAPHIC REVISION

2.1 Fungal contamination of food products

Fungi are a group of organisms that includes about 70 000 known species of a universe which is estimated to be 1.5 million. These organisms are ubiquitous, having evolved over time to occupy a wide range of ecological niches. They are present in virtually any environment such as soil, water, air and materials (Pelczar et al., 1996). Many fungi are harmful because they break down a variety of materials as they obtain food and can cause a number of diseases in plants and animals, including humans. However, contamination of food by microorganisms does not always result in its deterioration; many species have been used as a food source for humans, such as mushrooms and truffles and in fermentation of food commodities, as in the case of wine, beer, shoyu (soy sauce) and sake. Biotechnology also takes advantage of the metabolism of fungi making them to manufacture all types of special compounds with industrial applications (e.g. organic acids and enzymes) (Horn et al. 2009, Samson and Varga 2009).

Fungi have a great capacity for adaptation to different environmental conditions. On average, their water needs are lower than those of bacteria, and are more resistant to osmotic pressures due to strong presence in the midst of high concentrations of salt or sugar (Giorni et al., 2007). Fungi also tolerate the acidity and a wide range of temperature conditions (Magan, 2007).

Field fungi are those that can grow at 70 to 90% of relative humidity and temperatures around 20 to 25 °C. They usually require aW > 0.85 for active growth, and grow optimally at aW near 0.99. Examples of these fungi are *Alternaria*, *Cladosporium* and *Fusarium.* On the other hand, storage fungi are generally adapted to lower humidity levels and higher temperatures (Magan and Lacey, 1984). As showed in Figure 2.1, fungi like *Aspergillus* and *Penicillium* are major representatives of this latter group and, typically, *Aspergillus* grows at higher temperatures and at lower water activities (aW) than *Penicillium*. The minimal necessary aW for most *Aspergillus* and *Penicillium* species is 0.75-0.85, and they generally grow optimally at aW 0.93-0.98. *Aspergillus* requires aW as low as 0.73 for active growth demonstrating faster growth and producing spores that are often more resistant to light and chemicals, whereas *Penicillium* needs at least 0.78-0.80 (Magan, 2006).

Aspergillus is a large genus characterised by a distinctive round to elongate aspergilla bearing long chains of conidia which gives the fungus its characteristic morphology. It is composed of more than 180 accepted anamorphic species, with teleomorphs described in nine different genera (Rodrigues et al, 2007). Klich (2002) subdivided this genus in 7 subgenera, which in turn are further divided into Sections. Later, Peterson et al. (2008), subdivided this genus in 8 subgenera (Table 2.1).

Table 2.1 – Aspergillus taxonomy at a subgeneric level, adapted from Peterson et al. (2008)

Genus	Subgenus	Section
Aspergillus	*Aspergillus*	*Aspergillus*
		Restricti
	Circumdati	*Circumdati*
		Flavi
		Nigri
		Cremei
	Fumigati	*Fumigati*
		Clavati
		Cervini
	Nidulantes	*Nidulantes*
		Sparsi
		Usti
	Ornati	*Ornati*
	Candidi	*Candidi*
	Terrei	*Terrei*
		Flavipedes
	Warcupi	*Warcupi*
		Zonati

The genus *Aspergillus* has been described as ubiquitous for both substrate and geographical distribution and can be found in soil, where they contribute to the decomposition of dead organic matter, and can be found in commodities such as maize, peanuts, cotton, tree nuts, dried figs, sorghum, and other oil seeds (Ehrlich et al., 2007; Giorni et al., 2007; Iamanaka et al., 2007; Rodrigues et al., 2009). Fungi belonging to this genus can be either beneficial or harmful depending on the species and the substrate where they develop. Their ability to thrive in high temperatures and with relatively low aW makes them well suited to colonize a number of grain and nut crops.

Within this genus are some of the most important fermentation fungi, e.g., *A. sojae, A. oryzae* and *A. tamarii* that are used in oriental food fermentation processes and as hosts for heterologous gene expression (Campbell-Platt and Cook, 1989). In industry, isolates of *Aspergillus niger*, for instance, have a great economical and biotechnological interest and are extensively used for production of extracellular enzymes and organic acids such as citric acid (Schuster et al., 2002; Baker, 2006). *A. niger* and *A. oryzae* have been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government (Ward et al., 2006; Perrone et al., 2007; Pitt and Hocking, 2009).

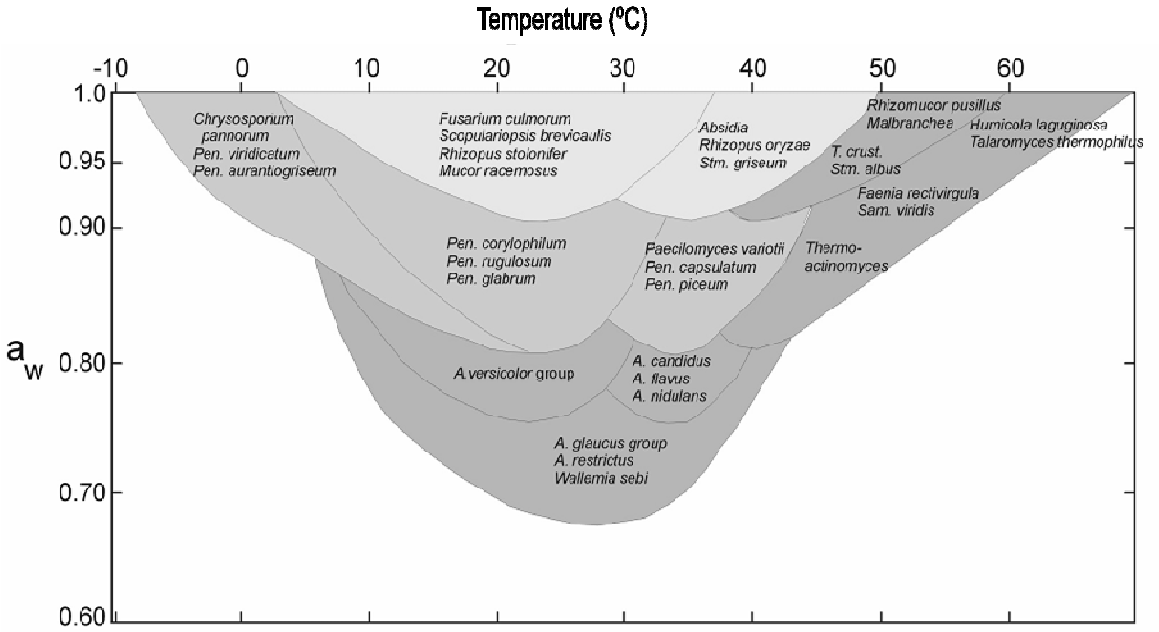


Figure 2.1- Prominent fungi and actinomycetes and their ability to grow at various temperatures and water activities (Serra, 2005).

Some *Aspergillus* can produce biologically active compounds, many of them toxic to plants and animals, including humans. These toxic compounds are also known as mycotoxins. The main mycotoxins produced by species belonging to *Apergillus* genus are: aflatoxins (B1, B2, G1, G2, M1, M2); sterigmatocystin; cyclopiazonic acid; ochratoxin A; patulin; aspergillic acid; citrinin; cytochasalin E; verruculogen and fumitremorgin A and B (Engenhart et al., 2002; Vaamonde et al., 2003; Sabater-Vilar et al., 2004; Magan et al., 2006; Frisvad et al., 2007).

Although the genus has been studied for several centuries, the systematic is complex and ever evolving. Most *Aspergillus* species have been described on the basis of morphological features which are based on the similarity of observable morphological (and usually physiological) characters, e.g., spore size and shape, or cultural characteristics (Samson and Varga, 2009). With the arrival of molecular technologies, there has been a tendency to overuse the genetic criteria to the description of species (Tibayrenc, 2006).

However, there isn't still a consensual approach and the trend is to use a polyphasic one, where all morphological, physiological, extrolite and DNA sequence data is considered together (Samson and Varga, 2009).

2.1.1 *Aspergillus* section *Flavi*

The group of *Aspergillus* subgenus *Circumdati* section *Flavi* has attracted much worldwide attention because of its industrial and mycotoxigenic potential. It is also one of the most studied because of the wide diversity of ecological niches that can occupy. Within this section, up until now, there are 34 species accepted (Table 2.1).

This group is divided into two subgroups of species, aflatoxins producing species which include *A. flavus*, *A. parasiticus* and *A. nomius* and another that does not include AFs producer species such as *A. oryzae*, *A. tamarii* and *A. sojae* (Hocking, 2006).

Section Flavi species occur in nature as saprophytes or as parasites of plants, insects and animals. *A. flavus* seems adapted to a wide variety of climates, habitats and substrates. While *A. flavus* is extremely widespread, the same does not apply to *A. parasiticus*. Aerial crops such as maize and cottonseed are infected predominantly with *A. flavus,* whereas peanuts, with their subterranean growth habit, and almonds are invaded by both *A. flavus* and *A. parasiticus* (Nesci and Etcheverry, 2002; Horn, 2005; Rodrigues, 2010). *A. nomius* is more common in tropical countries, being common in maize and brazil nuts (Ehrlich et al., 2007; Olsen et al., 2008).

Classic identification of *Aspergillus* section *Flavi* species has been based on morphological and biochemical characterization (Table 2.2). Most strains can be partly identified by conidial colour, seriation and conidial wall ornamentation; however, proper identification can only be done regarding the whole set of features.

In the aflatoxins producing group, both *A. flavus* and *A. nomius* can exhibit yellow-green colour, whereas *A. parasiticus* exhibits dark-green. *A. flavus* is predominantly biseriate, whereas *A. parasiticus* is uniseriate and *A. nomius* is predominantly uniseriate. *A. flavus* has smooth conidial walls whereas *A. nomius* has echinulate and *A. parasiticus* has rough conidial walls.

In the case of the non-aflatoxigenic goup, *A. oryzae* exhibits brown colour, *A. tamarii* dark-brown and *A. sojae* brown-green colour. *A. tamarii* can be either uniseriate or biseriate, this is a variable feature for *A. oryzae*, and *A. sojae* is uniseriate. Regarding the conidial walls ornamentation, *A. oryzae* has smooth walls, *A. tamarii* has echinulate and *A. sojae* has rough walls.

Further to the general morphological characteristics presented in Table 2.2, few species can still be divided in different chemotypes, according to variability within species, being the most relevant ones based on the mycotoxigenic profile or the sclerotia size in *A. flavus* strains.

In line with the mycotoxigenic profile five groups have been proposed: (i) chemotype I for AFBs and CPA producers; (ii) chemotype II for AFBs, AFGs and CPA producers; (iii) chemotype III for AFBs producers; (iv) chemotype IV for CPA producers and (v) chemotype V for non-producers (Vaamonde et al., 2003).

Based on morphological, genetic and physiological criteria, the aflatoxins producers from the *Aspergillus* section *Flavi* can also be divided into two types of strains. Isolates can be classified as L or S strains according to sclerotial morphology. S strains produce numerous small sclerotia (< 400 μm) and fewer conidia than L strains; L strains produce fewer, but larger sclerotia (Cotty, 1997). S strains produce relatively high levels of aflatoxins, while L strains produce only B1 and B2, or are atoxigenic (Egel et al. 1994).

Table 2.2 - Major morphological and biochemical characters used in the distinction of some species of *Aspergillus* within the section *Flavi*.

Species	Colony colour	Sclerotia	Conidia texture	Conidia size	Seriation[a]	AFBs[c]	AFGs[c]	CPA[c]
A. arachidicola	olive to olive-brown	-	echinulate	4.5-5	b or u	+	+	-
A. avenaceus	yellow to olive	+	smooth	4-5 x 3.2-4	b	-	-	-
A. beijingensis	olive-yellow	dnf[b]	verrucose	3.5-6.5	u	dnf	dnf	dnf
A. bombycis	green to bronze	dnf	rough	4-7	b	+	+	-
A. caelatus	olive to brown	+	coarse	5-6	u or b	-	-	-
A. coremiiformis	cream to brown	dnf	encrusted	6.9-9	b	dnf	dnf	dnf
A. flavus	yellow-green	+	smooth	3.5-4.5	b or b/u	+	-	+
A. lanosus	yellow	dnf	smooth	2.2-2.8	b	-	-	-
A. leporis	olive	+	smooth	3-3.5	b	-	-	-
A. minisclerotigenes	grayish-green	+	smooth	3-4	b	+	+	+
A.mottae	yellow-green	+	finely rough	3.3–4.3	b/u	+	+	+
A. nomius	yellow-green to olive-green	+	echinulate	4.5-6.5	u or u/b	+	+	-
A. novoparasiticus	yellow-green to olive	-	echinulate	3 – 7	u/b	+	+	dnf
A. oryzae	brown	-	smooth	4.5-8.0	variable	-	-	+
A. parasiticus	dark-green	+	rough	3.5-5.5	u	+	+	-
A. parvisclerotigenus	yellow-green	+	smooth	dnf	b	+	+	+
A. pseudocaelatus	olive to olive brown	-	echinulate	4.5–5	u or b	+	+	+
A. pseudonomius	dnf	-	echinulate	4–5	u	+	-	-
A. pseudotamarii	bronze to brown	+	rough	6.1-7.8	b or u	+	-	+
A. qizutongii	olive-yellow	dnf	smooth	5-6.5	u	dnf	dnf	dnf
A. robustus	dnf	+	echinulate	3.5-4.5 x 2.8-3.4	b	dnf	dnf	dnf
A. sergii	dark yellow-green	+	rough	3.3–4.3	u	+	+	+
A. sojae	brown-green	dnf	rough	5-6	u	-	-	-
A. subolivaceus	olive	+	smooth	4 x 3	b	dnf	dnf	dnf
A. tamarii	dark-brown	-	echinulate	5-8	u or b	-	-	+
A. terricola	olive to brown	dnf	echinulate	4.5-9	b or u	-	-	dnf
A. thomii	ochraceous	dnf	rough	3-5.5	b/u	dnf	dnf	dnf
A. transmontanensis	dark-green	+	echinulate	4.1–5.1	b/u	+	+	-
Petromyces albertensis	olive	+	smooth	2.3-3.5	b	-	-	-
P. alliaceus	brown	+	smooth	2.5-4	b/u	-	-	-

[a] u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate;

[b] dnf: data not found

[c] +: producer; - : non-producer

Table 2.3 – List of *Aspergillus* species belonging to the section Flavi, adapted from Rodrigues (2010).

Species	Type strain	Reference
A. flavus Link	CBS 100927	Link (1809)
A. oryzae (Ahlb.) E. Cohn	CBS 100925	Cohn (1883)
A. terricola É.J. Marchal	CBS 579.65	Marchal (1893)
A. parasiticus Speare	CBS 100926	Speare (1912)
A. tamarii Kita	CBS 104.13 (NRRL20818)	Kita (1913)
A. terricola var. *americanus*Marchal & É.J. Marchal	CBS 580.65	Thom & Church (1921)
A. avenaceus G. Sm.	CBS 109.46	Smith (1943)
A. thomii G. Sm.	CBS 120.51	Smith (1951)
A. sojae Sakag. & K. Yamada ex Murak.	CBS 100928	Sakaguchi & Yamada (1944) Murakami (1971)
A. alliaceus Thom & Church*	IMI 87209	Thom & Church (1945)
A. flavofurcatus Bat. & H. Maia	CBS 484.65	Batista & Maia (1955)
A. flavus var. *columnaris* Raper & Fennell	CBS 486.65	Raper & Fennell (1965)
A. subolivaceus Raper & Fennell	CBS 501.65	Raper & Fennell (1965)
A. leporis States & M. Chr.	CBS 151.66	States & Christensen (1966)
A. parasiticus var. *globosus* Murak.	CBS 260.67	Murakami et al. (1966)
A. lanosus Kamal & Bhargava	CBS 650.74	Kamal & Bhargava (1969)
A. coremiiformis Bartoli & Maggi	CBS 553.77	Bartoli & Maggi (1978)
A. robustus M. Chr. & Raper	CBS 428.77	Christensen & Raper (1978)
P. albertensis J.P.		Tewari Tewari (1985)
A. nomius Kurtzman, B.W. Horn & Hesselt.	CBS 260.88 (NRRL 13137)	Kurtzman et al. (1987)
A. caelatus B.W. Horn	CBS 763.97 (NRRL 25528)	Horn (1997)
A. beijingensis D.M. Li, Y. Horie, Yu X. Wang & R.Y. Li		Li et al. (1998)
A. qizutongii D.M. Li, Y. Horie, Yu X. Wang & R.Y. Li		Li et al. (1998)
A. bombycis S.W. Peterson, Yoko Ito, B.W. Horn & T. Goto	CBS 117817 (NRRL 26010)	Peterson et al. (2001)
A. pseudotamarii Yoko Ito, S.W. Peterson, Wicklow & T. Goto	CBS 766.97 (93MZ2D = IMI 86979 = NBRC 100702 = NRRL25397 = NRRL25517)	Ito et al. (2001)
A. parvisclerotigenus (Mich. Saito & Tsuruta) Frisvad & Samson	CBS 121.62 (NRRL A-11612 = IBT 3651 = IBT 3851)	Frisvad et al. (2005)
A. arachidicola Pildain, Frisvad & Samson	CBS 117610 (IBT 25020)	Pildain et al. (2008)
A. minisclerotigenes Vaamonde, Frisvad & Samson	CBS 115635 (IBT 27196)	Pildain et al. (2008)
Aspergillus novoparasiticus Gonçalves, Stchigel, Cano, Colombo & Guarro	CBS 126849 (LEMI 250)	Gonçalves e tal. (2011)
A. pseudocaelatus Varga, Samson & Frisvad	CBS 117616 (EF409242)	Varga et al. (2011)
A. pseudonomius Varga, Samson & Frisvad	NRRL 3353 (AF338643)	Varga et al. (2011)
A. mottae C. Soares, S.W. Peterson et A. Venâncio	CBS 130016 (MUM 10.231)	Soares et al. (2012)
A. sergii P. Rodrigues, S.W. Peterson, A. Venâncio & N. Lima	CBS 130017 (MUM 10.219)	Soares et al. (2012)
A. transmontanensis P. Rodrigues, S.W. Peterson, N. Lima & A. Venâncio	CBS 130015 (MUM 10.214)	Soares et al. (2012)

2.1.2 *Aspergillus* section *Nigri*

Species of the genus *Aspergillus* subgenus *Circumdati* section *Nigri* are widely distributed around the world with the capacity of developing in a vast variety of substrates. The black aspergilli are commonly found as soil organisms decomposing dead plant residues being pathogenic to several crops. Most of these fungi are associated with grapes, onions, maize, and peanuts, where they are cited as pathogens causing such diseases as peanut and maize seedling blight, and maize kernel rot (Palencia et al., 2010).

Many species are able to cause deterioration of food although some of them, as mention previously, are used in fermentation industries to produce organic acids, such as citric and gluconic acids, as well as hydrolytic enzymes like lipases and amylases (Abarca et al., 2004; Abrunhosa et al., 2010).

Aspergillus section *Nigri* is one of the more difficult group concerning classification and identification, and several taxonomic processes have been proposed. There is no doubt high biodiversity among this section as new molecular approaches are use, showing that species are occasionally difficult to recognise based solely on their phenotypic characters (Samson et al., 2007). This is particularly true within the *A. niger* aggregate, which consists of a group of morphologically indistinguishable species. According to Perrone et al. (2007) the most common isolates from grapes that constitute the *A. niger* aggregate are *A. niger*, *A. tubingensis*, *A. brasiliensis* and *A. foetidus*. In maize, *A. niger* is the most common isolate from the *Aspergillus* section *Nigri* followed by *A. awamori* (Magnoli et al., 2007) and by *A. japonicus*, in a very small percentage (Palencia et al., 2010).

Within the black aspergilli there were 19 species accepted by some authors (Samson et al., 2007; Noonim et al., 2008), but recently a few other species have been described and in a very recent revision by Varga et al. (2011b) the number of species in this section is higher: 28 species (Table 2.4).

Apart from their economic importance, black aspergilli are also important as mycotoxin producers, such as ochratoxin A (OTA) and more recently fumonisin B2 (FB2). Both mycotoxins occur in food and feed and have toxicological significance in human and animal diets (Astoreca et al., 2010; Abrunhosa et al., 2011b).

Several studies reported the presence of potential OTA producers *Aspergillus* section *Nigri* species in food and feed and consider *A. carbonarius* as the main OTA producer followed by *A. niger* (Palencia et al., 2010). Recent surveys indicate that these two black aspergilli are

the main source of ochratoxin A in major food products, including maize and wheat, in both tropical and subtropical zones of the world. The reported percentage of ochratoxigenic isolates belonging to the *A. niger* aggregate is much lower than *A. carbonarius* species (Khoury and Atoui, 2010). However, the incidence of *A. carbonarius* in comparison with *A. niger* is extremely reduced. *A. lacticoffeatus* and *A. sclerotioniger* are reported to also produce OTA (Abarca et al, 2001). As for FB2, up until now, only *A. niger* is known to produce it, and the studies have been centred mostly in strains isolated from grapes (Frisvad et al., 2007; Logrieco et al, 2009).

Table 2.4 – List of *Aspergillus* species belonging to the section *Nigri*.

Species	Type strain	Reference
A. niger Van Tieghem	CBS 554.65	Van Tieghem (1867)
A. japonicus Saito	CBS 114.51	Saito (1906)
A. awamori Nakaz.	CBS 557.65	Nakazawa (1915)
A. carbonarius (Bain.) Thom	CBS 556.65	Thom (1916)
A. foetidus Thom & Raper	CBS 121.28	Thom & Raper (1945)
A. aculeatus Iizuka	CBS 172.66	Iizuka (1953)
A. heteromorphus Batista & Maia	CBS 117.55	Batista & Maia (1957)
A. ellipticus Raper & Fennell	CBS 707.79	Raper & Fennell (1965)
A. tubingensis (Mosseray)Kozak.	CBS 134.48	Kozakiewicz, Z. (1989)
A. homomorphus Steiman, Guiraud, Sage & Seigle-Mur. ex Samson & Frisvad	CBS 101889	Samson et al. (2004)
A. piperis Samson & Frisvad	CBS 112811 (IBT 26239)	Samson et al. (2004)
A. sclerotioniger Samson & Frisvad	CBS 115572 (IBT 22905)	Samson et al. (2004)
A. lacticoffeatus Frisvad & Samson	CBS 101883 (IBT 22031)	Samson et al. (2004)
A. costaricaensis Samson & Frisvad	CBS 115574 (IBT 23401)	Samson et al. (2004)
A. vadensis Samson, R.P. de Vries, Frisvad & Visser	CBS 113365	De Vries et al. (2005)
A. coreanus S.B. Hong, Frisvad & Samson	CBS 119384	Hong et al. (2006)
A. ibericus Serra, Cabañes & Perrone	CBS 121593	Serra et al. (2006)
Aspergillus brasiliensis Varga, Frisvad & Samson	CBS 101740 (IMI 381727=IBT 21946)	Varga et al. (2007)
A. uvarum Perrone, Varga & Kozakiewic	IMI 388523 (CBS 127591=ITEM 4834=IBT26606)	Perrone et al. (2008)
A. aculeatinus Noonim, Frisvad, Varga & Samson	CBS 121060 (IBT 29077)	Noomin et al. (2008)
A. sclerotiicarbonarius Noonim, Frisvad, Varga & Samson	CBS 121057 (IBT 28362)	Noomin et al. (2008)
Aspergillus saccharolyticus Sørensen, Lübeck & Frisvad	CBS 127449 (IBT 28509)	Sørensen et al. (2011)
A. acidus Kozak	CBS 564.65	Varga et al. (2011b)
A. eucalypticola Varga, Frisvad & Samson	CBS 122712	Varga et al. (2011b)
A. fijiensis Varga, Frisvad & Samson	CBS 313.89	Varga et al. (2011b)
A. indologenus Frisvad, Varga & Samson	CBS 114.80	Varga et al. (2011b)
A. violaceofuscus Gasperini	CBS 123.27	Varga et al. (2011b)
A. neoniger Varga, Frisvad & Samson	CBS 115656	Varga et al. (2011b)

2.2 Natural occurrence of Mycotoxins

Mycotoxins can be defined as natural products that evoke a toxic response in higher vertebrates and other animals when fed in low concentrations. Mycotoxins can cause acute toxic, mutagenic, teratogenic, estrogenic and carcinogenic effects on animals. Over 300 mycotoxins have been identified but only those implicated in potential health risk for humans have been studied in detail (Bennett and Klich, 2003). According to the CAST report (CAST, 2003), there are 20 mycotoxins of concern: aflatoxins, trichothecenes (DON and T2 toxin), fumonisins, zearalenone, ochratoxin A, ergot alkaloids, cyclopiazonic acid, sterigmatocystin, gliotoxin, citrinin, penitrems, patulin, fusarin C, fusaric acid, penicillic acid, mycophenolic acid, roquefortine, PR toxin, and isofumigaclavines.

Mycotoxin production in crops is highly susceptible to environmental, chemical and biological factors (Figure 2.2). Conditions for mycotoxin production are usually more restrictive than those for fungal growth. Toxin-producing fungi can invade food at pre-harvest period, harvest-time or post-harvest during storage. Colonization and eventual contamination may occur in the same stage or a later one (Logrieco et al., 2003).

Contamination during crop storage may be affected by changes in temperature and water activity that allow ecological succession of different fungi. Different species hence require different conditions for optimal growth and mycotoxin production. The factors influencing the growth and toxin formation in fungi do not act singly but in a multiples. There is an interaction between the amount of inoculum, temperature, substrate moisture, physical condition of the substrate, and growth of other microorganisms. In a short period of time these conditions may be greatly altered with certain factors having more influence than others (Hesseltine, 1976).

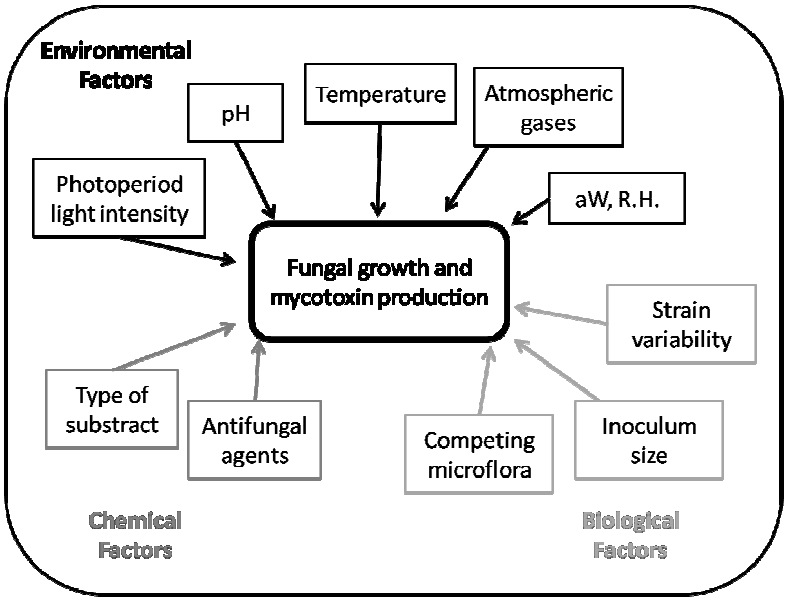


Figure 2.2- Principal factors influencing fungal growth and mycotoxin production (adapted from Martí, 2006).

In general, mycotoxins are produced optimally at 24-28 °C, but some mycotoxins require different temperatures. Table 2.5 shows the optimum temperatures and water activities, for the production of some mycotoxins. These are ranges, which are species dependent.

Table 2.5 – Optimum temperature and aW for mycotoxin formation.

Mycotoxins	Temperature for mycotoxin formation (°C)	Water activity (aW) for mycotoxins formation
Aflatoxins	30-33	0.99-0.996
CPA	25-30	0.98-0.996
OTA	20-31	0.98
Fumonisins	15-30	0.94-0.98
DON	15-25	0.97-0.99
ZEA	20	0.96-0.98
Patulin	20	0.95

Although there are geographical and climatic variations in the production and occurrence of mycotoxins, exposure to these substances occurs worldwide and it is estimated that many of the world's food is contaminated to some extent (Figure 2.3). Contamination of food with mycotoxins is especially relevant when a given population has as nutritional base one type of food product (eg, maize). If this source is contaminated, the population is continually exposed to the mycotoxin and can lead to the occurrence of serious chronic mycotoxicoses (Table 2.6).

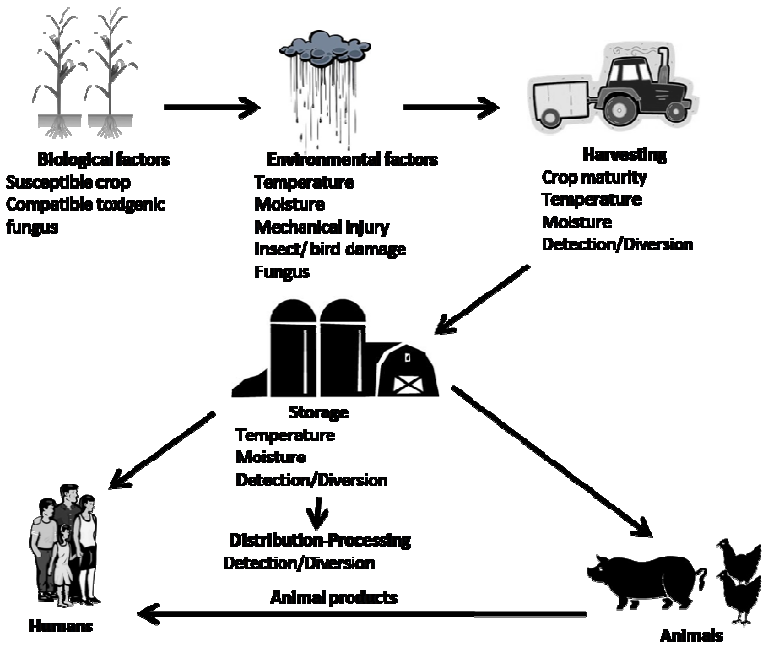


Figure 2.3 – Factors affecting mycotoxin occurrence in the food chain (adapted from Paterson and Lima, 2010).

Mycotoxicoses can be categorized as acute or chronic. Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time period, resulting in cancers and other generally irreversible effects (Bennett and Klich, 2003). Its severity depends on the type of mycotoxin, the extent of exposure such as duration and dose, age, nutritional status and health of the affected individual (Bhatnagar et al., 2002).

It was in the early 1960's, with the turkey X disease that mycotoxins attracted attention and their implications for human and animal health. Thousands of turkeys in England died, due to an event designated as turkey X, disease since the cause of death of the animals was unknown. Research efforts showed that the death was due to ingestion of feed contaminated with a toxic metabolite produced by a fungus, *Aspergillus flavus*. The chemical toxic compound was isolated, identified and named as aflatoxin. Subsequent studies have helped confirm that several human diseases were due to the ingestion of mycotoxins (CAST, 2003) such as alimentary toxic aleukia (ATA), whose main effects were due to the ingestion of flour contaminated with trichothecenes.

Nowadays, mycotoxins still contaminate the diet of a large proportion of the world's population (CAST, 2003). In a recent study, Wild and Gong (2010) considered mycotoxins as a

largely ignored global health problem. In their opinion this problem is more severe in low-income countries where agriculture practices and regulations to control human exposure are the least adapted to do so. In general, mycotoxin exposure is more likely to occur in parts of the world where poor methods of food handling and storage are common and where malnutrition is a problem (Bennett and Klich, 2003).

Table 2.6- Selected mycotoxins and their human and animal mycotoxicoses, adapted from Bhatnagar et al. (2002)

Mycotoxin	Main producing fungi	Toxic effect	Mycotoxicosis
Alternaria (tenuazonic acid, alternariol)	*A. alternate*	Apoptosis mutagen	Onyalai disease
Aflatoxin	*A. flavus* *A. parasiticus*	Apoptosis Mutagen Hepatotoxin Carcinogen teratogen	Acute aflatoxicosis Hepatocarcinogenesis, childwood cirrosis Rye's syndrome
Cyclopiazonic acid	*A. flavus* *Penicillium aurantiogriseum*	Nephrotoxin Cardiovascular lesion	Kodua poisoning
Ergot alkaloids	*Claviceps sp.* *A. fumigates* *P. chermesinum*	neurotrophy	St. Anthony's fire ergotism
Fumonisins	*Fusarium verticillioides* *A. niger*	Carcinogen Teratogen	Leukoencephalomalacia Pulmonary edema
Moniliformin	*Fusarium verticillioides*	Neurotoxin Cardiovascular lesion	Onyalai disease
Ochratoxin	*A. ochraceus* *A. westerdijkiae** *A. niger* *A. alliaceus* *A. terreus* *P. verrucosum* *P. nordicum*	Apoptosis Nephrotoxin teratogen	Balkan nephropathy Renal tumors
Penicillic acid	*P. aurantiogriseum* *A. ochraceus*	Neurotoxin mutagen	-
Roquefortine	*P.roqueforti*	neurotoxin	-
Rubratoxin	*P.rubrum* *P. purpurogenum*	Apoptosis Hepatotoxin teratogen	-
Sterigmatocystin	*A. versicolor* *A. nidulans* *A. parasiticus* *A. flavus*	Hepatotoxin Carcinogen	Hepatocarcinogenesis
Trichothecenes (T-2, deoxynivalenol)	*F. sporotrichioides* *Microdochium nivale* *S. atra*	Apoptosis Dermatoxin Neurotoxin Teratogen	Alimentary toxic aleukis, Akakabi-byo disease, stachybotryotoxicosis, esophageal cancer, red mold disease, yellow
Zearalenone	*F. graminearum*	Genitotoxin Estrogenic effects Mutagen	Cervical cancer Premature menarche

* Several isolates previously identified as *A. ochraceus*, including the original OTA-producing isolate (NRRL 3174) are now identified as *A. westerdijkiae*. Curiously, NRRL 3174 strain is now the *A. westerdijkiae* type strain.

2.2.1 Aflatoxins

Aflatoxins are the best characterized group of mycotoxins. They occur mostly in tropical and sub-tropical regions with high humidity and temperature and accumulate post-harvest when food commodities are stored under conditions that promote fungal growth.

The main naturally occurring aflatoxins are called B1, B2, G1 and G2. Aflatoxins are a group of polyketide-derived bis-furan-containing dihydrofuranofuranand tetrahydrofuran moieties (rings) fused with a substituted coumarin (Figure 2.4) and are detected by photophysical properties such as absorption or emission spectra. They show characteristic absorption at 360 nm which is the absorption maximum of AF. The “B” in AFB1 and AFB2 and “G” in AFG1 and AFG2 are abbreviations for blue (425 nm), and green-blue (540 nm) fluorescence respectively under UV irradiation (Do and Choi, 2007). AFB1 and AFG1 are much less fluorescent than AFB_2 and AFG2 and require to be converted into more fluorescent derivatives for sensitive detection (Muscarella et al., 2009). The most convenient system for analysis is the on-line post-column photochemical derivatization system (PHRED), which provides the derivative using UV light at 254 nm (Walking and Wilson, 2006).

Aflatoxin B1 (AFB1) is the most common mycotoxin found in human food and animal feed. AFB1 is a potent hepatocarcinogen that can induce tumors in many species of animals, including rodents, nonhuman primates, and fish. While the liver is the major target organ, under certain circumstances, significant numbers of tumors have been found in lung, kidney, and colon (Wang and Tang, 2005).

There are several outbreak reports of acute aflatoxins poisoning with severe repercussions to humans. In 1994, 400 Indians were affected with hepatitis and it was traced to heavily contaminated maize with *Aspergillus flavus* and containing high concentrations of aflatoxins. In Kenya, more recently, 375 people fell ill and 125 died, after consuming aflatoxin contaminated maize (Pitt and Hocking, 2009).

When aflatoxin B1 and B2 are ingested by lactating cows, a proportion (about 1.5%) is hydroxylated and excreted in the milk as aflatoxins M1 and M2 compounds of lower toxicity than the parent molecules, but significant because of the widespread consumption of cows' milk by infants (Pitt, 2000).

The most significant aflatoxin producing fungi are *Aspergillus* species belonging to section *Flavi.* These species are the most intensively studied due to the impacts on animal and human health. Aflatoxins are produced predominantly by some strains of *Aspergillus flavus*

and *Aspergillus nomius* and by most strains of *Aspergillus parasiticus* (Rodrigues et al., 2009). A number of other species, while toxigenic, are less commonly isolated, as it is the case of *A. pseudotamarii*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes* and *A. arachidicola*.

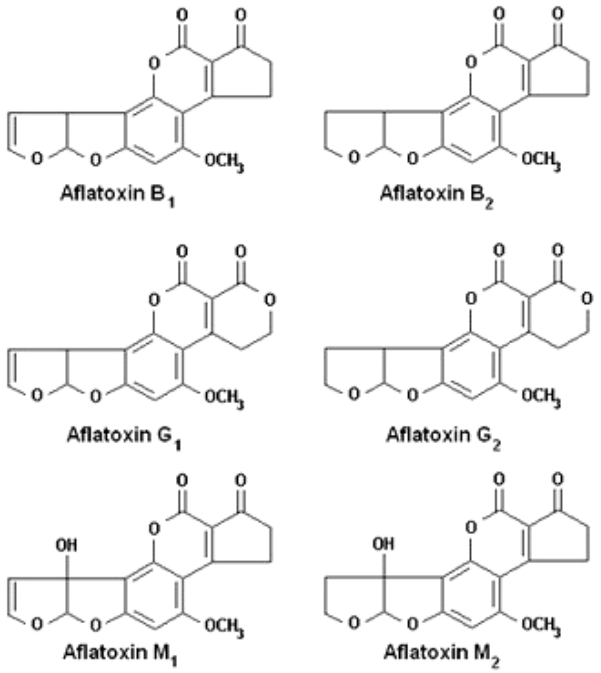


Figure 2.4 – Structure of aflatoxins (B1, B2, G1, G2, M1 and M2).

Aflatoxins are highly regulated, with rigorous limits imposed by European Union legislation where a maximum level of 2 µg/kg for aflatoxin B1 and of 4 µg/kg for total aflatoxin has been established in all cereals and all products derived from cereals with the exception of maize to be subjected to sorting or other physical treatment before human consumption, for which a maximum level of 5 µg/kg for aflatoxin B1 and 10 µg/kg for aflatoxin total has been established (Commission Regulation nº 165/2010). These limits can increase in countries outside the EU. In North America, for instance, the maximum allowed level for the sum of aflatoxins in maize is 20 µg/kg. The legislation, in most Asian, African and Middle East countries, focus on the presence of AFB1, being the limits superior to the ones imposed by the EU (van Egmond et al., 2007; www.knowmycotoxins.com/regulations.htm).

2.2.2 Cyclopiazonic acid

Cyclopiazonic acid (CPA) is produced predominantly by *A. flavus* strains. Other *Aspergillus* such as *A. tamarii* and *Penicillium* species are also known producers (Le Bars, 1979; Gqaleni et al., 1997; Frisvad et al., 2006; Maragos, 2009a). CPA occurs naturally in a wide variety of crop products alone or as a co-contaminant with AFs. It is an indole tetramic acid (Figure 2.5) and chromatographic detection involves measuring absorbance in the UV region, between 279 to 284 nm (Goto et al., 1996; Maragos, 2009a).

Although CPA toxicity data relative to humans are not available in the literature, the biological effects of CPA in several species have been thoroughly described elsewhere (Bryden 1991). Norred et al. (1988) reported a study where birds given CPA had reduced weight gains**,** and the severity of the effect was dose dependent. They assessed that, since CPA accumulates to a significant degree in edible tissues, further studies should be carried out on the effects of chronic exposure of animals and humans. CPA may be also transferred to milk and eggs after oral administration to lactating ewes and laying hens (Dorner et al. 1994), which makes the ingestion of such contaminated animal by-products a potential source of human exposure.

Together, CPA and AFs have been shown to cause health problems in animals and humans, resulting in important economic losses (Bamba and Sumbali, 2005; Lee and Hagler 1991; Urano et al., 1992). Their combination can produce degenerative changes, tremors, and liver, pancreas, spleen and kidney necrosis (Ohomomo et al., 1987; Gqaleni et al., 1997; Pinto et al., 2001). No specific regulations or official recommendations have yet been created for CPA.

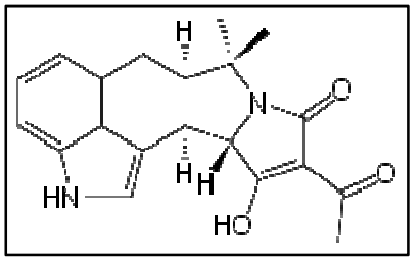


Figure 2.5 - Structure of cyclopiazonic acid.

2.2.3 Ochratoxin A

Ochratoxin A (OTA) has been reported as naturally occurring in almost all cereals including wheat, maize, barley, oats and cereal products (Jorgensen et al, 1996). OTA has also been found in many other food commodities such as coffee, wine, beer, dried fruits, spices and herbs (Pittet, 1998). Cereals and cereal based food and feed are the main contributors to OTA intake in humans and animals, since OTA is stable under normal food processing operation conditions and it is carried-over from raw-materials to processed products (Magnoli et al., 2007). OTA is a potent nephrotoxin and has teratogenic, immunosuppressive and carcinogenic properties, belonging, therefore, to the 2B group, of substances potentially carcinogic to humans (Cabañes et al. 2002; IARC, 1993a).

The economically most important producers belong to *Aspergillus* sections *Circumdati* and *Nigri*. OTA is believed to be produced in nature by three main species of fungi, *Penicillium verrucosum, Aspergillus westerdijkiae* (previously *A. ochraceus*) and several related *Aspergillus* species, and *A. carbonarius* with a small contribution by *A. niger*, where only a minor percentage of isolates are able to produce OTA. These three groups of species differ in their ecological niches, in the commodities affected, and in the frequency of their occurrence in different geographical regions (JECFA, 2001). *P. verrucosum* incidence is more common in the cool damp conditions of northern Europe, at temperatures below 30 °C, while *A. westerdijkiae* is more important in warmer climatic regions of the world (Magan and Aldred, 2005). The ability to produce this mycotoxin by some species of *Aspergillus* section *Nigri*, being *A. carbonarius* the main producer, extends the concern from the temperate zone to the tropical and sub-tropical zones due to the distribution of these black species. Because of *A. carbonarius* black spores, it is highly resistant to sunlight and survives sun-drying. Conditions for OTA production are at maximum detected at 20 °C, for *A. carbonarius*. High values of aW (0.95-1 aW) favours OTA production by all species of black aspergilla being rarely detected below 0.90 aW (Martí, 2006).

Other species like *A. alliaceus*, *A. steynii* and *P. nordicum* are also consider major OTA producers in food and feed products (Abrunhosa et al., 2010).

Structurally, OTA consists of a para-chlorophenolic group containing a dihydroisocoumarin moiety that is amide-linked to L-phenylalanine (Figure 2.6).

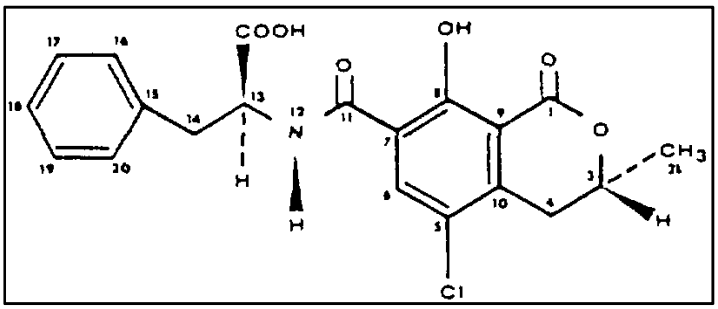


Figure 2.6 – Structure of ochratoxin A (OTA).

OTA is regulated with limits imposed by EU legislation where the maximum levels depend of the targeted commodity. In unprocessed cereals the maximum level is of 5 µg/kg, being reduced in cereal products to a maximum level of 3 µg/kg. In cereal product destined for children consumption the maximum level is of 0.5 µg/kg (Commission Regulation (EC) nº 1881/2006).

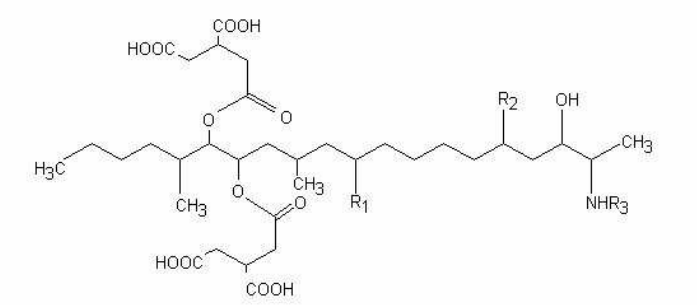
2.2.4 Fumonisins

It was in 1988 when fumonisins were first identified and isolated and so far there are 28 fumonisin analogues known. Fumonisins are divided into four groups: Series A, B, C and G. With regard to their toxicity the B-type fumonisins represent the most important ones. In naturally contaminated food and feed fumonisin B1 represents about 70% - 80% of the total fumonisin content (Rheeder et al., 2002). Of the currently identified fumonisins, the B1 (FB1), B2 (FB2) and B3 (FB3) are the most frequently detected in fungal cultures or in naturally contaminated maize (Orsi et al., 2000). Fumonisin B2, when compared to fumonisin B1, is less cytotoxic (Gutleb et al., 2002).

Fumonisins are widely distributed geographically, and their natural occurrence in maize has been reported in many areas of the world. These toxins are capable of causing significant disease in horses and swine and have been shown to be carcinogenic in rats and mice. The risk of fumonisins to humans was evaluated by the International Agency for Research on Cancer in 1993, and the toxins produced by *F. verticillioides* were evaluated as "Group 2B carcinogens," i.e., probably carcinogenic to humans (Cast, 2003; IARC, 1993b; Gelderblom et al., 1993; Sanchis et al., 1995).

Fumonisins are a group of toxins produced mainly by *Fusarium verticilloides* and *F. proliferatum*. These species grow over a wide range of temperatures, but only at relatively high water activities (about 0.9 aW), being more frequently associated with field contamination and not as hazardous as *Aspergillus* or *Penicillium* species in storage commodities. However, recent reports state that *Aspergillus niger* can also produce fumonisin B2 (FB2) (Frisvad et al., 2007; Logrieco et al., 2009; Mogensen et al., 2010), making FB2 a mycotoxins of concern in more broader conditions. Mogensen et al. (2009) showed that FB2 production by these species is higher while growing in low water activity (aW) culture media, when comparing with its production by *Fusarium* producing species.

Fumonisins are a group of structurally related compounds. FB1 is the diester of propane-1,2,3-tricarboxylic acid and 2S-amino-12S,16R-dimethyl-3S,5R,10R,14S,15R-pentahydroxyeicosane in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3 tricarboxylic acid. FB2 is the C-10 deoxy analogue of FB1 in which the corresponding stereogenic units on the eicosane backbone have the same configuration (Figure 2.7).



Fumonisina	R1	R2	R3
B1	OH	OH	H
B2	H	OH	H
B3	OH	H	H
B4	H	H	H
A1	OH	OH	CH3CO
A2	H	OH	CH3CO

Figure 2.7 – Basic structure of fumonisins series A and B, and list of known analogs with the corresponding side chains.

Fumonisins are regulated with limits imposed by EU legislation where a maximum level for the sum of FB1 and FB2 in unprocessed maize is imposed in 4 mg/kg, reducing considerably when the maize products are for direct human consumption (0.8 mg/kg) or for children (0.2 mg/kg) (Commission Regulation (EC) nº 1881/2006).

2.3 Economic Importance of Maize

2.3.1 World Economic Importance

Maize (*Zea mays* L.) is a cereal crop that is grown widely throughout the world in a range of agroecological environments. No other grain is produced annually as much as maize. About 50 species exist and consist of different colours, textures and grain shapes and sizes. White, yellow and red are the most common types. Maize is a rich source of starch (60-68 %), protein (8-12 %), fat (3-5 %) and minerals (1-2 %). The cultivation of corn is thought to have most probably originated in Central America, particularly in Mexico, from where it spread northward to Canada and southward to Argentina (FAO, 1992).

There has been continuous increase in the demand of maize mainly owing to increase in the demand from meat and starch sector. There is also a growing requirement of maize in poultry sector, which uses maize as feed. In some parts of the world, maize is used as food grain for human consumption. In Industry, maize is used in refineries for producing products such as maize oil, gluten for animal feed, maize starch, syrup, dextrose (used mainly by the pharmaceutical industry as a starting material for manufacturing Vitamin C and penicillin), alcohol for beverages, ethanol, fructose maize syrup used mainly by the soft drink industry, biodegradable chemicals and plastics, rubber, paper, textiles, ready-to-eat snack foods and breakfast cereals, cornmeal, flour and additives in paint and explosives. It is estimated that maize yields 4000 industrial products (Vishwanatha, 2005).

Significant increases in world maize production is in part due to additional land being cultivated, but more significantly as a result of the use of genetically improved cultivars, more efficient field practices and fertilizer application, and the introduction of more highly reproductive maize varieties (FAO, 1992).

The world average production of maize was, in 2010, around 671.37 million tonnes covering an average area of 143.74 million hectares with an average yield of 4,670 kilogram per hectare.

Among the major exporters of maize, USA dominates the international trade followed by China, the European Union, Argentina and Brazil. Major importing nations of maize are Japan, Korea, Taiwan, Mexico, Egypt, Malaysia, European Union and Colombia. World's major producers are showed in Figure 2.8.

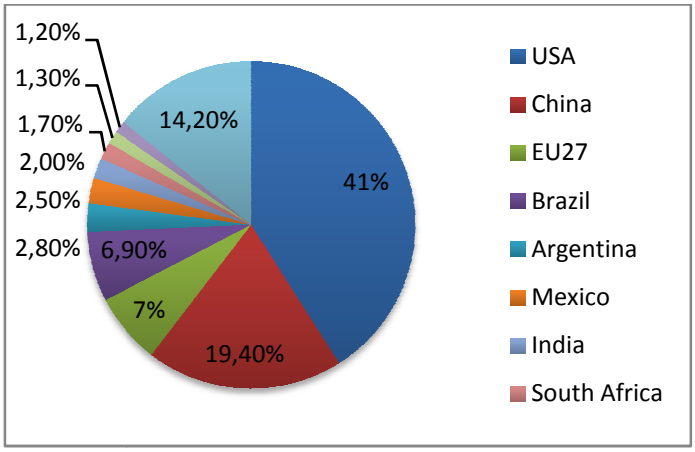


Figure 2.8 – World maize production in 2009/2010 (Adapted from http://www.grains.org/corn).

2.3.2 Economic Importance in Portugal

Maize is a crop with great tradition in Portugal and, as can be seen in Table 2.2, is the first grain crop in terms of production. Most maize production occurs in Alentejo region, soon followed by the Centre and North regions (Figure 2.9). In the autonomous regions of Azores the production is almost nonexistent and it is not produced in the Region of Madeira.

In 2009, low soil moisture content and reduced water for irrigation purposes delayed spring crops. This, together with a scenario of falling maize prices and rising production costs, has contributed to the smallest area of maize harvested for grain in the most recent decades, with a 12.0% decline from 2008 (INE 2010a, INE 2010b).

Nevertheless, maize continues to have a major role in the economy of the country, even though the production is insufficient and imported maize is needed.

Table 2.7 – Values of production surface (ha) and production (tonnes) of the most important cereals in Portugal in 2006, 2008 and 2010 (INE 2008, INE 2010a, INE 2011).

Cereal	2006		2008		2010	
	Production area (ha)	Production (tonnes)	Production area (ha)	Production (tonnes)	Production area (ha)	Production (tonnes)
Wheat	104 684	249 610	88 313	203 332	57 727	82 577
Maize	**102 746**	**534 700**	**109 640**	**699 666**	**95 656**	**657 960**
Oats	53 674	87 108	55 232	92 422	61 748	66 145
Barley	44 154	105 547	43 080	99 824	20 224	30 620
Rice	25 392	148 673	26 334	150 680	29 120	170 216
Rye	23 476	23 802	21 324	22 213	20 441	18 202
Triticale	19 228	40 236	na	na	24 487	25 871

na - Not available

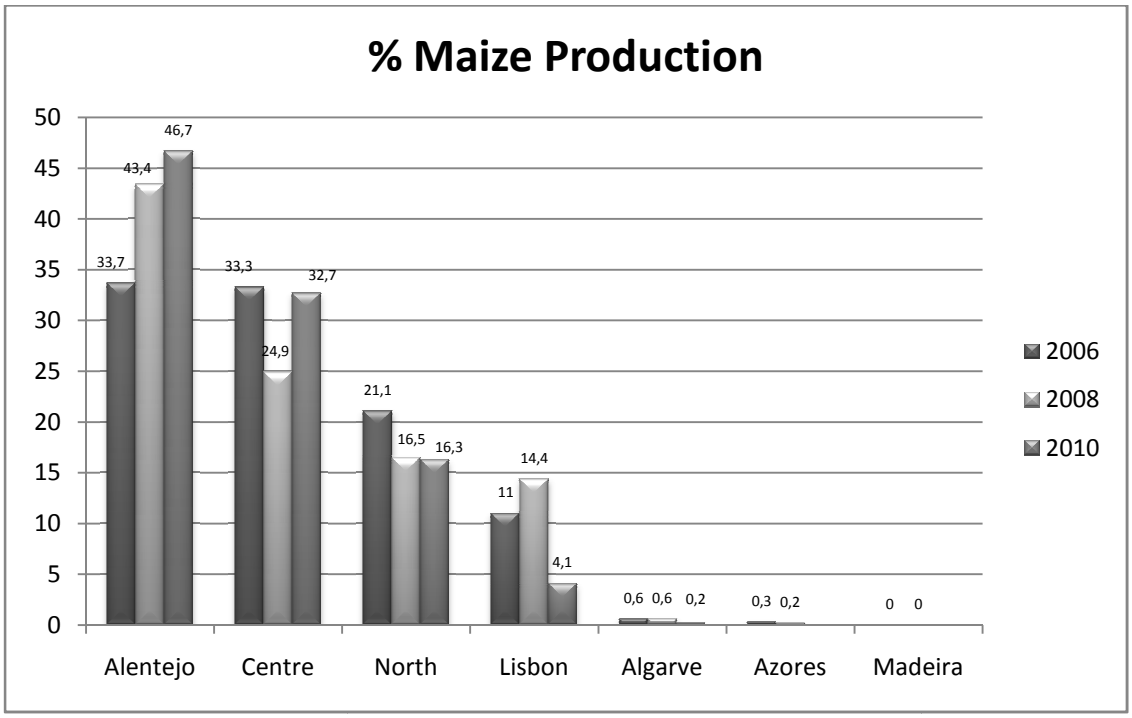


Figure 2.9- Distribution of maize production (% of national production) between regions, in 2006, 2008 and 2010 (INE).

2.4 Mycotoxin contamination of maize

As previously referred, maize constitutes one of the worldwide most important crops, being, however, a good substrate for growth, development and activity of filamentous fungi (Janardhana et al., 1999; Oyebanji and Efiuvweywere, 1999). It is associated with a large number of fungal species belonging to the genders of *Aspergillus, Fusarium,* and *Penicillium*, that can cause spoilage and mycotoxin contamination (Kumar et al., 2008; Marín et al., 1998; Richard et al., 2009).

Concerns about mycotoxin occurrence in maize are usually associated to *Aspergillus flavus*, for its ability to produce aflatoxins, and to *Fusarium verticillioides* and *F. proliferatum*, for the production of fumonisins (Giorni et al., 2007; Chulze, 2010). Other fungi associated with this commodity are the ochratoxigenic ones - *A. westerdijkiae, Penicillium verrucosum* and black *Aspergillus* (Shotwell et al., 1969; van der Merve et al., 1965, Palencia et al., 2010).

A synergistic relationship was found between *A. flavus* and *F. verticillioides* when they were inoculated on maize ears (Zummo and Scott, 1992). Whereas *F. verticillioides* affected *A. flavus* aflatoxin production, *A. flavus* did not affect fumonisin production by *F. verticillioides*. However, aflatoxin production was significantly lowered on ears co-inoculated with both isolates compared to those inoculated only with *A. flavus*. Wicklow et al. (1988) suggested that the presence of competing fungi on some kernels and not all, could explain why kernels with high aflatoxin levels may be located next to toxin-free kernels.

Pre-harvest invasion by fungi occurs primarily by plant host-fungus and other biological interactions (e.g. insects), while post-harvest fungal growth is ruled by crop (nutrients), physical (temperature, moisture) and biotic factors (insects, interference competition).

2.4.1 Pre-harvest contamination

Fusarium species are predominantly considered as field fungi and fumonisins are formed in maize before harvest or during the early stage of drying. Except under extreme conditions, the concentrations of fumonisins do not increase during grain storage (JEFCA, 2001).

A. flavus can infect maize pre- and post-harvest, which means that under some circumstances maize can be already contaminated with aflatoxins at harvest time. In a

seasonal survey of the mycoflora associated with maize grains in standing maize crops, it was shown that the environmental conditions had a marked effect on the quality and density of fungi. In the winter, when the moisture ranged between 22% and 30% species of *Fusarium* were dominant. The *A. flavus* incidence was comparatively lower (17%) in colder months. However, with the rise in temperature during summer, when grain moisture ranged between 18% and 22%, *A. flavus* dominated and had 72% of incidence (Sinha and Bhatnagar, 1998). *A. flavus* spores can germinate on the stigma surfaces of the plants and the germ tube penetrates the developing embryo in a manner mimicking pollen germ tubes. Under stress, like drought, significant levels of aflatoxins can be produced in the plant tissue during growth in the field (Moss, 2002).

2.4.2 Post-harvest contamination

There is a profound change in the grain ecosystem at harvest, from the environmental extremes of the field to the comparatively steady environment of storage. It also supplies an opportunity for the redistribution of fungal inoculums in the grain and for the introduction of further inoculums (Magan and Lacey, 1984).

Stored maize is a man-made ecosystem in which quality and nutritive changes can occur due to the interactions among physical, chemical and biological factors (Figure 2.10). The deterioration and loss of quality in stored grain is usually a result of the action of fungi, insects and mites - acting individually or in different combinations at various times of the storage period (Samapundo, 2006). The environmental factors of temperature, water availability and gas composition influence not only the rate of fungal spoilage but also the production of mycotoxins (Magan, 2003).

The *Aspergilli* are generally considered as the most important fungal contaminators of maize during storage (Bankole and Mabekoje, 2004). This has been attributed to their ability to grow and produce mycotoxins at lower water availability conditions than *Fusarium* spp., which dominate the pre-harvest period (Magan and Lacey, 1988).

Although the *Fusarium* spp. are predominantly considered as field fungi, Marín et al. (2004) reported that fumonisin production may occur post-harvest when the storage conditions are inadequate. In a wide range of water activities (0.968, 0.956, 0.944, 0.925) and temperature (25 °C and 30 °C) there can be an increase in FB1 and FB2 concentration with

time (Marín et al., 1995). This can be prevented if there is no delay in drying maize to the desired moisture content of 14-15%.

Stored maize is also highly susceptible to aflatoxins if the phases of drying and storage are poorly managed. In order to reduce aflatoxins levels, prevention is the key point. Sorting cobs that are damaged, insect infested or moulded grains from the rest of the grain can help preventing an additional increase in aflatoxins levels (Hell et al., 2008).

Opposite to the pre-harvest conditions, post-harvest handling of grain presents many more opportunities for controlling fungal growth and its consequences. In the case of aflatoxins, for example, it is not formed until at least 48 hours after spore germination. This delay means that maize, with a moisture level optimal for the growth of A. *flavus,* will not be dangerous even when heavily inoculated if the maize can be dried to below 13% within 48 hours (Hesseltine, 1976).

Hence, careful drying of grains and good storage management may minimize post-harvest fungal growth and, consequently, mycotoxin production (Degirmencioglu et al., 2005). This is not the case in developing countries, where traditional storage practices lacking suitable storage structures for maize and where the absence of storage management technologies are more common. Tefera et al. (2011) refer the use of metal silos as a way to minimize post-harvest losses and thereby increase food production efficiency. Metal silos are hermetically sealed structures and air tight. Besides this, have the advantage of, according to FAO (2008a), being relatively cheap and needing very little maintenance. Generally silos lasts about 20 years, and a well-maintained one can last up to 30 years.

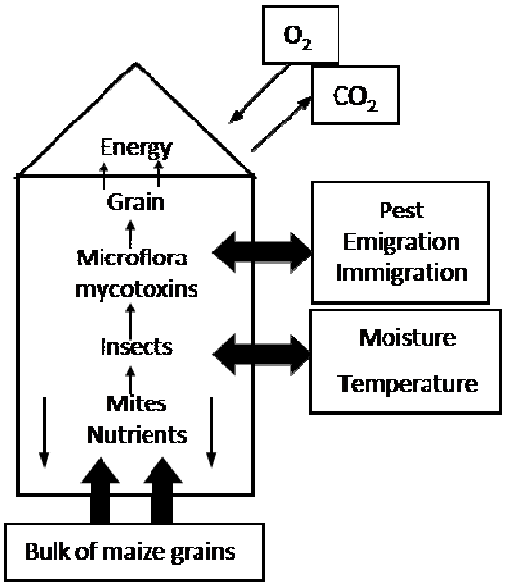


Figure 2.10 – Interactions between biotic and abiotic factors in stored grain ecosystems (adapted from Magan et al., 2003).

In Portugal maize is normally harvested with a water content between 18–22% (aW 0.90–0.93), so that to reduce or prevent the production of most mycotoxins, drying should take place as soon after harvest and as rapidly as possible, to values between 13-15%. Traditional practices, such as maize drying in threshing floors and storage in wooded structures, are common for small farmers where the entire production is for internal consumption. Major producers, however, store in metal silos, where they can control temperature and water content, so the product can have a longer shelf-life and be safely commercialized.

2.4.3 Post-harvested control strategies

The only way to minimize the impact of pre-harvest natural contamination is through the application of post-harvest processing techniques, which will reduce subsequent entry into the food and feed chain. Post-harvest prevention strategies can only be effective for mycotoxins that are formed during this component of the food chain (Magan and Aldred, 2007). Good storage is vital to minimise post-harvest losses and although moisture content is the most important property affecting stability of the grain during storage, temperature and duration of storage are also important factors (Weinberg et al., 2008).

Storage is associated with a range of hazards. Mould spoilage, pest infestations and grain germination (which can occur if sufficient moisture is present, *e.g.* condensation due to temperature differences) are the main problems.

2.4.3.1 Modified atmospheres in stored grain

Modified atmosphere (MA) storage is one of the food preservation methods that maintain the natural quality of food products. The storage life of food products is considerably extended by modifying the atmosphere surrounding the food, thus reducing the respiration rate of food products and the activity of insects or microorganisms in food (Jayas and Jeyamkonda, 2002). MA may be efficient in controlling fungal growth and mycotoxin production in stored maize. Disinfestation of stored grain using MA involves the alteration of the natural storage gases such as carbon dioxide (CO_2), oxygen (O_2) and nitrogen (N_2), by reducing O_2 in the atmosphere and increasing CO_2 and N_2, to render the atmosphere in the stores lethal to pests.

The maize storing in a plastic silo-bag is a common practice especially in developing countries. Here the respiration of grain, fungi, insects and other live organisms consumes O_2 and generates CO_2, creating a self-regulated atmosphere, which has effects on the seeds, insects and fungi (Castellari et al., 2010; Weinberg et al., 2008).

Being the fungal species involved in the deterioration of stored maize aerobes, the need of O_2 is required. The problem arises due to the fact that some species can tolerate high levels of CO_2, being able to survive and grow in niches where other species cannot grow and thus dominate specialised grain ecosystems (Chulze, 2010). The tolerance to low O_2 and high CO_2 is also influenced by interactions with grain type and water availability (aW). The treatment is more effective if the grains have low aW (Magan and Aldred, 2007). Janardhana et al. (1998) studied the effect of modified atmosphere storage systems (MAS) having 60% CO_2, 40% CO_2 and 20% CO_2 in combination with N_2 (30%, 50%, 70%, respectively) and 10% O_2 on freshly harvested maize grains. The results showed that, visible moulding was postponed by 15 days under 60% CO_2 and 40% CO_2 atmospheres in grains with 20% moisture content, and no mould growth was visible on maize kernels with 15% of moisture content in all MAS treatments tested throughout the storage period.

In the studied regions, the maize moisture content is insured through an air rotation system with convection movements, preventing the water condensation inside the silo. If not

done, there could be an increase of moisture, especially on the top kernels, elevating the probability of fungal developing and mycotoxin production.

2.4.3.2 Ozone

The application of ozone is attracting attention because of its advantages. It can be generated electrically on site at the time of use, eliminating the need to store and dispose pesticide packages, and it leaves no residue (Sousa et al. 2008).

Ozone has also been evaluated to control fungal populations in stored maize. Kells et al. (2001) showed that 50mg/cm^3 of ozone in the air reduced the cfu/g of *Aspergillus parasiticus* in stored maize.

2.4.3.3 Insect pests control

Contamination of stored grain with insects and insect fragments is a major concern in the grain industry. In the past, prevention of insect pests has been achieved mainly by a chemical strategy, but the intensive use of chemical compounds has resulted in the evolution of resistant populations. Pesticides are being withdrawn from use because of high costs to re-register or develop new ones, and environmental considerations have made the manufacture of certain pesticides illegal, such as methyl bromide (recognized as an ozone-depleting chemical). Thus, alternative, preferably non-residual, methods of effective pest control are needed for the post-harvest handling, storage, and processing of grains (Mason and Strait, 1998).

Because the previously popular fumigant methyl bromide has been phased out in most countries under the Montreal Protocol, phosphine became almost the only widely used and cost effective fumigant. It is an effective fumigant for disinfection of storage maize and other commodities in most developed countries, having the advantage of not leaving harmful residues in the environment nor in treated products (Fields and White, 2002; Horn and Horn, 2006). However, there are disadvantages, as phosphine acts slowly in killing insects and is less effective at lower temperatures, taking much longer to control pests (Liu, 2011). Also, pests developing high levels of resistance toward phosphine have become common in Asia, Australia and Brazil. High level resistance is also likely to occur in other regions, but may not have been as closely monitored. Essential oils offer an alternative to phosphine to control insect pests. Contact and fumigant insecticidal actions have been demonstrated for a range of essential oil constituents (Chulze, 2010).

2.4.3.4 Post- harvest control with preservatives

Weak acids are used in moist grain specifically destined for animal feed to prevent fungal spoilage. The most common weak acid preservatives used are sorbic acid, benzoic acid, and propionic acid.

In maize, there are several studies about the use of butylated hydroxyanisole (BHA) and propyl paraben (PP) to control the growth of *F. verticillioides* and *F. proliferatum* and to decrease fumonisin production in naturally contaminated maize and on irradiated maize (Farnochi et al., 2005; Torres et al., 2003). Reynoso et al. (2002) showed that low concentrations of both antioxidants may be more effective together than the two alone. Marín et al. (2000), showed that at 0.85 aW, a 0.1% concentration of proprionates is very effective at controlling *Penicillium*, *Aspergillus*, *Eurotium* and *Fusarium* species, but at 0.90-0.95 aW there was no effect on these species, except on the *Penicillium* species.

These preservatives are fungistats which imply the need of a proper coverage of the grain to prevent under-treated pockets. Poor coverage can lead to growth of spoilage fungi, especially mycotoxigenic fungi which can sometimes metabolise these aliphatic acids (Magan and Aldred, 2007).

CHAPTER THREE: SAMPLE COLLECTION

3.1 Introduction

3.1.1 Climate Change: Implications for Food Safety

In an interpretative review, Adams et al. (1998) considered that recent research has advanced understanding of the sensitivity and vulnerability of agricultural systems to climate change. However, the author considered that indirect effects of climate changes, which include the changes in the incidence and severity of agricultural pests and diseases, have not been fully assessed.

Paterson and Lima (2010) consider that climate represents the key agro-ecosystem driving force of both fungal colonization and mycotoxin production, and whereas there are several factors involved in mycotoxin contamination, climate is the most important.

Climate related factors can impact food safety through many ways such as changes in temperature and precipitation patterns, increased frequency and intensity of extreme weather events, ocean warming and acidification, and changes in the transport pathways of complex contaminants. Temperature increases and changes in rainfall patterns have an impact on the occurrence of microorganisms and the patterns of their corresponding foodborne diseases (Tirado et al., 2010).

Although the impact of climate change on fungal colonization has not been fully addressed, factors such temperature, humidity and precipitation are known to have an effect on toxigenic fungi and on their interaction with the plant hosts. Usually, fungi have temperature ranges within which they perform better and the increasing average temperatures could lead to changes in the range of latitudes at which certain fungi are able to compete (FAO, 2008b). There is, therefore, reason to believe that increased climate variability associated with climate change trends may result in higher pre-harvest levels of mycotoxins (Wu et al., 2011).

3.1.2 Influence of climate in maize contamination

In maize, fungi that produce aflatoxins and fumonisins are more likely to infect and produce toxins in warmer temperatures. In general, warmer temperatures combined with greater extremes in precipitation or drought increase plant stress, further predispose maize to fungal infection and mycotoxin contamination (Wu et al., 2011). This encourages the fungal

partner to develop more than under favourable plant conditions with the expectation of greater production of mycotoxins (FAO, 2008).

The Mediterranean zones have been identified as being very susceptible to climate changes and extreme changes in temperature, CO_2 and rainfall patterns are expected. This raises the probability of migration of pathogens in response to warmer, drought-like climatic conditions (Magan et al., 2011). This way, mycotoxins outbreaks, more common in tropical and warmer countries, may start to become more common in this particular region of the globe. In fact, in 2003, a prolonged hot and dry weather in Europe caused an outbreak of aflatoxins contamination, with consequent problems.

Aflatoxins are specifically expected to become more prevalent with the foreseen climate change. One of the reasons, is due to the fact that *A. flavus* has a wider range of temperature tolerance (19-35°C) when comparing to *Fusarium* (25-30 °C) with about 28°C optimum for growth and 30-33°C for aflatoxin production. Nevertheless, the same conclusion could be taken for *F. verticillioides*, a producer of the fumonisin toxins and the most common species on maize in Southern Europe, since fumonisins have been associated with dry weather during grain fill and late season rains (Miraglia et al., 2009).

Very recently, in 2012, EFSA (European Food Safety Authority) published a report where it predicts the emergence of aflatoxins in cereals in the European Union (EU) due to climate change. This report modelled and predicted that the increase in temperature in +2 °C, would lead to higher levels of contamination in the areas where maize is currently grown, whereas an increase of +5 °C, would lead to levels of contamination lower even though the risks are expected to be wider and enlarge towards northern EU countries (Figure 3.1)

In Portugal, climatic extremes have been more frequent in recent years (SIAM, 2001). During the sampling year of this work, climate was characterised by intense cold and ice in the winter, associated with low precipitation and wind (in critical timings of winter cereals development) and also by high temperatures since April. This has hampered the regular development of the crops: blossom occurred too early and ear development and grain filling were defective, definitely jeopardising yield (INE, 2009b).

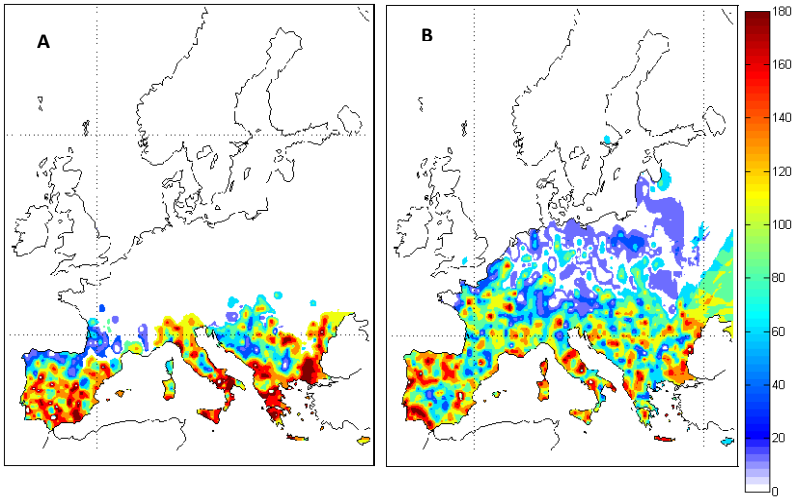


Figure 3.1 – Maps reporting the risk for aflatoxin B1 contamination in maize in the +2 °C climate change scenario (A) and in the +5 °C climate change scenario (B) (adapted from EFSA, with permission).

To fully understand if climate can influence the occurrence of certain fungi and the mycotoxin production, in this chapter, it is described the choice of regions where maize was sampled and it is characterized their climate, as well as the materials and the methodologies used during sampling. Results and discussion of the sampling plan are also assessed.

3.2 Materials and Methods

3.2.1 Sample Origin

With the collaboration of the National Association of Maize and Sorghum Producers (ANPROMIS) a sampling plan was established. Three regions with either Agricultural Cooperatives or farmer organizations belonging to ANPROMIS were selected, based on their geographic placement and climate. Samples were taken in the regions of Beira Litoral, Ribatejo and Alto Alentejo between November of 2008 and April of 2009 (Figure 3.2).

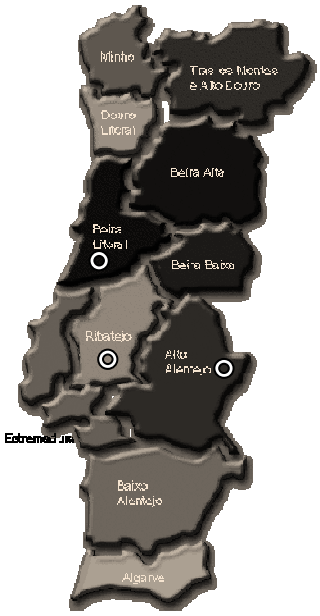


Figure 3.2– Map of Continental Portugal divided in regions.
◎Regions where the samples were taken (picture taken from the URL: debbyeric.blogs.sapo.pt).

3.2.2 Climate and Regional characterization

The latest available results of the climatological normals 1971-2000 by the Meteorological Institute, allows the identification of the different types of weather in Continental Portugal, according to the Köppen-Geiger classification. The results obtained by this classification map, serve to confirm that in most of the territory the climate is temperate continental, Type C, with subtype Cs (temperate climate with dry summer) and in a small area Arid climate-Type B Subtype BS (steppe climate) (Figure 3.3).

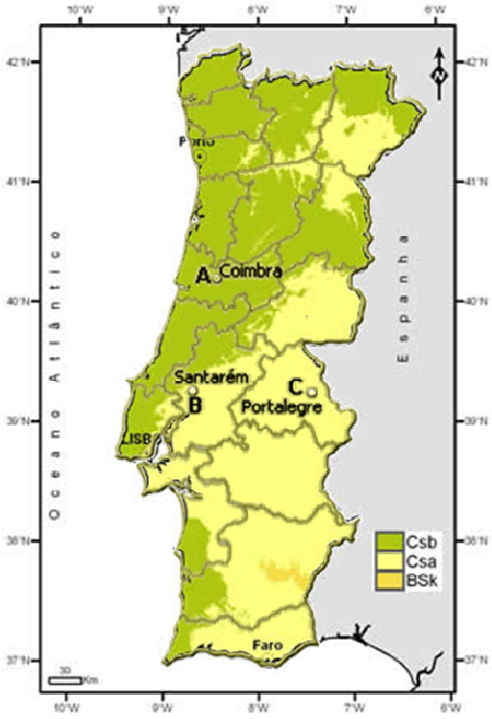


Figure 3.3– Map of Continental Portugal with the climate classification according to Köppen-Geiger and the districts where the samples were taken. Csa, temperate climate with warm summer and dry in the interior regions of the Douro Valley (part of the district of Bragança), as well as in regions south of the Montejunto-Estrela mountain system (except on the west coast of Alentejo and Algarve). Csb, temperate climate with dry mild summers, in almost all regions located northern of the Montejunto-Estrela mountain system and in the west coast of Alentejo and Algarve. BSk, cold steppe climate of mid-latitude in a small part of the region of Alentejo, in the district of Beja.

3.2.2.1 Beira Litoral

Beira Litoral coastal region has 7793 km^2 and it is bounded on the North by the Douro basin, on the East by the Sierras Caramulo, Buçaco and Lousã, and Southwest by the Serra de Aire and Candeeiros. The climate is transitional between Atlantic and Mediterranean with annual precipitation of 984 mm in Coimbra (Figure 3.4, page 40). The annual average temperatures are very moderate, 14.6 °C in Coimbra (Table 3.1). In higher areas the annual precipitation can easily exceed 1000 mm. Even though this region is characterized by the predominance of small farming and diversified cultures, maize prevails as the main source of agricultural income in most of the region, while the development of dairy products is more evident. Other important crops are rice and wine (GDE, volume III).

Table 3.1– Air temperature. Climate Normal - Coimbra, 1971/2000. Data obtained in the URL: https://www.meteo.pt/pt/oclima/clima.normais/006/

Month	Average of minimum temperature (°C)	Average temperature (°C)	Average maximum temperature (°C)
January	4.6	9.6	23
February	5.9	10.9	25.5
March	6.9	12.6	29.5
April	8.4	13.9	32.5
May	10.8	16.2	35
June	13.5	19.4	41.6
July	15	21.6	40.2
August	14.4	21.5	40
September	13.4	20.2	40
October	10.9	16.6	34.6
November	7.7	12.9	27.6
December	6.3	10.8	25.2

3.2.2.2. Ribatejo

Ribatejo is a central region of Portugal with an area of 7500 km^2 that extends within the Tejo basin, between Abrantes and Tejo estuary. Its borders are between the regions of Beiras in the north, of Estremadura in the west and southwest, and Alentejo in the east and south. The climate is temperate south-Mediterranean, influenced by the river that runs through. The annual average temperatures are very moderate, 15 °C in Santarém (Table 3.2), with an annual precipitation of about 500-600 mm (Figure 3.4). Ribatejo's agriculture enjoys a vast region of fertile soils based on Tejo alluvium. The variety of landscape is more pronounced in the Northwest, with predominance of small farming and diversified cultures, while in the Southwest dominates extensive and industrial agricultural. The main agricultural products are wheat, maize, rice, tomatoes, wine and vegetables (GDE, volume VIII).

Table 3.2– Air temperature. Climate Normal - Santarém, 1971/2000. Data obtained in the URL: https://www.meteo.pt/pt/oclima/clima.normais/018/

Month	Average of minimum temperature (°C)	Average temperature (°C)	Average of maximum temperature (°C)
January	4.9	9.6	20.6
February	6.2	11	24.8
March	7.3	12.9	29
April	8.6	14.1	31.6
May	10.6	16.5	36
June	13.3	20	43.5
July	15.2	22.6	43
August	15.3	22.7	40.5
September	14.1	21	40.2
October	11.6	17.1	33.6
November	8.4	13.2	26.5
December	6.5	10.8	23

3.2.2.3 Alentejo

Alentejo is a region of southern Portugal with an area of 25,160 km^2 and it is bordered to the north by the Tejo River, the east by the river Guadiana and the Spanish border to the south by Algarve mountain system, and to the west by the Atlantic and the rivers Tejo and Sado. The Alentejo is divided in Alto Alentejo (12,420 km^2) and Baixo Alentejo (13,740 km^2). The Alentejo has a uniform relief predominating a wide plain, whose altitude varies between 300 m and 200 m though punctuated with some hills. The region of Alentejo has a Mediterranean climate feature of continental, i.e. hot and dry. The average annual temperature exceeds 17 °C, and in the month of June temperatures above 30 °C are recorded throughout the eastern region (Table 3.3). Average annual precipitation is less than 600 mm (Figure 3.4). Economically, Alentejo is predominantly an agricultural region. Farming and ranching is practiced on the plantation scheme, subsisting small and medium-sized farms in the more fertile and moist areas. The main crops are wheat, barley, oats and rye (GDE, volume I, INE, 2008).

Table 3.3 – Air temperature. Climate Normal - Portalegre, 1971/2000. Data obtained in the URL: https://www.meteo.pt/pt/oclima/clima.normais/006/

Month	Average of minimum temperature (°C)	Average temperature (°C)	Average of maximum temperature (°C)
January	5.7	8.5	20.4
February	6.2	9.4	22.5
March	7.6	11.5	25.5
April	8.2	12.3	29.6
May	10.6	15.3	32.3
June	14.4	19.9	39.4
July	17.3	23.5	40.4
August	17.2	23.5	39.1
September	16.1	21.2	39.5
October	12.5	16.2	31
November	9.1	12.1	25.7
December	6.8	9.5	23.2

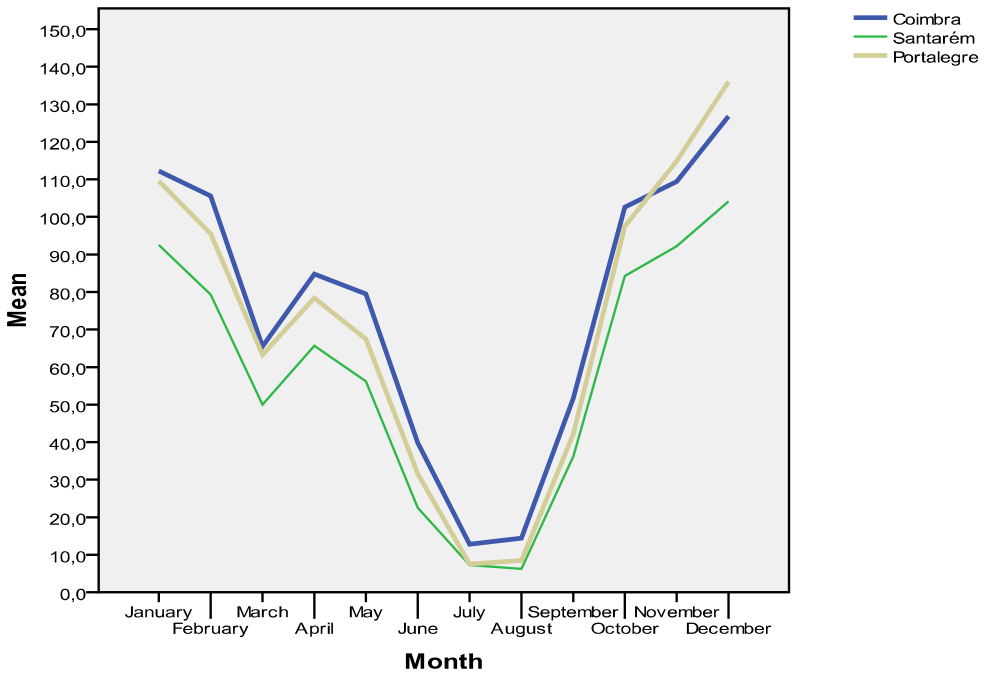


Figure 3.4 – Average precipitation (mm) levels between the years 1971-2000 in the three sampling districts.

3.2.3 Sampling methodology

Sampling general guidelines were given to ANPROMIS being subsequently modified according to the specific characteristics of the sampling place. It was agreed that samples would be taken from three distinct stages of the storage chain (reception, after drying and after storage). Sample size was defined to be 100 g, and these were collected in paper bags. Just before collection, the moisture level was determined and recorded.

3.2.3.1 Maize storage chain

Maize reception

After the crop season, producers take their maize batches to the respective Cooperative or Organization. Here a preliminary test is made to determine maize moisture content (Figure 3.5). With gathered information, the time needed to dry the kernels to the humidity level required for storage is computed.

Figure 3.5 – Sampling of maize, prior entering the farmer's organization AGROMAIS (Riachos, Ribatejo), for the measurement of water content.

Storage in threshing-floor

Between reception and drying, maize kernels are stored in threshing-floor (Figure 3.6). Kernel batches with similar water content levels are put side by side.

Figure 3.6 – Tractor pouring maize kernels in threshing-floor. Maize removal to the dryer and subsequent storage is made as quick as possible, avoiding to expose it longer than needed to the natural elements (Riachos, Ribatejo).

Drying of maize kernels

An important step prior to storage is drying, to remove excess water from the grain, since it reduces or prevents the production of most mycotoxins. A range of different types of driers may be used (diesel, wood, sun). High-temperature driers are capable of drying large quantities of grain quickly. The critical water content for safe storage is around 13-14% (Chulze, 2010).

Storage in silos

Silos (Figure 3.7) have temperature and water content measure sensors. To prevent water condensation inside, an air rotation system, in convection movements, insures that maize decreases its humidity inside the silo. Drying of maize till its final moisture content is achieved during storage in silos.

Figures 3.7 A, B – Picture from inside of a silo, where bottom maize is being thrown on the top of the pile (A). Outside view of Cersul´s silos, Elvas, Alto Alentejo (B).

Expedition

The last step is selling the commodity. They must ensure that the product has quality until this final step transferring the subsequent product quality to the buyer's responsibility.

3.2.3.2 General guidelines

The general sampling procedure was the one described below:

- 10 samples of maize were taken at three stages of the storage chain (reception, drying and storage). These samples were taken during two consecutive days;
- Samples were collected into paper bags to prevent water condensation with the indication of the date, water content and the kind of dryer;
- These samples were transported to the laboratory within 24 hours.

Beira Litoral

Cooperativa Agrícola de Coimbra, S. Silvestre, Coimbra

Maize samples were taken in an Agricultural Cooperative that receives dried maize directly from the producers. The quantity of green maize (not dried) is very small and no samples of it were collected. Therefore, the sampling in this region is slightly different from the original guidelines, since only dried kernels were sampled. The way each producer dried its product differ, some used dryers heated with wood or with diesel, whereas others, in a more traditional fashion, dried their maize in the sun by placing it in threshing-floors. One of the tasks in this region was to compare fungi and mycotoxin presence in the different types of dryers.

Ribatejo

AGROMAIS, Riachos, Santarém

The samples were taken into the farmer's organization – AGROMAIS – that receives exclusively green maize (not dried) from producers. The same dryer (heated with diesel) is used for all batches. The task in this region was to compare the fungi and mycotoxin presence in the different sampling moments.

Alto Alentejo

CERSUL, Santa Eulália, Elvas

This farmer organization (CERSUL), similar to AGROMAIS, receives exclusively green maize (not dried) from the producers. The same dryer is used for all batches. The task in this region was to compare the fungi and mycotoxin presence in the different sampling moments.

3.3- Results and Discussion

There is a dynamic interaction between temperature and precipitation. Temperature influences both the timing and duration of the growing season. Both of these factors will affect the balance between the amount of precipitation received by a crop and potential evapotranspiration (PET) from the crop surface (Kenny and Harrison, 1992).

The three sampling regions have slight different climates. Beira Litoral climate is characterized as transitional between Atlantic and Mediterranean, whereas Ribatejo and Alto Alentejo as Mediterranean. However, the diversity of the average temperatures of these regions, in 2008/2009, was not considerably different (Figure 3.8).

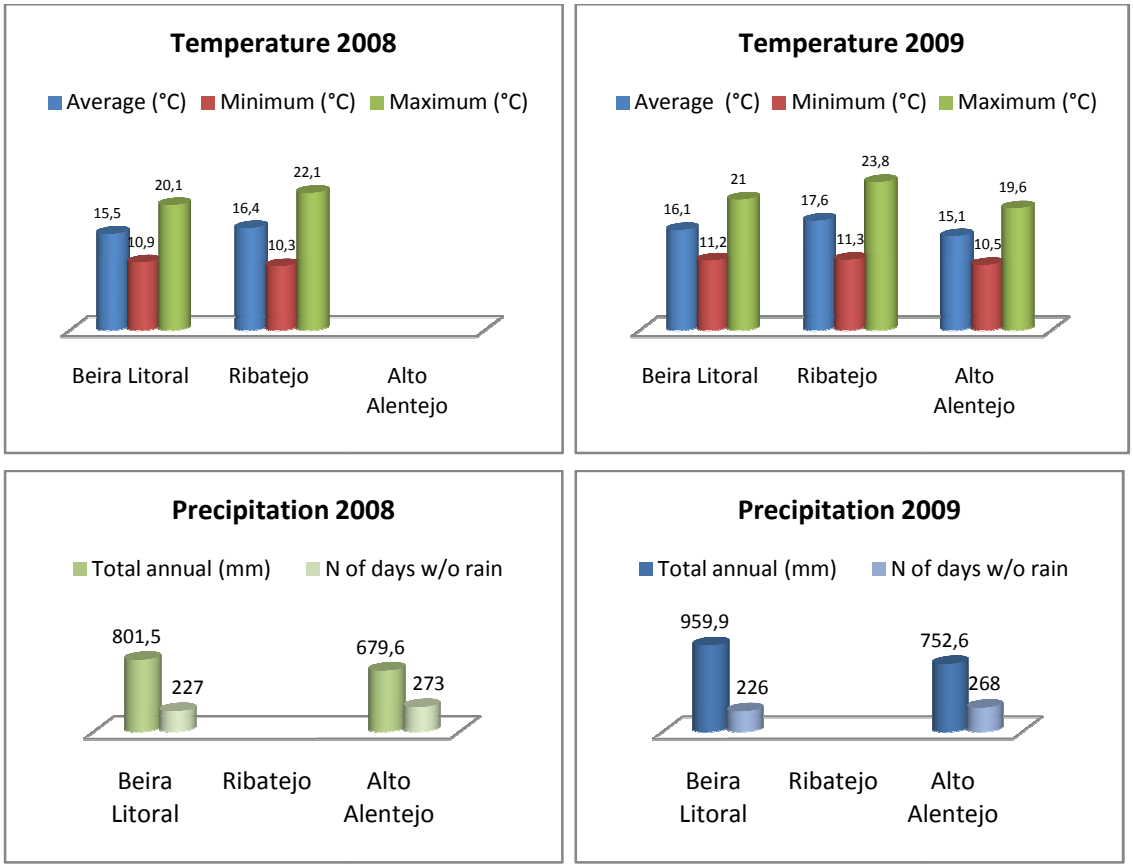


Figure 3.8 – Average air temperature and precipitation in the years of 2008 and 2009. Data taken in three meteorological stations at the district capitals: Coimbra (Beira Interior), Portalegre (Alto Alentejo) and Santarém (Ribatejo) (INE 2010a; INE 2010b). No temperature data was available for Portalegre in 2008. No precipitation data was available for Santarém. N of days w/o rain: number of days without rain in the year.

In these two years and according to the producers, maize prices were extremely low, thus the different harvesting season in the regions were more influenced by economical issues than by climate and ripening times.

Nevertheless, Ribatejo was the region where the campaign first started. Ribatejo was the region with the higher average of air temperature when compared with the other two regions in 2009 and with Beira Litoral in 2008. According to Meza et al. (2008) within a range between 10 and 35 °C, increases in ambient temperature accelerate the rate of development, allowing maize to complete its phonological stages in shorter periods of time. Even though there was no precipitation data available for this region, it is expected that in those years the precipitation levels were the lowest in comparison with the other two regions. This conclusion can be taken when looking at the average precipitation levels between the years 1971-2000 (Figure 3.4, page 42).

Both average air temperatures and precipitation in Alto Alentejo were lower than in Beira Litoral, but the harvesting campaign started earlier in this region followed by Beira Litoral, where they only started to received already dried maize in November. The October samples from Beira Litoral were of stored maize from the previous year (Table 3.4). The meteorological station of Alto Alentejo is situated at the city of Portalegre, fairly high on a hillside of São Mamede Sierra, maybe 150 or 200 meters above the downtown area. The city of Portalegre is about 400 meters and the meteorological station at about 590 meters above sea level, which explains why, opposite to what could be expected, for Alto Alentejo, the recorded average temperatures are lower than in Beira Litoral. However, maize is grown at a lower altitude, being expected to be exposed to higher temperatures.

Generally, meteorological annual data recorded in 2008 and 2009 (Figure 3.8) followed the climate normal trends recorded from 1971 to 2000 in the same places (Tables 3.1 to 3.3). On the other hand, in a study about climate changes in Portugal (SIAM, 2001), it was concluded that between 1971 and 2000, there was an increase in the average air temperature since 1976, being the last 6 years the warmest in the last 12 years. The precipitation levels also show a different trend since 1976, with a significant precipitation reduction during spring together with less clear variations during the other seasons. This suggests that there is a tendency for the reduction of the rainy seasons' duration. In fact, climate data shows that, in the last 10 years, there is an increase of the frequency in which there is extreme and severe drought, especially in the south regions.

Table 3.4 – Sampling months and number of samples taken per region, between 2008 and 2009.

	Region			
Data	Beira Litoral	Ribatejo	Alto Alentejo	Total
September	0	10	0	10
October	12*	10	10	32
November	20	0	10	30
February	0	10	0	10
March	0	10	3	13
Total	32	40	23	95

* These samples collected in October correspond to stored maize from the previous year compaign.

Unfortunately, sampling methodology was not strictly followed due to different factors, which created less homogeneity for data analysis. In Beira Litoral region, for instance, producers dried the maize kernels before taking it to the Agricultural Association, using different types of dryers, being wood dryer the most common (Table 3.5).

Table 3.5 – Number of samples taken at reception, dryer and storage.

Region	Origin					
	Reception	Association's Gas Dryer	Storage	Producer's Gas Dryer	Producer's Wood Dryer	Producer's Diesel Dryer
Beira Litoral	0	8	10	1	12	1
Ribatejo	10	10	20	0	0	0
Alentejo	10	10	3	0	0	0
Total	20	28	33	1	12	1

As previously stated, good post-harvest practices can minimize pre-harvest natural contamination. Post-harvest colonization of various fungi on food commodities reduces their shelf life and market value. Being aW an important factor for fungal development proper water content measurement is required.

Samples water content of green maize was measure in the reception and had values between 19–23% (aW 0.90–0.93), after drying samples had values between 14–15% and during storage the values reduced to 10–13% (Figure 3.9). The storage values are as recommended in the literature (Chulze, 2010; Magan and Aldred, 2007), being adequate to prevent both fungal growth and the production of mycotoxins. It is needed to insure accurate and regular moisture measurements to ensure that safe thresholds are not breached.

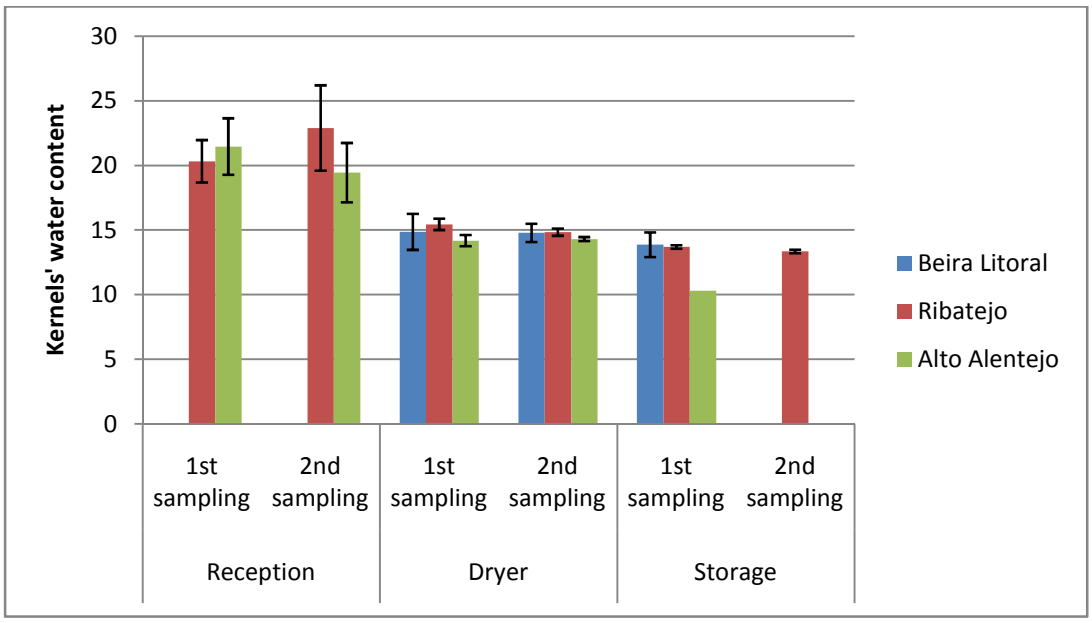


Figure 3.9 – Average of the samples humidity content taken at each region in different moments of maize storage chain. Both in Alto Alentejo and Ribatejo two sets of samples were taken in reception and dryer. In Beira Litoral two sets of samples were taken after dryer. In storage one set of samples was taken in A. Alentejo and in Beira Litoral, and two were taken in Ribatejo.

3.4 Conclusions

In conclusion, climate changes and the different agroecological conditions were not the main factors that influenced the harvest campaign timeline.

Cereal production in Portugal has dropped, in recent years, due to both adverse weather conditions and the market prices. Despite this, maize production has maintained its levels of productivity.

The two Producer's Society and the Agricultural Cooperative showed to have good practices, by reducing the water content before storing and by constant monitoring of the temperature and humidity inside the silos.

CHAPTER FOUR: ISOLATION AND IDENTIFICATION OF *ASPERGILLUS* SPECIES

4.1 INTRODUCTION

Aspergillus comprises more than 250 recognized species, with 12 distinct teleomorph genera, being all members of the family *Trichocomaceae* of the order *Eurotiales*. Due to the different emerging identification techniques (e.g. molecular), various rearrangements of the species groups, sections and subgenera were made. The taxonomy of *Aspergillus* and its teleomorphs has recently been re-investigated by using a polyphasic approach in order to examine the variability within the species (Peterson et al., 2008; Rodrigues et al., 2011; Samson et al., 2007; Varga et al., 2011a,b). The methods used by many researchers to identify the *Aspergillus* species include morphological and physiological characters, molecular data, production of metabolites and more recently matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS).

4.1.1 Polyphasic approach for species identification in genus *Aspergillus*

4.1.1.1 Phenotypic identification

The defining characteristic of the genus *Aspergillus* is the aspergillum-like structure. It consists of conidiophores which enlarge at its apex to form a rounded, elliptical or club shaped vesicle. The fertile area of the vesicles gives rise to a layer of cells called phialides that produce long chains of mitotic spore called conidia or conidiospores. The vesicles can bear either one or two palisade of cells. An *Aspergillus* with phialides borne directly on the vesicle is referred to as “uniseriate”. An *Aspergillus* with two cells layers is called “biseriate”. The second layer of cells, located between the phialides and vesicle are called metulae. The stipe is often thick walled and can have only a few thin septa or none at all (Susca et al., 2010b).

The morphological keys to common mycotoxigenic and food born *Aspergillus* species are based on macromorphology features like mycelium and conidia colour, colony diameter on different culture media and growth temperatures, reverse colour, presence of exudates, sclerotia and cleistothecia. Sclerotia and cleistothecia are closed and usually round structures about the size of a poppy seed that may be abundant in a colony. Sclerotia are rounded masses of mycelium with an outer melanised rind that macroscopically resemble cleistothecia but do not contain sexual spores. Cleistothecia are the sexual reproductive stage and contain the meiotic ascospores born within asci (Bennet, 2010).

4.1.1.2 Molecular identification

Identification of the *Aspergillus* species based exclusively in morphological characteristics might be difficult due to the level of expertise that is needed and morphological characters can vary. In this respect, the use, in recent years, of molecular tools such as Restriction Fragment Length Polymorphisms (RFLP), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and DNA sequencing have proved to be useful in the detection and identification of fungi within this genus. Furthermore, DNA sequence characters provide the best means for inferring relationships among organisms, because it is possible to sample very large numbers of variable characters (Geiser et al., 2007).

However, the information obtained from a single locus cannot be relied on to return the species phylogeny nor can it solve questions of species boundaries (Lutzoni et al. 2004, Godet and Munaut 2010). Frisvad et al. (2005) used ITS (internal transcribed spacer) sequences and RAPD comparisons in section *Flavi*, Pildain et al. (2008) used beta tubulin (ß-tubulin) and calmodulin sequences for analysis of new section *Flavi* species, and Varga et al. (2009) used ß-tubulin sequences for sectional analysis.

The sequence of ITS region along with single-copy conserved genes such as calmodulin and ß-tubulin is now commonly used to identify and to the phylogenetic analyses of important food spoilage and mycotoxigenic fungi (Pitt and Hocking, 2009). Such was the case of the revision of the sections *Nigri* and *Flavi* made by Vargas et al. (2011a,b). Multilocus DNA sequence analysis can avoid the subjectivity of determining species limits through the use of concordance analysis of several genes (Baum and Shaw, 1995) and provides reliable information for fungal species identification (Taylor et al. 2000, Dettman et al. 2006, Peterson 2008, Samson and Varga, 2009).

The vast majority of *Aspergillus* teleomorphs use a homothallic (self-fertile) system for crossing. Anamorphic species (LoBuglio and Taylor 2002) have long been thought to have some cryptic or undiscovered means of genetic recombination. In ascomycetes, sexual compatibility is determined by mating type genes designated *MAT1-1* and *MAT1-2*. Sequence analysis of mating type genes can show homo- and heterothallism and identify potentially compatible isolates (Ramirez-Prado et al. 2008) for mating experiments. Horn et al. (2009) have demonstrated teleomorphs in several section *Flavi* species but ascospores mature only after prolonged incubation.

4.1.1.3 Mycotoxigenic profile

Aspergillus isolates can be characterized by their profiles of secondary metabolites. Practically all species can produce a unique combination of different types of small organic compounds (Samson and Varga, 2009), which means that these metabolites can be used in species recognition because they have high species specificity (Larsen et al., 2005). In traditional identifications, secondary metabolites have only been used indirectly such as the observation of the colour of diffusible pigments and the odour of cultures. With the development of separation methods such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC), and of advanced detection methods it is now possible to detect, identify and quantify the individual secondary metabolite profile (Larsen et al., 2005).

4.1.1.4 Spectral analysis by MALDI-TOF ICMS

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is rapidly attracting the interest of microbiologists because of its powerful features that allow rapid and reliable identification of microorganisms. MALDI-TOF MS is a powerful method to detect and identify proteins by molecular weight determination of individual, specific fragments. The method is accurate and easy to use, allowing quick determination of molecular weights of proteins with minimal sample requirements. MALDI-TOF MS is now widely used for the identification and characterization of clinical important microorganisms (Benagli et al., 2010).

MALDI-TOF MS consists in subjecting a sample covered with an UV-absorbing matrix that functions as an energy mediator, to a pulsed nitrogen laser. The matrix transfers the absorbed photoenergy from the irradiation source to the surrounding sample molecules, resulting in minimum fragmentation (Santos et al., 2010). The ability of monitoring ions over a broad *m/z* range (mass spectra) forms the basis of taxonomic identification (Marvin et al., 2003).These mass spectra function as “fingerprints” or “spectral signatures”, which are unique and representative for individual microorganisms, and unknown sample identification can be performed from comparison with previously constructed databases.

Hettick et al. (2008) showed that MALDI-TOF ICMS data may be used for identification of members of the genus *Aspergillus* at the species and strain levels. In this study the authors conclude that to identify correctly unknown species and strains, a comprehensive database of the 180 species of *Aspergillus* is required. Rodrigues et al. (2011), in a polyphasic approach of species identification belonging to *Aspergillus* section *Flavi*, compared the results obtained in

the different methodologies (phenotypic, MALDI-TOF ICMS, molecular) obtaining a very good agreement between different methodologies. It demonstrated that MALDI-TOF ICMS-based fingerprinting methodology is sensitive and accurate for the discrimination between species and strains of section *Flavi.*

This technique has the advantage of being objective and fast, and inexpensive in terms of labour and consumables when compared to other biological techniques.

4.2 MATHERIALS AND METHODS

4.2.1 Plating and Isolation of *Aspergillus* species

Direct plating is the preferred method for detecting and isolating of fungi from grains and nuts (Pitt and Hocking, 2009). It was decided not to disinfect maize kernels prior to the plating. Sterile Petri dishes triple ventilated, of 94 mm diameter and 16 mm high were used as described below:

4.2.1.1 Direct plating with no previous disinfection

Twenty five grains chosen randomly from each sample were picked with disinfected forceps and placed in 5 Petri dishes with 15 mL of culture medium (five grains per plate as showed in Figure 4.1). Maize kernels were disposed to insure that the colonies had growing space. This process was carried out under sterile conditions.

Figure 4.1 – Plates with 5 maize kernels with 5 days of incubation.

4.2.1.2 Culture medium

Culture medium used for the isolation was malt extract agar with 10% of sodium chloride (MEA10). Commercial MEA base (HIMEDIA) was often used and sodium chloride of analytical grade (Fisher Scientific) was added. Extremely rich in nutrients this medium is adequate for growing filamentous fungi. It is used to identify species belonging to *Penicillium* and *Aspergillus* genus (Santos et al, 1998). With the addition of salt, water activity (aW) is reduced allowing the growth of xerofilic fungi. This medium was sterilized at 121 °C for 15 minutes.

MEA10: malt extract agar with 10% of sodium chloride

20 g of malt extract powder (HIMEDIA)

1 g of peptone (HIMEDIA)

20 g of glucose (HIMEDIA)

20 g of agar (HIMEDIA)

100 g of NaCl (Fisher Scientific)

Distilled water to fill up to 1000 mL

Mix all the components except the malt extract. Add distilled water and stir. Heat until all agar is dissolve. Add the malt extract and stir again.

4.2.1.3 Growth conditions

Plates were kept in the dark at 25 °C for 7 to 15 days, facing up and not sealed.

4.2.1.4 Detection of Aspergillus species

Aspergillus strains were detected after observing the plates under a stereomicroscope (Hund Wetzelar) and considering general morphological characteristics such as colony colour and conidial head shape.

4.2.1.5 Isolation from maize kernels

Isolations were carried under stereomicroscope observation and by picking a small sample of hyphae or conidia and placing this sample on a fresh plate (Figure 4.2). All strains belonging to the gender *Aspergillus* were isolated with a needle sterilized at flame. To cool the needle tip, it was dip in semi-solid agar, kept in eppendorfs, allowing also an easier adherence of conidia. Conidia were then transferred to malt extract agar (MEA) and incubated for 7 days at 25 °C. Colony purity was verified by visual observation under the stereomicroscope. When required, for the description of new species, monosporic cultures were prepared, as described later (page 64). Isolates were designated with the code yyXASnn, where yy means the year of isolation, X refers to the commodity (e.g. A = almond, M = maize), AS refers to the genus *Aspergillus* and, nn is the isolate number.

Culture media formulations used for identification purposes are described in the next section.

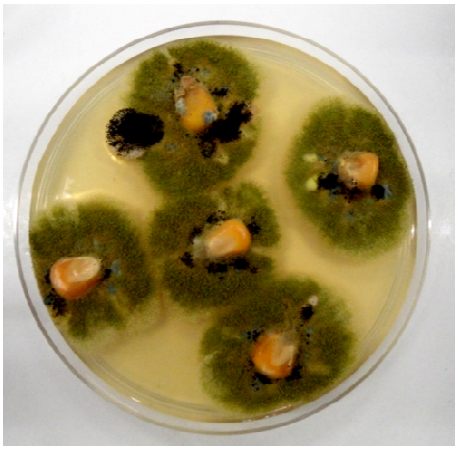


Figure 4.2 – View of Petri dish with 5 maize kernels with fungi growth after 10 days of incubation.

4.2.2 Identification and characterization of *Aspergillus* isolates

Identification and characterization of the isolates was carried out by morphological characterization, following the taxonomic keys and guides available for the *Aspergillus* genus (Klich, 2002; Pitt and Hocking, 2009). Extrolite characterization was also performed by accessing mycotoxin production (*e.g.*, AF, CPA, OTA).

Given that some of the isolates belonging to the *Aspergillus* section *Flavi* could not be described with accuracy up to the species level, molecular analysis were also performed to eight of these strains.

4.2.2.1 Morphological observation

The direct exam of the isolates allowed the determination of the colony colour and other characteristics, such as the quantity of spores, the presence or absence of sclerotia and type of reproductive structures. This exam was performed by direct observation under fluorescence light and under a stereomicroscope.

Growth conditions

Isolates were incubated at 25 °C in the dark for 7 days and sometimes longer in order to better determine the colony colour.

Identification of Aspergillus species

In order to identify the different *Aspergillus* isolates three point inoculations were made on different culture media such as CYA, CY20S and MEA. The same isolates were also grown on *Aspergillus flavus* and *parasiticus* agar (AFPA, FLUKA). This is a selective medium for the enumeration in foods of the aspergillic acid producing fungi and allows distinguishing strains belonging to the groups of *A. flavus* and *A. parasiticus*, through the developing of orange reverse colour after 2 to 3 days of incubation at 25 °C (Pitt et al., 1983).

Culture media

MEA, CYA and CYA20S formulation can be found in the manuals of Santos et al. (1998) and Pitt and Hocking (2009). All media were autoclaved for 15 minutes at 121 °C.

MEA : malt extract agar

20 g of malt extract powder (HIMEDIA)

1 g of peptone (HIMEDIA)

20 g of glucose (HIMEDIA)

20 g of agar (HIMEDIA)

Distilled water to fill up to 1000 mL

Mix all the components except the malt extract. Add distilled water and stir. Heat until all agar is dissolved. Add the malt extract and stir again.

AFPA: Aspergillus Differentiation Agar (Base- Fluka)

10 g of peptic digest of animal tissue

20 g of yeast extract

0.5 g of ferric ammonium citrate

0.002 g of dichloran

20 g of agar

1000 mL of distilled water

Suspend 21 g in 1 litre distilled water. Heat to boiling to dissolve the medium completely.

CYA: Czapek Yeast Autolysate agar

30 g of sucrose (Fisher Scientific)

5 g of extract of Autolysed Yeast Cells (Difco)

1 g of K_2HPO_4 (Pronalab)

10 mL of Czapek Concentrate

15 g of agar (HIMEDIA)

Distilled water to fill up to 1000 mL

Czapek Concentrate

30 g of $NaNO_3$ (AppliChem)

5 g of KCl (Riedel-de-Haën)

5 g of $MgSO_4.7H_2O$ (CALBIOCHEM)

0.1 g of $FeSO_4.7H_2O$ (Merck)

0.1 g of $ZnSO_4.7H_2O$ (Merck)

0.05 g of $CuSO_4.5H_2O$ (Panreac)

Distilled water to fill up to 100 mL

Mix all the medium components. Add distilled water and stir.

CY20S: Czapek Yeast Autolysate agar with 20% of sucrose

200 g of sucrose (Fisher Scientific)

5 g of extract of autolysed yeast cells (Difco)

1 g of K_2HPO_4 (Pronalab)

10 mL of Czapek Concentrate

15g of agar (HIMEDIA)

1000 mL distilled water

Mix all the medium components. Add distilled water and stir. Heat until all agar is dissolved.

Semi-solid agar medium (0, 2%)

0.1 g of agar (HIMEDIA)

0.025 g of tween 80 (Riedel-de-Haën)

50 mL of distilled water

Heat until all agar is dissolved.

Microscopic preparations for optical microscope

Microscope mounts were made by removing, with a sterile needle, a small portion of the colony with sporing structures grown in MEA, or for less sporulating species in MEA10. Being most *Aspergillus* freely sporing fungi with little mycelium, the portion of colony was taken near the hedge where fruiting structures are young and with less spores. These were placed in a slide with 96% alcohol to remove excess conidia and coloured with cotton blue and sometimes with lactophenol cotton blue. A cover slip was added. Microscopic examinations

were performed with a Leica ATC 2000 microscope. For specific cases where measures of the fungal structures were needed, a Leica DMR microscope with bright field, phase contrast and DIC optics was used. A Leica EC3 camera was mounted on the microscope for photomicrography.

Lactophenol cotton blue solution (100mL)

50 mg of Methyl Blue (Cotton Blue, Aniline Blue)

25 g of Phenol

25 g of L(+)-Lactic Acid

50 g of Glycerol

Add all the ingredients, boil and stir. Let it cool and filtrate to remove particles. Keep it in the dark.

Cotton blue solution (stock solution)

Add 0.5g of cotton blue to 30 mL of lactic acid, boil and stir. Let it cool and filtrate to remove particles. Keep it in the dark.

Cotton blue (mount solution)

Add a few drops of stock solution to 20 mL of lactic acid.

Microscopic preparations for scanning electron microscopy

SEM micrography was performed in a NanoSEM-FEI Nova 200 (FEG/SEM); EDAX-Pegasus X4M (EDS/EBSD) equipment. Samples were prepared by rubbing a standard SEM aluminium stub across a growing colony and coating it with gold. Colonies were grown in CYA and CYA20S.

Determination of the fungal frequencies

Fungal frequency of the isolates per *Aspergillus* section (FAS) was calculated as the ratio of the number of isolates per *Aspergillus* section and the total number of isolates. The value was converted in a percentage for easier perception.

FSC is the fungal frequency at the three storage stages (reception, dryer and storage). It was calculated by dividing the number of isolated strains belonging to the different *Aspergillus* sections at each sample stage by the total number of samples at each storage stage.

The relative fungal frequency of each *Aspergillus* sections per region and per sampling point (FAR) was calculated by dividing the number of strains belonging to a defined *Aspergillus* section and isolated at a determine point of the storage chain at each region, by the total number of strains belonging to a defined *Aspergillus* section at each point of the storage chain.

The fungal frequency of the isolated *Aspergillus* sections at each dryer at Beira Litoral (FD) was calculated as the ratio between the number of strains belonging to a defined *Aspergillus* section and isolated from a specific dryer, and the total number of samples taken from a specific dryer.

Isolates preservation

Many freely sporing fungi survive for several months when stored at 4 °C. Representative strains belonging to the gender *Aspergillus* were initially isolated and preserved in tube slants at this temperature for further studies (morphological and mycotoxigenic). This preservation technique has the disadvantage of taking too much space and not being recommended for long time preservation, therefore preservation at -18 °C and -80 °C in criovials was adopted for future studies.

Preservation at 4 °C

Sterile 15 mL centrifuge tubes containing MEA slants were inoculated with a loop full of spores and incubated for 7 days at 25 °C. The tubes were then put in the refrigerator.

These tubes were stored for 8 months, being prepared fresh cultures every 2 months.

Preservation at -18 °C

Criovials with beads Commercial beads were washed with abundant water and put into 2% HCl solution for 12 hours. After this step, beads were once again washed, immersed in distilled water and dried in the incubator. After being completely dried, beads were sterilized at 121 °C, for 15 minutes, in 20 ml flasks. Screw cap criovials (Nalgene) were filled until ¼ of its capacity with the beads in sterile conditions.

Solution of 10% glycerol A solution of 10% glycerol (87%, Merck) was made and autoclaved in 20 mL flasks at 121 °C for 15 minutes.

Spore suspensions Spore suspensions were prepared by adding 1 mL of sterile 10% glycerol with a sterile tip from a freely sporulating sector of a pure colony. These suspensions were then transferred to criovials. These were put at 4 °C for 24 hours and then transferred to the freezer at -18 °C. Colonies with fewer spores were preserved by cutting a piece of agar and then transfer to a crioval. Glycerol solution was added until the agar was covered as described in Serra (2005).

Preservation at -80 °C

Fungal preservation at -80 °C was performed as described for -18 °C with the difference of the glycerol solution. Serra (2005) described that a 10% glycerol solutions is enough for the continuous viability of the fungal specimens, but Pitt and Hocking (2009) suggest the use of 60-80 % glycerol solution as cryoprotectant.

Solution of 60% glycerol A solution of 60% glycerol (87%, Merck) was made and autoclaved in 20 mL flasks, at 121 °C, for 15 minutes.

Spore suspensions Spore suspensions were prepared by adding 1 mL of sterile 60% glycerol with a sterile tip from a freely sporulating sector of a pure colony. These suspensions were then transferred to criovials. These were transferred to *Cryo Freezing Container*, a recipient with 2-propanol that allows a progressive cooling with a controlled rate of 1 degree

per minute in the -80 °C chest freezer. Colonies with fewer spores were preserved by cutting a piece of agar and transfering to criovials as described before.

Monosporic cultures

This procedure was used whenever it was needed to confirm the purity of the fungal isolate, for unequivocal identification purposes. From a sporulating culture, a spore suspension was prepared in a sterile 0.05% Tween 80 solution. This suspension was homogenised by vortexing for a few minutes and diluted twice by successive 10-fold dilutions. The spore dispersion and dilution was confirmed on the microscope with the aid of a Neubauer counting chamber. The suspension showing a reduced number of spores ($1x10^5$ spores/mL) was used to inoculate 1 Petri dish (9 cm diameter) containing a very thin layer of MEA. The plate was inoculated with 100 µL of spore suspension. The culture was incubated at 25 °C over night. After incubation, germination was checked at the stereomicroscope. A well isolated spore was collected with the aid of an inoculation needle and transferred to a new Petri dish with MEA, in order to obtain a monosporic culture.

4.2.2.2 Molecular characterization of Aspergillus section Flavi isolates

Molecular characterization was performed to fully identify *Aspergillus* section *Flavi* strains that could not be properly identified with the classical methods.

Thirty four strains, including type strains, isolates from maize (collected under the scope of this thesis), and isolates from almonds (previously collected in our laboratory) were molecularly characterized. Sequences were deposited at GenBank under accession numbers listed in Table A.3.1. Additional type strains were used in the phylogenetics studies with sequences taken from GeneBank (Table 4.1).

This characterization was made in the United States Department of Agriculture (USDA) laboratories, Peoria. Strains were sent by mail to this institution and, for transportation purposes, were firstly inactivated as described below:

- A loop full of spores was suspended in 0.2% agar and used for inoculation on slants in 15 mL centrifuge tubes with MEA. After 7 days growth in the dark at 25 °C, a 1% solution of thimerosal was added in sufficient amount to cover cultures, and left for 24 hours to inactivate cultures.

Table 4.1 – *Aspergillus* type strains and *Aspergillus* isolates from maize and almonds used in this study.

Strain	Species	Substrate
NRRL 4181^{T}	*Petromyces alliaceus*	Soil
NRRL 517^{T}	*A. avenaceus*	Seed peas
NRRL 26010^{T}	*A. bombycis*	Silk worm excrement
NRRL 25528^{T}	*A. caelatus*	Peanut field soil
NRRL 13603^{T}	*A. coremiiformis*	Soil
NRRL 1957^{T}	*A. flavus*	Cellophane diaphragm of an optical mask
NRRL 28986	*A. flavus*	Peanut field soil
NRRL 28987	*A. flavus*	Peanut field soil
NRRL 28992	*A. flavus*	Peanut field soil
MUM 10.206	*A. flavus*	Almond
MUM 10.232	*A. flavus*	Maize
NRRL 3648^{T}	*A. lanosus*	Forest soil
NRRL 5108	*A. lanosus*	
NRRL 28998	*A. minisclerotigenes*	Peanut field soil
NRRL 29000	*A. minisclerotigenes*	Peanut field soil
NRRL 29002	*A. minisclerotigenes*	Peanut field soil
MUM 10.226	*A. minisclerotigenes*	Maize
MUM 10.227	*A. minisclerotigenes*	Maize
MUM 10.228	*A. minisclerotigenes*	Maize
MUM 10.229	*A. minisclerotigenes*	Maize
MUM 10.230	*A. minisclerotigenes*	Maize
MUM 10.203	*A. minisclerotigenes*	Almond
MUM 10.231^{T}	*A. mottae*	Maize
MUM 10.231	*A. mottae*	Maize
MUM 10.214^{T}	*A. transmontanensis*	Almond
MUM 10.205	*A. transmontanensis*	Almond
MUM 10.211	*A. transmontanensis*	Almond
MUM 10.221	*A. transmontanensis*	Almond
MUM 10.222	*A. transmontanensis*	Almond
MUM 10.223	*A. transmontanensis*	Almond
MUM 10.208	*A. sergii*	Almond
MUM 10.219^{T}	*A. sergii*	Almond
NRRL 13137^{T}	*A. nomius*	Wheat

Table 4.1 – *Aspergillus* type strains and *Aspergillus* isolates from maize and almonds used in this study.

Strain	Species	Substrate
NRRL 502^{T}	*A. parasiticus*	Insect, Mealybug on sugar cane
MUM 10.225	*A. parasiticus*	Almond
MUM 10.212	*A. parasiticus*	Almond
MUM 10.224	*A. parasiticus*	Almond
MUM 10.215	*A. parasiticus*	Almond
NRRL 25517^{T}	*A. pseudotamarii*	Field soil collected in tea fields
NRRL 20818 ^{T}	*A.tamarii*	Activated carbon
NRRL 447^{T}	*A. orzyae*	Unknown source
NRRL 3751^{T}	*A. kambarensis*	soil
CBS 110.55^{T}	*A. fasciculatus*	air
NRRL 506^{T}	*A. effuses*	
NRRL 4818^{T}	*A. flavus* var. *columnaris*	Butter
CBS 121.62^{T}	*A. parvisclerotigenus*	*Arachis hypogaea*
CBS 822.72^{T}	*A.toxicarius* Murak.	*Arachis hypogaea*
CBS 117610^{T}	*A. arachidicola*	*Arachis glabrata* leaf
CBS 117611	*A. arachidicola*	*Arachis glabrata* leaf
CBS 117612	*A. arachidicola*	*Arachis glabrata* leaf
CBS 117615	*A. arachidicola*	*Arachis glabrata* leaf
NRRL 4868^{T}	*A. chungii*	Air

DNA extraction

A portion of biological material was transferred from the dead seven day old culture into a 1.5 mL tube containing 450 µL CTAB buffer and 0.5mm-diameter glass beads (Sigma, St. Louis, MO, USA). After vortexed for 5 min at maximum speed, polysaccharides and proteins were precipitated by adding 450 µL chloroform, mixing by inversion and separated by centrifugation at maximum speed for 10 minutes in a microcentrifuge. Cleaned supernatant was transferred to a new tube (350 µL) and the same volume of isopropanol was added. This solution was gently mixed by inversion and centrifuged at maximum speed for 2 min to collect precipitated nucleic acids. The DNA pellet was washed with 200 µL of 70% ethanol, centrifuged at maximum speed for 2 min and air dried after gently discarding the supernatant. DNA was

resuspended in 100 µL of sterile deionized water, placed in heat block for 10-60 minutes at 55 °C, to resuspend pellet, and diluted 1:100, for amplifications.

CTAB buffer

10 mL of 1M Tris-Cl pH 8.4

8.18 g of NaCl

5 mL of 0.5M EDTA pH8.0

2g of CTAB

Distilled water to fill up to 100 mL

Dissolve CTAB by heating in 55 °C waterbath

Pre-preparation of the primers

A solution containing 10 µL of green juice, 6 µL of sterile distilled water, 1 µL of primer (forward) and 1 µL of primer (reverse), for each primer set (table 4.2), was made according to the number of samples.

Table 4.2 - Details of the primers used and target zone.

Primer pair	Target zone	Sequences	Reference
Bt2a-Bt2b	Beta tubulin gene	f 5'GGTAACCAAATCGGTGCTGCTTTC3' r 5'ACCCTCAGTGTAGTGACCCTTGGC3'	Glass and Donaldson (1993)
Cf1-Cf4	Calmodulin gene	f 5'GCCGACTCTTTGACYGARGAR3' r 5'TTTYTGCATCATRAGYTGGAC3'	Peterson (2008)
I5-D2r	ITS and partial 1su-rDNA	f 5'GGAAGTAAAAGTCGTAACAAGG3' r 5'TTGGTCCGTGTTTCAAGACG3'	White et al. (1990), Peterson (2008)
Mf-Mr	*Mcm7* gene	f 5'ACIMGIGTITCVGAYGTHAARCC3' r 5'GAYTTDGCIACICCIGGRTCWCCCAT3'	Schmidt et al. (2009)
M1f-M1r	Mating type gene	f 5'ATTGCCCATTTGGCCTTGAA3' r 5'TTGATGACCATGCCACCAGA3'	Ramirez-Prado et al. (2008)
M2f-M2r	Mating type gene	f 5'GCATTCATCCTTTATCGTCAGC3' r 5'GCTTCTTTTCGGATGGCTTGCG3'	Ramirez-Prado et al. (2008)
5F-7R	RNA polymerase	f 5'GAYGAYMGWGATCAYTTYGG3' r 5'GAYTGRTTRTGRTCRGGGAAVGG3'	Liu et al. (1999)
Tsr1-Tsr2	*Tsr1* gene	f 5'CCACGC TCA TTC AA ATCTTC T 3' r 5'CCGGTAGTTCGACCACTCGCATA3'	Schmidt et al. (2009)

f – forward; r- reverse

Amplification

After pouring 18 µL of the primer solution into a PCR plate, 2 µL of DNA was added. The amplification processed with an ABI GeneAmp PCR System 9700 Thermal Cycler, ramp rate, °C/sec up to 3.5 (heating), and consisted as described in table 4.3. Purified PCR products were used as a sequencing template.

Table 4.3 – Description of the amplification process for each primer set.

Primer pair	Predenaturation	Denaturation	Annealing	Extension	Final extension	Number of cycles
Bt2a-Bt2b	94 °C/2 min	96 °C/30 s	51 °C/1 min	72 °C/1 min	72 °C/5 min	35 cycles
Cf1-Cf4	94 °C/2 min	96 °C/30 s	51 °C/1 min	72 °C/1 min	72 °C/5 min	35 cycles
I5-D2r	94 °C/2 min	96 °C/30 s	51 °C/1 min	72 °C/1 min	72 °C/5 min	35 cycles
Mf-Mr	94 °C/2 min	96 °C/30 s	56 °C/1 min	72 °C/1 min	72 °C/5 min	40 cycles
M1f-M1r	94 °C/2 min	96 °C/30 s	56 °C/1 min	72 °C/1 min	72 °C/5 min	40 cycles
M2f-M2r	94 °C/2 min	96 °C/30 s	56 °C/1 min	72 °C/1 min	72 °C/5 min	40 cycles
5F-7R	94 °C/2 min	96 °C/30 s	51 °C/1 min	72 °C/1 min	72 °C/5 min	35 cycles
Tsr1-Tsr2	94 °C/2 min	96 °C/30 s	53 °C/1 min	72 °C/1 min	72 °C/5 min	35 cycles

Agarose gel electrophoresis

Agarose gel electrophoresis is the easiest way to look if the amplification was successful. After primer set amplification, an agarose gel was prepared, poured into the electrophoresis tank and after cooling covered with TAE buffer. The samples were then placed (2 µL) and run for 5 minutes. After electrophoresis the gel was placed on a light box, and exposed to ultraviolet radiation. A photograph with a digital camera was taken.

Stock Solution of 0.5 M EDTA

Weight 93.05 g of EDTA disodium salt. Dissolve in 400 mL deionized water and adjust pH with NAOH to 8.0. Top the solution to a final volume of 500 mL.

Stock Solution of 50X TAE

Weight 242 g of Tris base. Dissolve in approximately 750 mL deionized water. Carefully add 57.1 mL glacial acid and 100 mL of 0.5 M EDTA (pH8.0) and adjust the solution to a final volume of 1000 mL.

Working Solution of 1X TAE buffer

Dilute the TAE stock solution by 50X in deionized water.

Gel Agarose 1.2%

1.2 g of agarose

25 mL of TAE with Gel Red

75 mL Buffer TAE

Clean-up of the samples before sequencing

The PCR product was purified with ExoSAP-IT® (USB, Affymetrix, Inc.) following the supplier's protocol; briefly, 5µL of diluted EXOSAP-IT (1:5 with sterile water) was added to 20 µL of reaction products. The cleaning program consisted of degradation of remaining primers and nucleotides step at 37 °C/45 min, followed by an inactivated step at 80 °C/15 min and cooling to 4 °C at the end of the reaction (Figure 4.3).

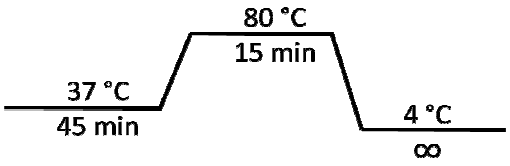


Figure 4.3 - Cleaning program setting.

Sequencing

A solution containing 0.5 µL of BigDye Terminator Mix, 2.2 µL of sterile distilled water, 2 µL of 5xBuffer and 0.3 µL of each primer, was made according to the number of samples.

After pouring 5 µL of this solution in a sequencing plate, 5 µL of cleaned DNA was added. Thermal cycler conditions were set as described in Figure 4.4.

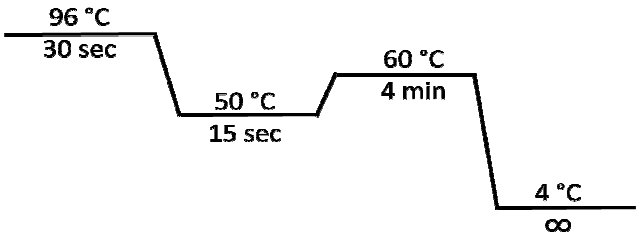


Figure 4.4 - Thermal cycle setting for the sequencing program.

DNA sequencing was performed with dye terminator technology (3.1) and an ABI 3730 sequencer, both from Applied Biosystems (Foster City, California). PCR products were sequenced in both directions.

Data analysis

Sequencing errors were detected and corrected using Sequencher (Gene Codes, Ann Arbor, Michigan). DNA sequences were aligned for phylogenetic analysis with ClustalX (Larkin et al. 2007). After alignment the leader elements were trimmed off in Textpad. PAUP* 4.0b10 (Swofford 2003) was used to conduct parsimony analysis and to generate phylogenetic trees for single gene alignments as well as on combined alignment. Bootstrapping (bs) was performed in PAUP* with maximum parsimony criterion and TBR branch swapping for 1000 replicates. MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) was used to calculate Bayesian posterior probabilities (pp) of branches. Mostly default settings were used. *Mcm7*, *Tsr1*and *RPB2* datasets included only protein coding whereas mating type, *BT2* and *CF* loci included proteins coding and intron regions, and were accordingly partitioned into intron and exon regions. Markov chain Monte Carlo (MCMC) analysis was conducted for up $5x10^6$ generations until the chains converged. Exclusionary principle of Baum and ShaW (1995) and genealogical concordance phylogenetic species recognition concepts (Taylor et al. 2000) were used for concordance analysis. Congruence was based on strongly supported branches with bootstrap values and posterior probability above 90% and 0.90, respectively.

Mating type determination

Type strains and all isolates in our study were submitted to a screening in order to determine their mating type. Using both primer pairs for each MAT locus in a PCR reaction, the result is obtained by the migration band. The smaller band migration indicates that is *MAT1-1*, the further band migration indicates that the strain is *MAT1-2* (Figure 4.5).

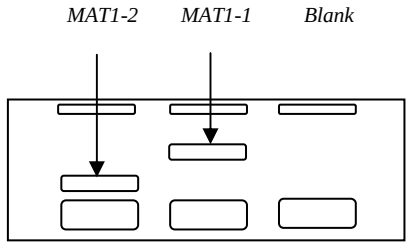


Figure 4.5 – Scheme of mating type determination.

Holotype

The holotype is a single physical example of an organism, designated by the original describer at the time of publication, when the species was formally described. Since under the scope of this Thesis a new species was described, its holotype was prepared and stored in MUM.

The holotype methodology was provided by Stephen W. Peterson and was adapted according to the strains characteristics. The methodology consisted in pre-measuring and marking 90 mm plates (55-60 mm) with MEA and CYA. A loop full of spores was suspended in 0.2% agar and used for inoculation in the centre point of the plate. After 5 days growth in the dark at 25 °C the culture medium was cut following the marks with a sterile scalpel (Figure 4.6); the cut sample was placed in a card slide with water soluble glue, dried for 10 hours at 37 °C, and placed in a plastic bag.

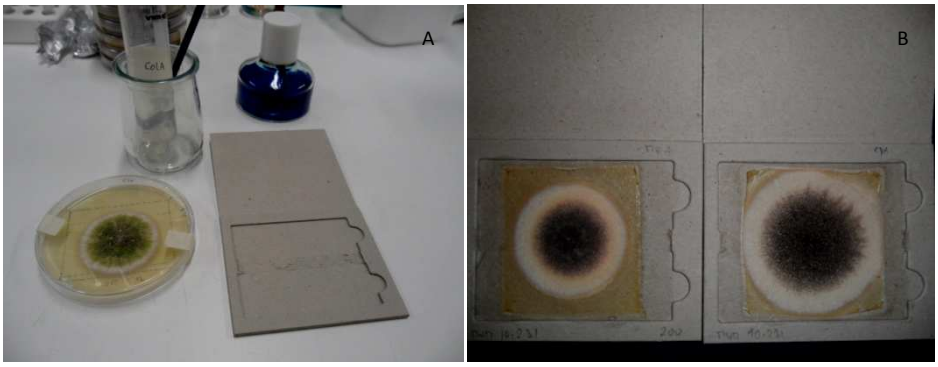


Figure 4.6 – A. Making of the holotype. B. Holotype of the strain MUM 10.231 in MEA and CYA.

4.2.2.3 Mycotoxin production Aspergillus strains

Some media seem to be more mycotoxin producing inducers than others. For the different mycotoxins in study different media were used being made three point inoculations on CAM, CYA and YES media. Samples were taken as positive in CAM if fluorescence showed under UV light and in the other media by HPLC when a peak was obtained at a retention time similar to each standard, with a height five times higher than the baseline noise.

Fluorescence in Coconut Agar Medium (CAM)

For a preliminary screening for AF and OTA production, strains were inoculated at a central point on a 60 mm diameter Petri dish containing 10 ml of Coconut Agar Medium (Davis et al., 1987) and incubated for 5 days in the dark at 25 °C. Cultures were observed for fluorescence under long-wave UV light (365 nm) after 3 and 5 days (figure 4.7). Results obtained in CAM were compared with results obtained by the more reliable chromatographic methodology.

CAM: coconut agar medium

200 mL of commercial coconut milk

8 g of agar (HIMEDIA)

pH 7

Mix all the components and stir. Heat until all agar is dissolve. Adjust pH 7 with a concentrated NaOH solution. Autoclave at 121 °C for 15 minutes.

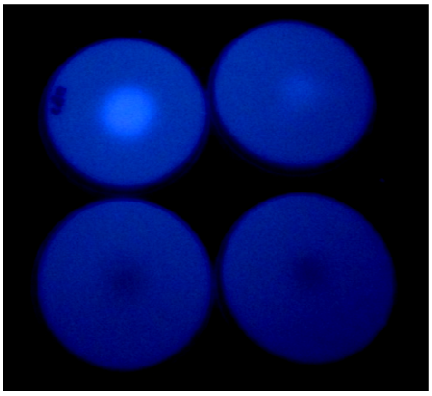


Figure 4.7 - Inoculated CAM plates under UV light with 3 days growth. Two plates are negative (bottom) and two are positive (above).

Aflatoxins production

All strains were tested for AF production in aflatoxin-inducing Yeast Extract Sucrose agar medium (YES). Strains were inoculated on 90 mm diameter plates and incubated at 25 °C for 7 days in the dark. Extraction methodology described by Bragulat *et al.* (2001) was employed: briefly, 3 agar plugs were removed from one colony, and placed into a 4 mL vial, where 1 mL of methanol was added. After 60 minutes, the extract was filtered through 0.45 µm filters, evaporated and further dissolved in 1 mL of mobile phase.

Samples were analysed by HPLC with a Jasco FP-920 fluorescence detector (365 nm excitation wavelength; 435 nm emission wavelength), using a photochemical post-column derivatization reactor (PHRED - Aura Industries, USA). Chromatographic separations were performed on a reverse phase C18 column (Waters Spherisorb ODS2, 4.6 mm x 250 mm, 5 µm), fitted with a precolumn with the same stationary phase. The mobile phase was water:acetonitrile:methanol (3:1:1, v/v) pumped at 1.0 mL/min . The injection volume was 50 µL.

Aflatoxins standard was supplied by Biopure (Austria). A mix of aflatoxins, containing 2 mg/L each of AFB_1 and AFG_1, and 0.5 mg/L each of AFB_2 and AFG_2 was used.

YES: yeast extract and sucrose agar

10 g of extract of autolysed yeast cells (Difco)

75 g of sucrose (Fisher Scientific)

10 g of agar (HIMEDIA)

500 mL of distilled water

Mix all the components. Add distilled water and stir. Heat until all agar is dissolve.

CPA production

Aspergillus section *Flavi* strains were inoculated on 90 mm diameter plates containing CYA and incubated at 25 °C for 14 days in the dark (Gqaleni *et al.*, 1997), and extracted as described for aflatoxin analyses.

Samples were analysed using a HPLC equipped with a Varian 2050 UV detector (285 nm). Chromatographic separations were performed on a EuroSpher 100 NH_2 column (Knauer, 4.6 mm x 250 mm, 5 µm), fitted with a precolumn with the same stationary phase. The mobile phase used was acetonitrile: 50 mM ammonium acetate (3:1, v/v), pH 5 pumped at 1.0 mL/min. The injection volume was 50 µL.

CPA standard was supplied by Sigma (St. Louis, MO, USA).

OTA production

Aspergillus niger strains were tested for OTA production in YES. Strains were inoculated on 90 mm diameter plates and incubated at 25 °C for 7 days in the dark. The used extraction methodology was the one described for aflatoxin analyses. Samples were analysed by HPLC with a Jasco FP-920 fluorescence detector (333 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 x 4.6 mm, 5 µm) fitted with a precolumn with the same stationary phase. The mobile phase was water:acetonitrile:methanol (99:99:2, v/v) pumped at 0.8 mL/min. The injection volume was 50 µL.

OTA standard was supplied by Biopure (Austria).

The concentration of the working solution was checked by UV spectrophotometry [Equation 4.1]. The concentration of this working solution was found to be 177 ng/mL and was used to prepare HPLC standards.

$$[OTA] = \frac{A_{max} \times M \times 1000}{\varepsilon \times \delta} \qquad [4.1]$$

Where:

[OTA] represents the concentration of OTA in solution expressed in μg/mL;

A_{max} is determined at the absorption maximum of the absorption curve (= 331 nm)

M is the relative molecular mass of OTA (M=403,8 g/mol)

ε is the molar extinction coefficient of OTA in this solvent mixture (=5440 m^2/mol)

δ is the optical path in cm

FB2 production

Strains were tested for FB2 production in CYA. Strains were inoculated on 90 mm diameter plates and incubated at 25 °C for 7 days in the dark. The used extraction and derivatization methodology consisted in removing 5 agar plugs from one colony, and placed into a 4 mL vial, where 1 mL of methanol:water (75:25) was added. After sonication for 50 minutes, the extract was filtered through 0.45 μm filters, evaporated and further derivatized as described in Abrunhosa et al. (2011b). Briefly, 200 μL of methanol, 200 μL of borate buffer, 100 μL of sodium cyanide and 100 μL of NDA were added into the dry extracts following the mentioned order. The samples were then placed in heat block for 15 minutes at 60 °C and diluted with 1.4 mL of acetonitrile/water (3:2, v/v).

Samples were analysed by HPLC with a Jasco FP-920 fluorescence detector (420 nm excitation wavelength; 500 nm emission wavelength). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 x 4.6 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase was acetonitrile:water:acetic acid (60:40:1, v/v) pumped at 1.0 mL/min. The injection volume was 50 μL.

Fumonisin B2 standard was supplied by Sigma (USA).

Borate buffer (0.05 M)

3.61 g sodium borate decahydrate ($B_4Na_2O_7.10H_2O$) (Sigma-Aldrich ®)

100 mL distilled water.

Stir until complete dissolution of the solid and adjust pH to 9.5 with HCl (6N). Store at 4 ° C.

Sodium cyanide solution (0.13 mg/mL)

0.13 g Sodium cyanide (Sigma-Aldrich ®)

100 mL distilled water

Mix the sodium cyanide with 100 mL of distilled water to obtain a 1.3 mg/mL solution. Homogenize solution and transfer 10 mL of this first solution to a 100 mL volumetric flask. Add distilled water. Homogenize to be stored at 4 ° C.

NDA (0.25 mg/mL)

2 mg NDA (Sigma-Aldrich ®)

8 mL methanol

Homogenize and store at 4 ° C for a maximum of 5 days.

Simultaneous detection of aflatoxins and CPA

Alternatively to the individual analyses of aflatoxins and CPA by *Aspergillus* section *Flavi* isolates, a methodology based on the photolysis of CPA was developed for the simultaneous detection of these mycotoxins. The strains were simultaneously tested for AF and CPA production in CYA. All strains were inoculated on 90 mm diameter plates and incubated at 25 °C for 12 days in the dark (Gqaleni *et al.*, 1997) and extracted as described above.

Extracts were analysed by HPLC equipped with a Jasco FP-920 fluorescence detector (372 nm excitation; 462 nm emission), using a photochemical post-column derivatization reactor (PHRED - Aura Industries, USA). Chromatographic separations were performed with C18 column (Waters Spherisorb ODS2, 4.6 mm x 250 mm, 5 µm) with a precolumn of the same stationary phase. The mobile phase was methanol: 4mM zinc sulphate (65:35, v/v), pH 5 pumped at 0.8 mL/min (Hayashi and YoshizaWa, 2005; Oliveira et al., 2008). The injection volume was 50 µL.

4.3 Results and Discussion

4.3.1 Isolation and characterization of *Aspergillus* species

The results regarding fungal isolation from the three steps of maize storage chain (reception of green maize, after drying and storage) are described in this chapter. Being the main interest of this study the isolation of *Aspergillus* strains, only isolates of this gender were isolated and identified to the section level. Around ninety one percent of the maize samples were contaminated with strains of *Aspergillus*. These were subsequently grouped into five sections: *Flavi* (423 isolates) followed by *Nigri* (270 isolates) and *Wentii* (214 isolates). Two other sections were rarely isolated: *Circumdati* (15 isolates) and *Candidus* (2 isolates). The section *Aspergillus* was also observed regularly, but no isolations were made. In total, nine hundred and eighteen *Aspergillus* strains were isolated from the ninety five samples obtained in the three regions (Figure 4.8).

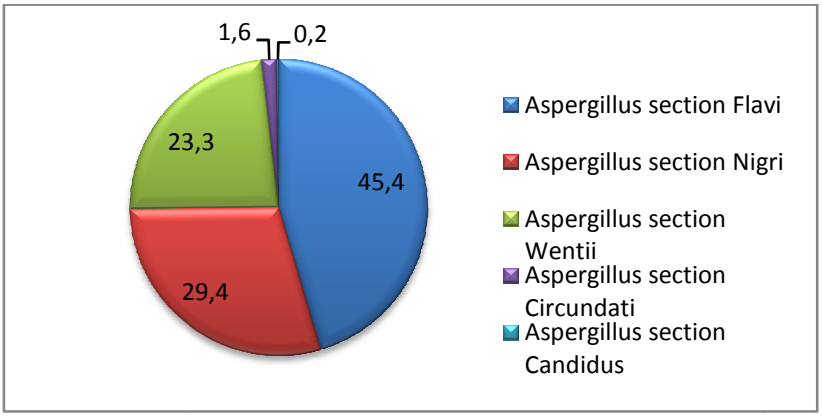


Figure 4.8 – Fungal frequency (*FAS*) of *Aspergillus* isolates belonging to different sections.

Other species, such as *Penicillium* spp., were also observed in the plates but were not isolated as well.

The fungal frequency of isolations obtained from the samples belonging to the three steps of the maize storage chain differs (Figure 4.9). The lowest frequency is located at the reception followed the dryer. The highest frequency occurs at storage.

At reception maize samples had high values of humidity, ranging between 19-23%. Even though the maize kernels were plated in a xerophilic medium (MEA10), some of the

samples had excessive fungal growth of other genders (like *Rizhopus*); these species, taking advantage of the high humidity in the grains, avoided the growth of *Aspergillus* species. In these samples, the incidence of *Aspergillus* strains may have been determined by defect. As the maize kernels dried, the incidence of Aspergillus strains increases, since it is more adapted to the xerophilic conditions.

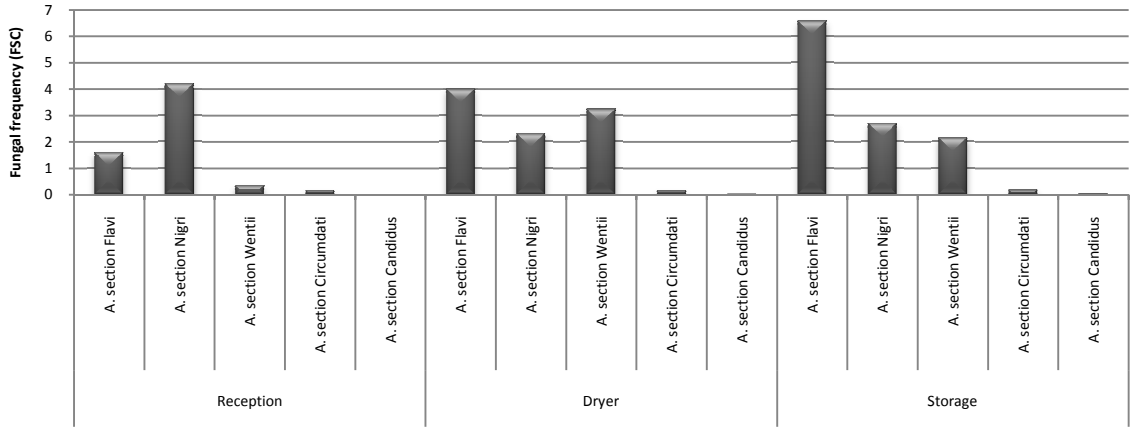


Figure 4.9 – Fungal frequency (*FSC*) at the three storage steps divided into the five *Aspergillus* sections.

Dividing the fungal incidence into the isolated sections, *Aspergillus* section *Flavi* is the most common section isolated from both the dryer and storage. At reception, the frequency of isolates belonging to *Aspergillus* section *Nigri* is higher than the *Flavi* section. At the dryer the incidence of black *Aspergillus* drops to the third place, below *Aspergillus* section *Wentii*. However, the higher incidence of the latter section is due to its higher occurrence in one of the studied regions, as it may be concluded from Figure 4.10. Regional differences are observed during the three stages of the storage chain.

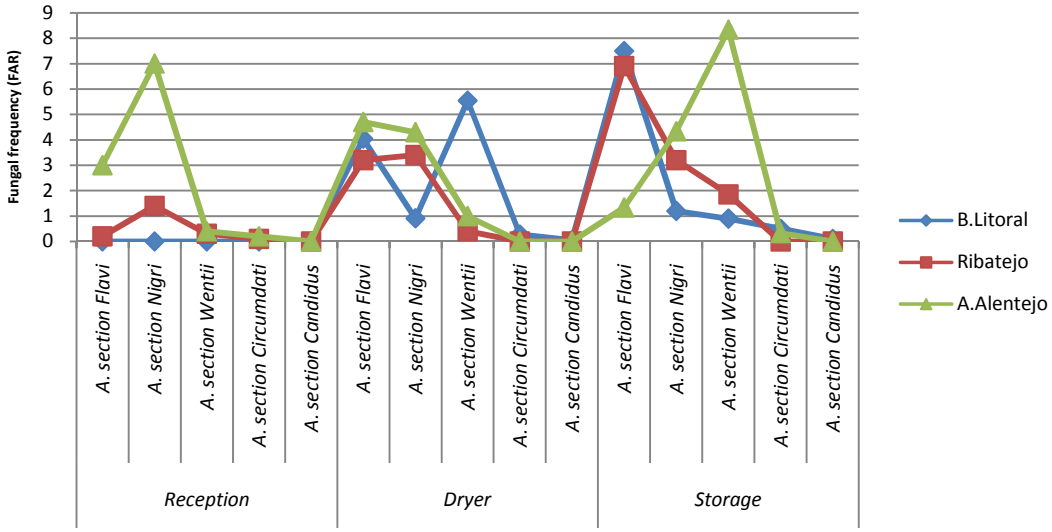


Figure 4.10 – Fungal frequency (*FAR*) divided into the isolated *Aspergillus* sections per region.

Beira Litoral

As previously referred, no reception samples were analysed in Beira Litoral region. All samples obtained were already dried, using different types of dryers (Figure 4.11). From twenty two dried samples, two hundred and thirty eight *Aspergillus* species were isolated. Most of these isolates belong to two sections: *Flavi* and *Wentii*.

Fungal frequency was calculated according to the [Equation 4.4] for each type of dryer. The gas dryer from the Agriculture Cooperative showed higher fungal frequency (16.2) in comparison with the other dyers. This frequency is followed by the diesel dryer (11.0), the wood dryer (7.5) and finally by the gas dryer from the producers (7). Opposite to both gas dryers, the wood and diesel dryers have more incidence of *Aspergillus* section *Wentii* than *Aspergillus* section *Flavi*.

At storage, the higher frequency belongs to the *Aspergillus* section *Flavi* (7.5), followed by the sections *Nigri* (1.2), *Wentii* (0.9), *Circumdati* (0.5) and finally by the section *Candidus* (0.1). Some of these samples were dried in threshing floors before storage, but no major difference is observed when compared with the other types of drying processes. Overall, the frequency of *Aspergillus* section *Nigri* is relatively low when compared with the other two regions.

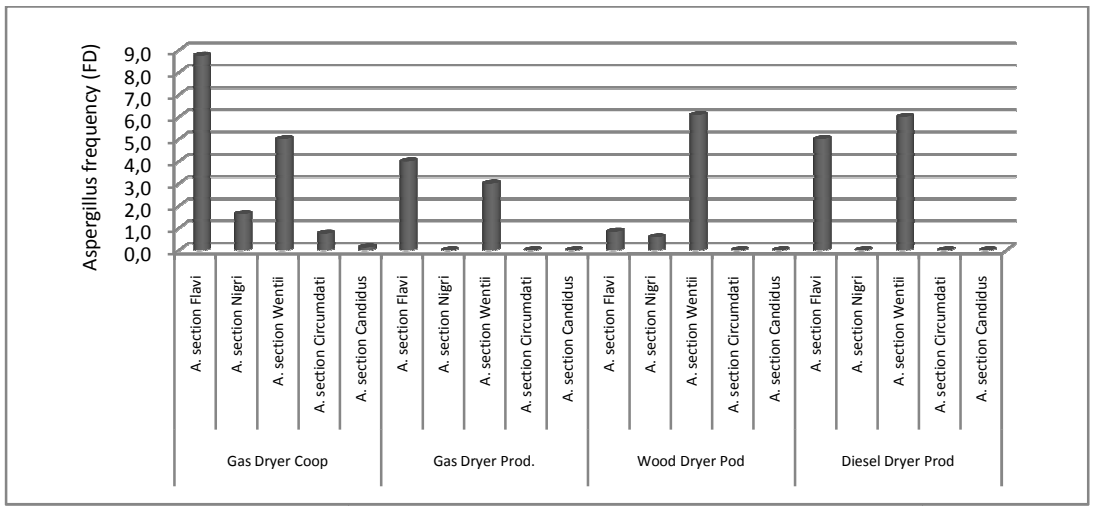


Figure 4.11 – *Aspergillus* frequency (*FD*) at each type of dryer used to dry the maize kernels in the region of Beira Litoral.

Ribatejo

In Ribatejo region the sampling scheme was followed as planned, where 40 samples were obtained. Being the first region where the sampling started the contamination problems with other genders was more evident preventing the isolation of *Aspergillus* strains in its first sampling at reception. In the second sampling at reception a better isolation scheme was considered allowing the isolation of *Aspergillus* strains. Therefore, the fungal frequency at this stage was considerably low (2.0), being the black *Aspergillus* the most common.

After drying, the fungal frequency increased (7.0). The two sections with higher frequencies at this stage were the *Flavi* (3.2) and *Nigri* (3.4).

The major difference between the storage and the dryer samples was the distribution of isolated strains between sections. Even though the quantity of fungi isolated was similar (6.0) at storage, the *Aspergillus* section *Flavi* isolates was isolated at higher frequencies than of other sections (Figure 4.10).

Alto Alentejo

The green maize samples in Alto Alentejo were taken after the first trial in Ribatejo, which allowed the isolation of *Aspergillus* strains in both sampling moments, being the fungal frequency high (10.5). Similarly to Ribatejo, at this stage of the sampling chain, the black

Aspergillus (7.0) were the ones with higher frequency, and its frequency was also very similar to the one of the section *Flavi* at the dryer (4.7), even if slightly lower (4.3).

Opposite to the other regions, at storage, the frequency of *Aspergillus* section *Wentii* is considerably higher (8.0) when comparing to the other sections. Here, the section *Flavi* has a much lower frequency, occupying the third place (1.0).

Similar to the other regions the section *Circumdati* has low frequency, and no strains of the section *Candidus* were isolated.

4.3.2 Identification and characterization of *Aspergillus* section *Flavi* isolates

From the ninety five samples of maize kernels obtained in the three Portuguese regions, *Aspergillus* section *Flavi* isolates were found in seventy four samples (78%). From these, four hundred and seventeen strains were isolated (Table 4.4). This result is slightly higher than the ones obtained by Bankole and Mabekoje (2004), where *A. flavus* was detected in 65% of maize samples in Nigeria, and by Gao et al. (2007) where 58% of the samples, taken in China, showed growth of *Aspergillus* section *Flavi* strains.

The incidence of *Aspergillus* section *Flavi* strains differed among regions being present in 69% of the samples from Beira Litoral and 83% of the samples from both Ribatejo and Alto Alentejo.

Table 4.4 - Number of isolates and samples from three Portuguese Regions.

Region	Number of samples with isolations per total number of samples	Number of *Aspergillus* section *Flavi* isolates
Beira Litoral	22/32	164
Ribatejo	33/40	172
Alto Alentejo	19/23	81
Total	74/95	417

Classical identification methods of *Aspergillus* section *Flavi* strains were performed by examining several macroscopic (*e.g.*, colony colour and cultural characteristics) and microscopic phenotypic characteristics (*e.g.,* spore shape). Extrolite characterization was also performed by accessing mycotoxin production (*e.g.*, AF and CPA).

Given that some of the isolates belonging to the *Aspergillus* section *Flavi* could not be described with accuracy up to the species level, molecular analysis was also performed to these strains.

The polyphasic approach revealed the presence of five distinct species: *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus tamarii*, *Aspergillus minisclerotigenes* and a not yet identified species, here described as *Aspergillus mottae* (Table 4.5).

Table 4.5 – Number of isolates of *Aspergillus* section *Flavi* from three Portuguese Regions.

Region	Species				
	A.flavus	*A.parasiticus*	*A.tamarii*	*A.minisclerotigenes*	*A. mottae*
Beira Litoral	152	1	13	1	0
Ribatejo	163	1	4	0	1
Alentejo	68	1	5	6	1
Total	383	3	22	7	2

The majority of the isolates belong to the *A. flavus* species (91.8%) and 0.7% were identified as *A. parasiticus*, being consistent with the results obtained in similar studies (Giorni et al., 2007; Gao et al., 2007) where 93% and 99% of the isolates were identified as *A. flavus*, and 7% and 1% were identified as *A. parasiticus*, respectively, showing that *A. parasiticus* is not a major cause of aflatoxin contamination in the field and during storage of maize.

Besides *A. flavus* and *A. parasiticus*, no other species were reported in these former studies, whereas in our study other species belonging to the section *Flavi* were identified (Figure 4.12).

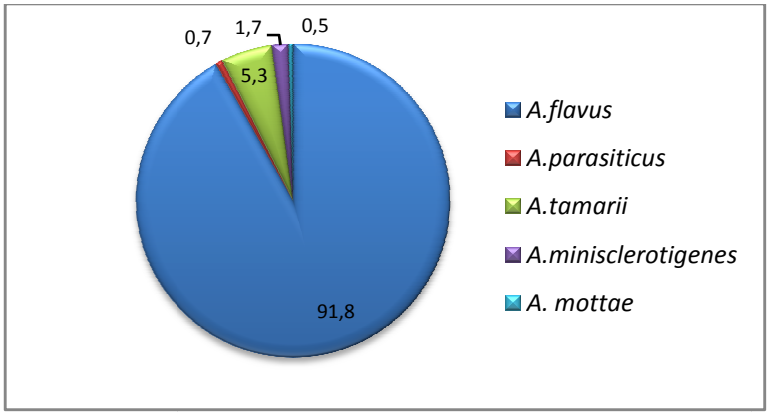


Figure 4.12 – Percentage of *Aspergillus* section *Flavi* species, isolated from maize kernels.

4.3.2.1 Phenotypic identification

Within this section several morphological characteristics were observed in order to identify the isolated strains. The full list of isolated strains, with the main traits used in their identification is given in annex 1 (Table A1).

The colony colour was registered after inoculating in CYA for 7 days, at the dark, at 25 °C. The colour pallet was the one used in Pitt and Hocking (2009), where yellow green was used to describe the light green colour common within the species of *A. flavus* but also seen in *A. minisclerotigenes* (Figure 4.13), dark green was used to describe the common colour observed in *A. parasiticus* species and olive brown was used to refer the *A. tamarii* species. The majority of the isolated strains showed yellow green colour, only three showed dark green and twenty two showed olive green colour.

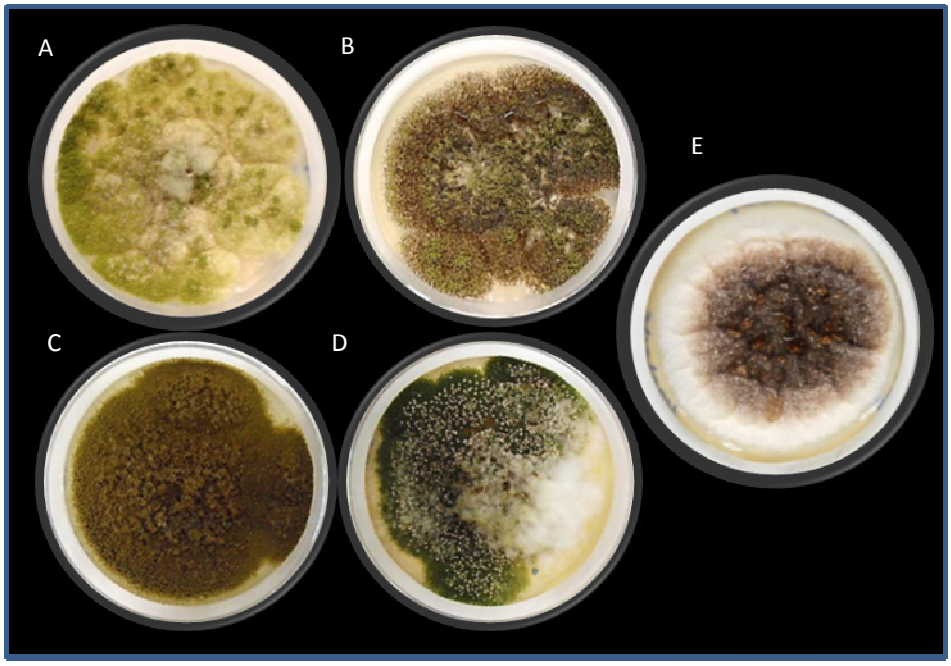


Figure 4.13 – *Aspergillus* section *Flavi* strains, CYA at 25 °C after 7 days. A- *A. flavus* (MUM 10. 232); B- *A. minisclerotigenes* (08MAS570); C- *A. tamarii* (09MAS68); D- *A. parasiticus* (08MAS169); E- *A. mottae* (MUM 10.233).

Sclerotia production was also registered. The difference in sclerotia production allows species to be subdivided into groups of *A. flavus* according to the morphology. The large strain (L) has sclerotia >400 µm in diameter and the small strain (S) has sclerotia < 400 µm (Horn, 2003). According to Geiser et al. (2000), the *A. flavus* group can be divided into two, where

group I is composed with type L and S strains and only produce AFBs and the group II is composed of S strains that produce AFBs or AFBs and AFGs. This division was made through genetic studies.

In our study, 53.5% of the *A. flavus* isolates produced sclerotia and 46.5% were non-sclerotia strains. Only one strain (0.5%) had sclerotia under 400 µm and produced the four aflatoxins. Abbas et al. (2005) in a study with isolates from soil and different commodities did not found a single S type strain in maize and had similar percentage of (L) sclerotia strains and non-sclerotia strains. Barros et al (2005) reported the same amount of isolates with L phenotypes. Giorni et al. (2007) reported that 62% of the strains isolated from maize kernels had sclerotia, and similar to our study only one strain had S phenotype.

All *A. minisclerotigenes* strains had sclerotia with sizes below 400 µm, as described by Pildain et al. (2008) and *A. mottae* produces numerous small dark sclerotia, like *A. minisclerotigenes*, *A. parvisclerotigenus* and some strains of *A. flavus. A. parasiticus* isolates showed the production of abundant, large sclerotia, whereas *A. tamarii* isolates did not show any sclerotial production.

Molecular characterization of *BT2*, *CF*, *RPB2*, *MCM7*, *TSR1* genes

The multiple loci and increased number of nucleotides in the combined dataset improves statistical support compared to single locus studies. Knowing the broad relationships in *Aspergillus*, it allows to put other data (e.g. phenotypic, extrolite) into phylogenetic perspective (Peterson, 2008).

The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the nuclear ribosomal RNA genes, such as internal transcribed spacers (ITS). Single-copy conserved genes, namely β-tubulin (*BT2*) and calmodulin (*CF*) have also been extensively used as targets for taxonomic studies in the genus.

The ITS sequences were not used in this phylogenetic study but are available at GenBank (Table A.3.1). Even though they are useful information for identification purposes, it did not show resolution up to species level, which happened with the other targeted genes.

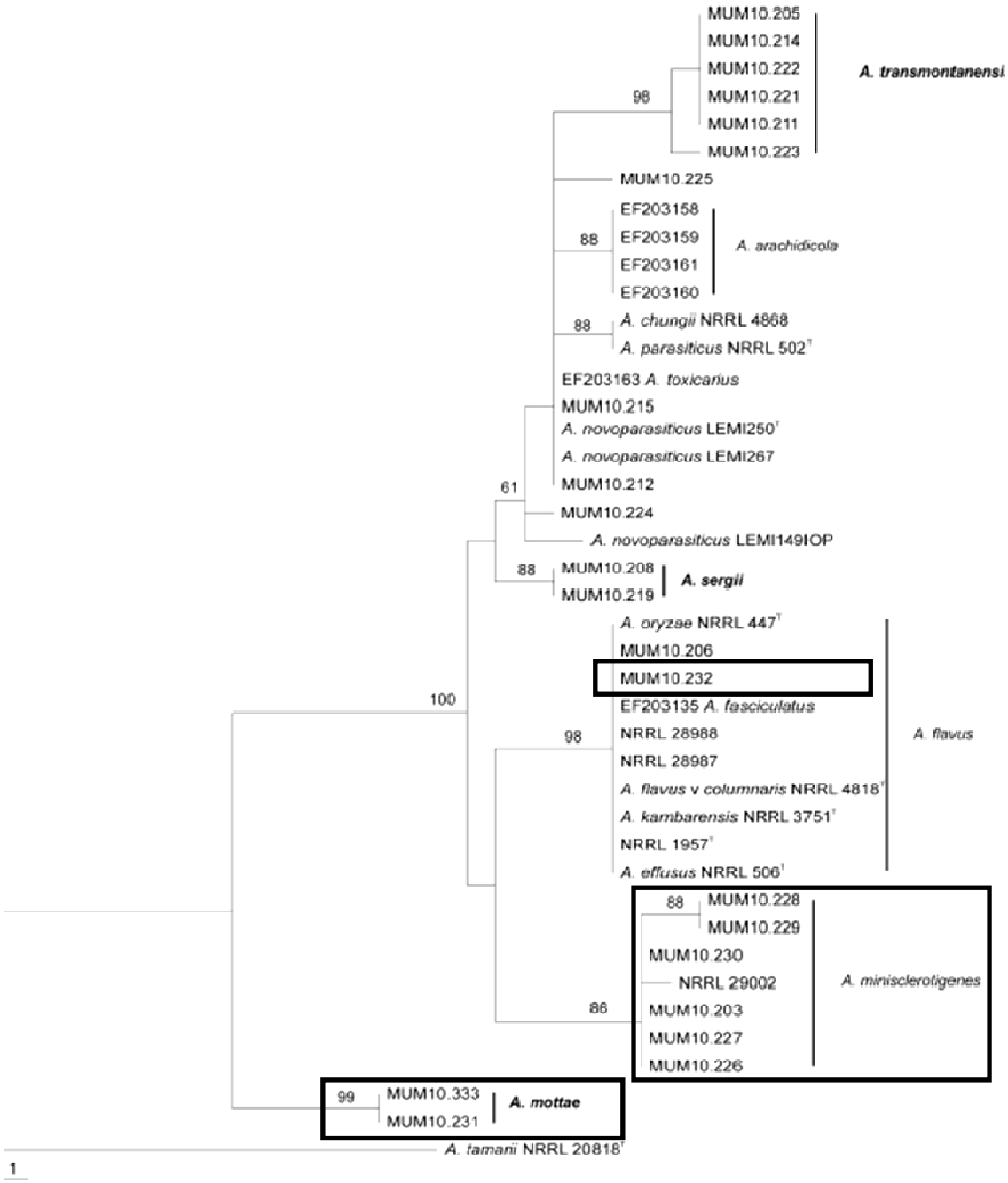


Figure 4.16 – Beta-tubulin locus 552 characters, 488 are constant, 28 are variable parsimony non-informative, 16 are parsimony informative; >10.000 equally most parsimonious trees, CI=0.9565, RC=0.9399.

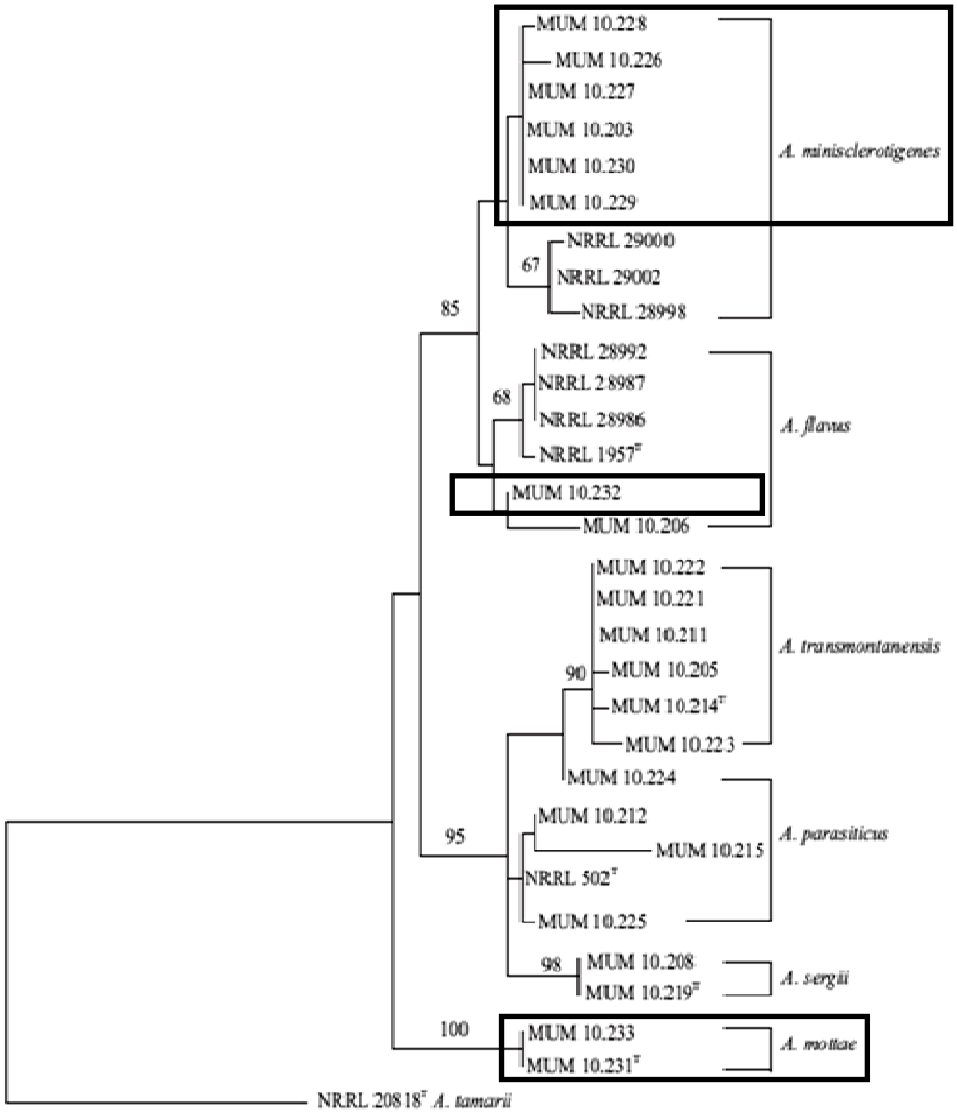


Figure 4.17 – *CF* locus, 752 characters: 651 are constant, 60 are parsimony-uninformative, 41 are parsimony-informative: CI = 0.9068, RC = 0.8569.

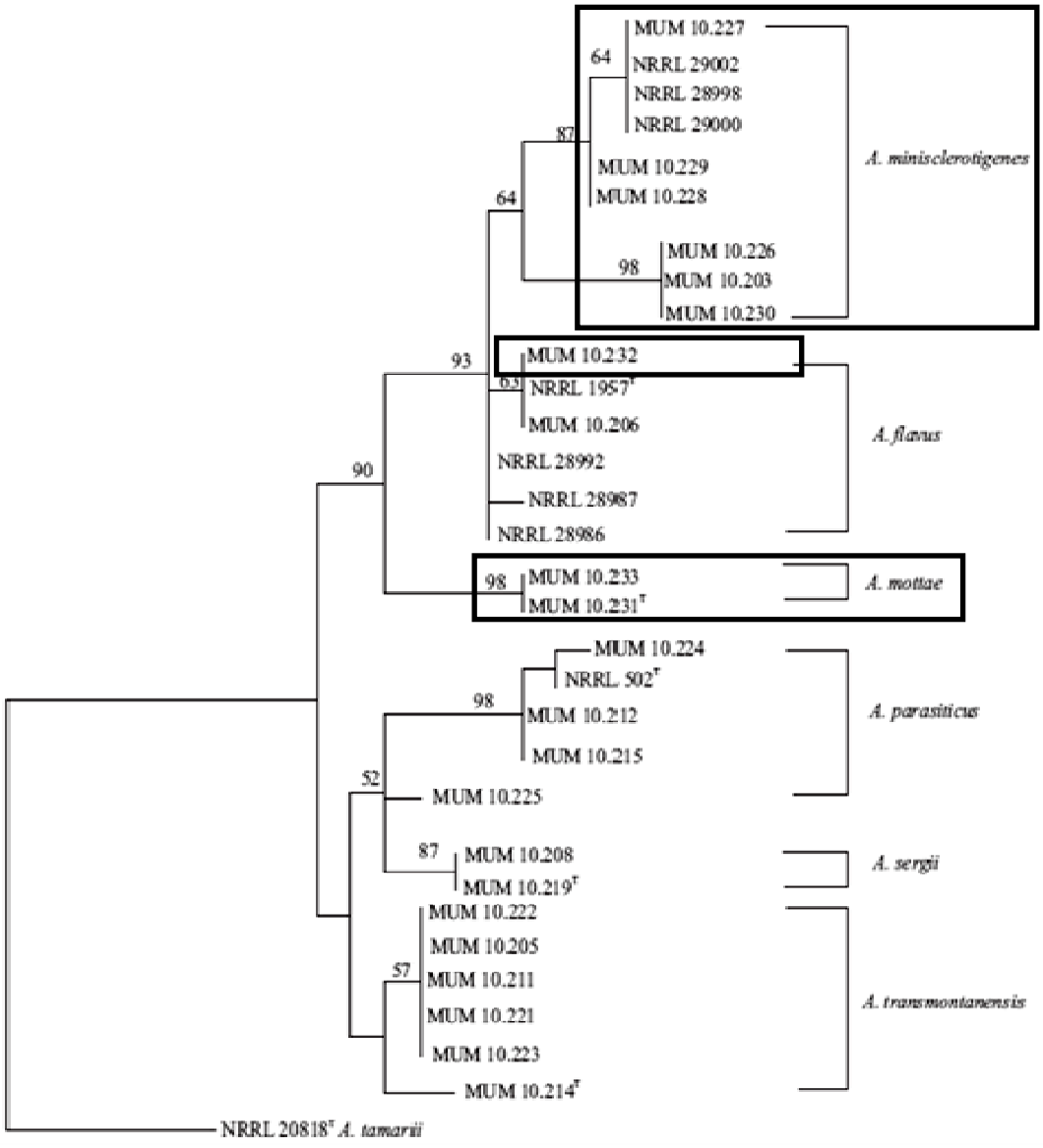


Figure 4.18 – *Mcm7* locus, 580 characters, 536 constant 14 characters are parsimony-uninformative, 30 parsimony-informative; CI = 0.9184, RC = 0.8935.

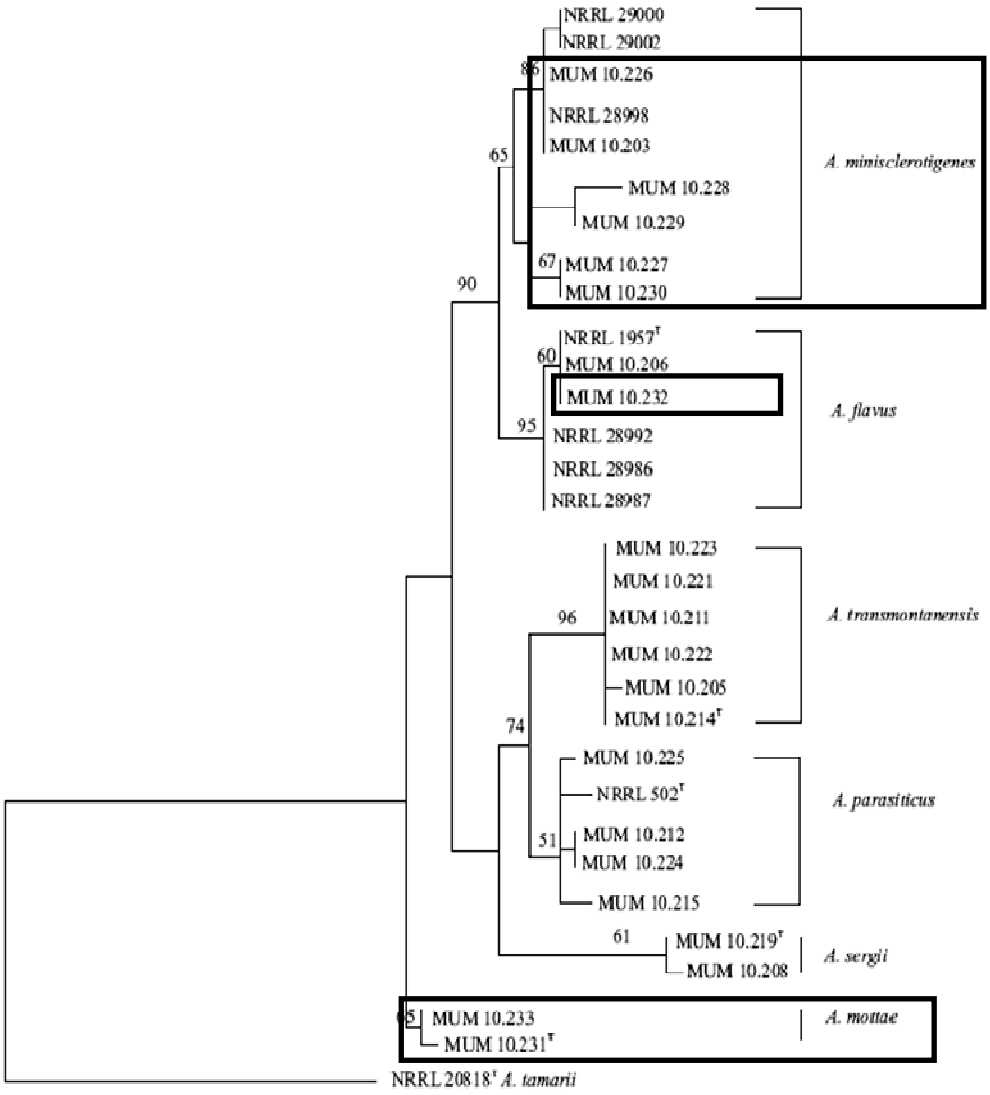


Figure 4.19 – *RPB2* locus, 1015 characters: 919 constant, 66 parsimony-uninformative, 30 parsimony informative; CI = 0.9245, RC = 0.8765.

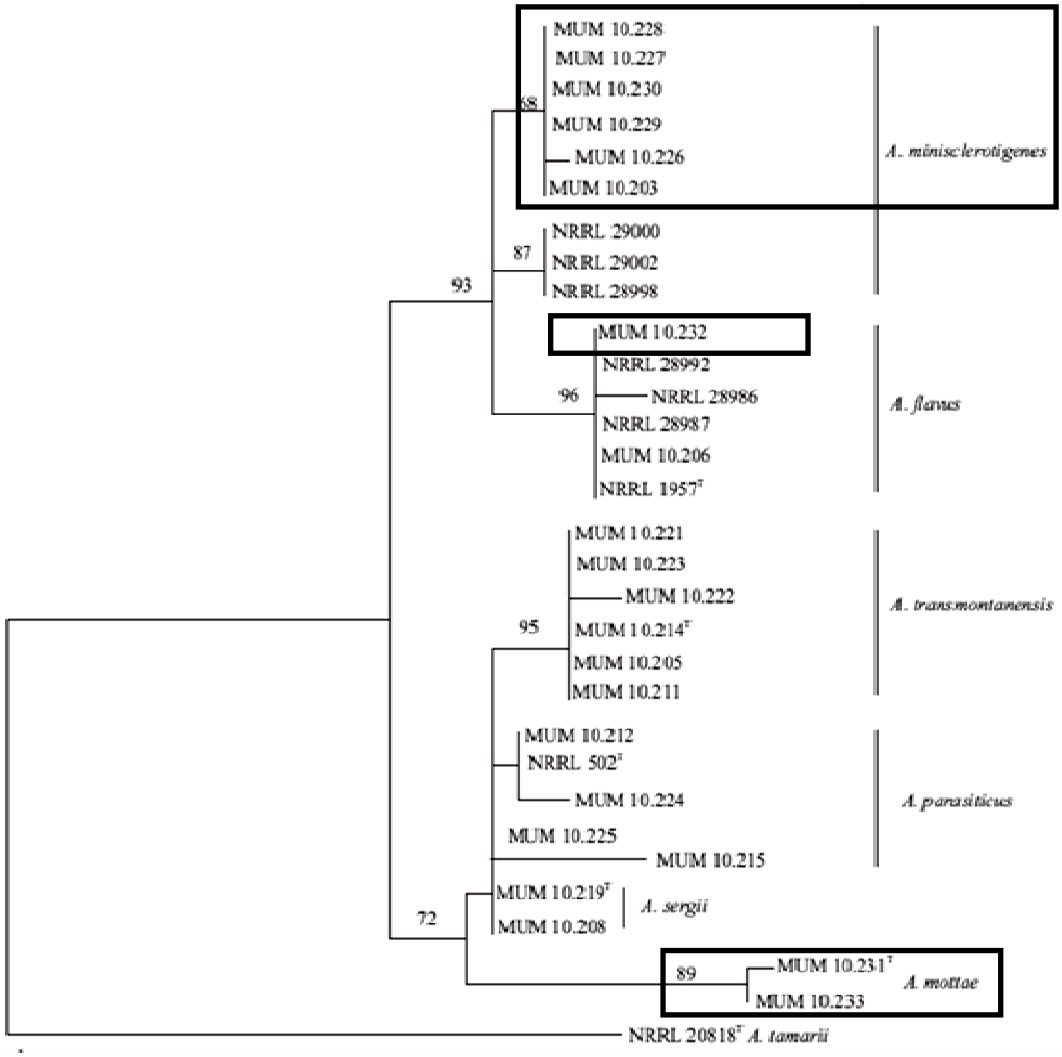


Figure 4.20 – *Tsr1* locus, 742 characters, 663 constant, 59 parsimony-uninformative, 20 parsimony informative, CI = 0.9881, RC = 0.9803.

The combined data (*BT2*, *CF*, *Mcm7*, *RPB2*, *Tsr1*) tree (Figure 4.21) showed that most of the sequenced isolates fell into two main clades represented by *A. flavus* and *A. parasiticus*. A third clade ancestral to *A. parasiticus* and *A. flavus* clades was formed by the two isolates (MUM 10.231 and MUM 10.233) belonging to the new species *A. mottae* (Figure 4.21). This is supported by 97–100% of the bootstrap sample and 1.00 Bayesian probability.

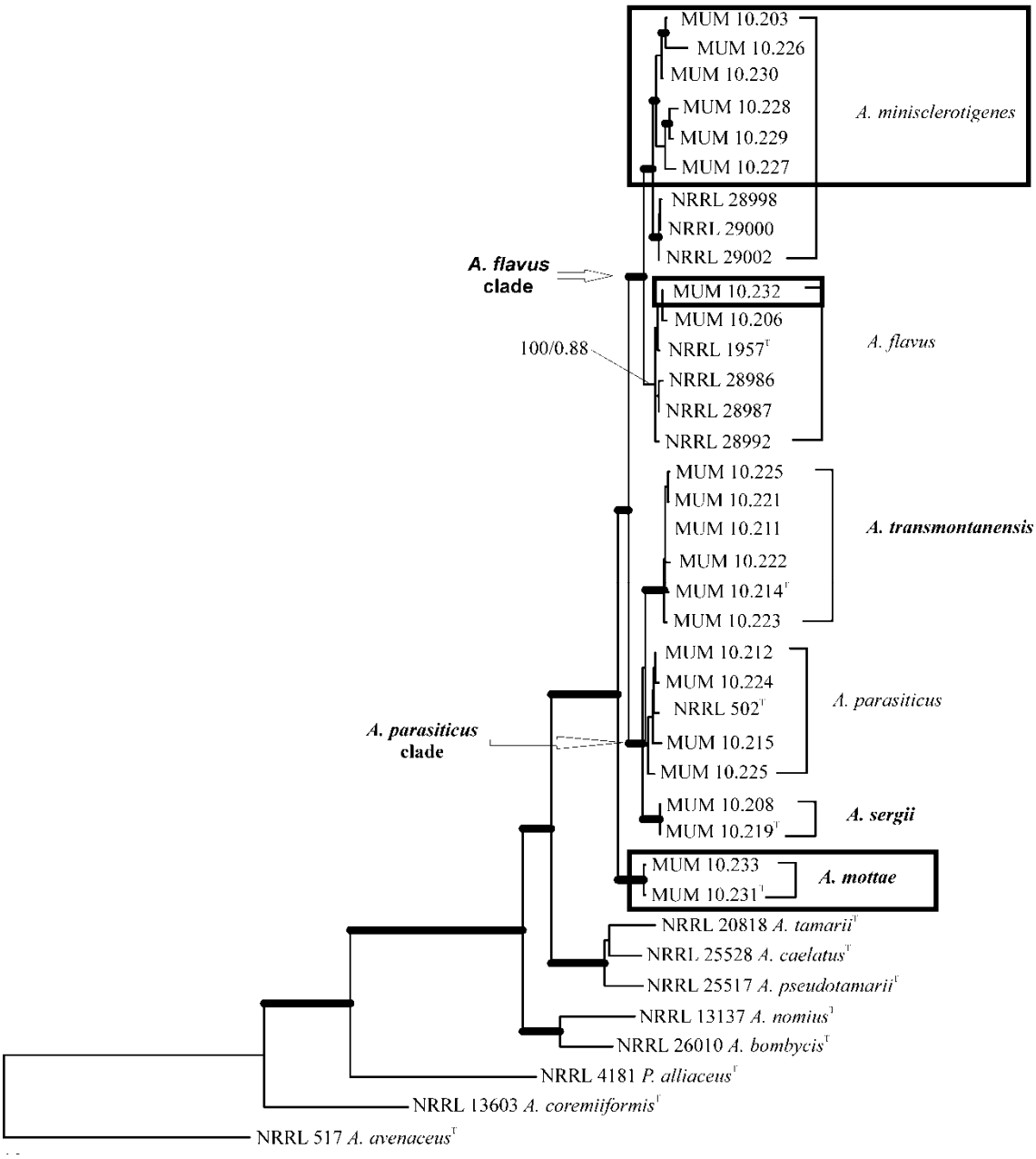


Figure 4.21- Phylogenetic tree calculated from combined *BT2*, *CF*, *Mcm7*, *RPB2* and *TSR1* data. Of 3746 total characters 2485 are constant, 618 variable characters are parsimony-uninformative, 643 characters are parsimony informative. There were more than 100 most parsimonious trees with CI = 0.7692 and RC = 0.5980. Boostrap values/Bayesian posterior probabilities are placed above internodes, thick branches have bootstrap values>90%. Bootstrap values below 70 were not considered.

To further comparison, additional sequences of beta tubulin and calmodulin of the isolates were aligned with homologous section *Flavi* sequences obtained from GenBank and

used to generate the maximum parsimony tree (Figure 4.22). A branch with >95% bootstrap proportion leads to a number of isolates.

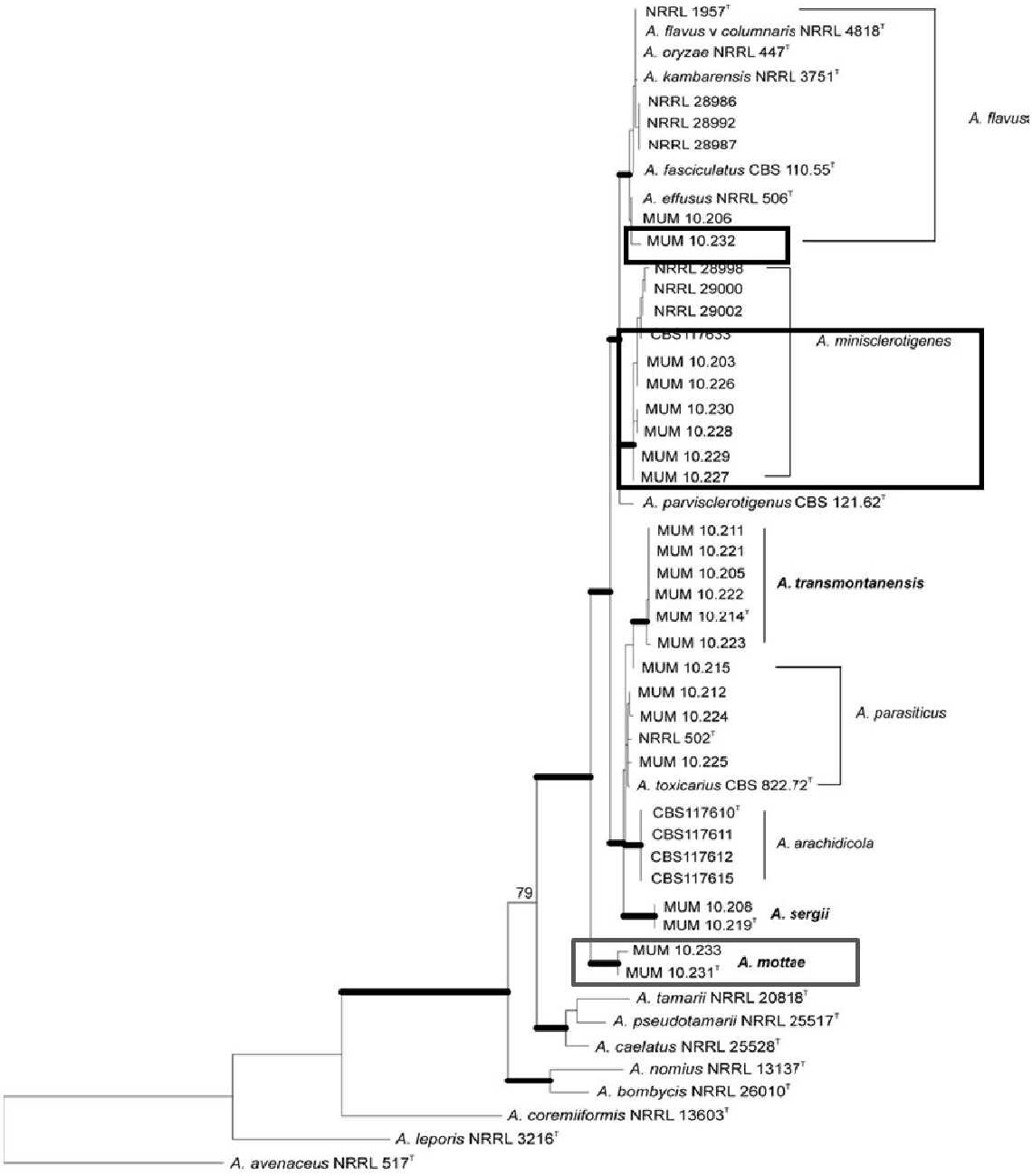


Figure 4.22 - Combined beta tubulin and calmodulin loci dataset: of 1318 total characters 799 are constant, 253 are variable but parsimony-uniformative and 268 parsimony-informative; CI=0.7998 RC=0.6585; 1 of >100 most parsimonious trees.

The *A. minisclerotigenes* isolates form a very strongly supported clade (100% bs/1.00 pp) with a subclade containing three isolates from Australia. The maize isolates of *A. flavus (*MUM 10.232) and *A. minisclerotigenes* (MUM 10.226, MUM 10.227, MUM 10.228, MUM 10.229, MUM 10.230) are strongly supported as sibling species.

A. mottae isolates were isogenic at three of five loci and in each case formed a distinct clade with greater than 85% bs support at 4 of the 5 loci. In both Figures 4.21 and 4.22, *A. mottae* is basal in the tree relative to the *A. flavus* and *A. parasiticus* clades with very strong statistical support.

The genealogical concordance phylogenetic species concept is based on recognizing the boundaries of species by concordance of the tree diagrams from different unlinked loci and the intraspecific variation of the isolates of each species. *A. mottae* had insufficient isolates to apply the concordance species concept, but is on a unique branch that is statistically distinct from other accepted species. *Aspergillus mottae* is separated from *A. minisclerotigenes* by relatively minor phenotypic differences, and the two isolates available left some doubts about how well these distinctions will separate the species once additional isolates are known. However, the DNA sequence distinctions have high statistical support. The acquisition of additional isolates of *A. mottae* will enable concordance analysis and testing. *A. mottae* is well supported by phylogenetic analysis of the multilocus combined data. Its branches are fully congruent as the same group of isolates always occurs as a terminal group at each locus and there is strong statistical support for that grouping, either by bootstrap analysis or Bayesian posterior probability analysis (Peterson 2008). *Aspergillus mottae* shares a most recent common ancestor with both the *A. flavus* and *A. parasiticus* clades. For this reason, study of *A. mottae* might be useful in analyzing the evolution of characters and toxins in the *A. flavus* and *A. parasiticus* clades (Figure 4.22). Donner et al. (2009) reported the existence of a strain (SBG), isolated from maize from Nigeria, that produces high levels of AFGs and AFBs and numerous small sclerotia. These are some of the characteristics of the new proposed species *A. mottae,* but we have not had access to SBG isolates.

In the combined data tree (Figure 4.21) and in some individual loci (Figure 4.17; 4.19; 4.20) the *A. minisclerotigenes* isolates from Australia are strongly supported as a distinct clade, but at other loci Australian and Portuguese isolates reside in a single clade that is contradictory to the combined data tree.

Mating type determination

Type strains and all isolates in our study were submitted to a screening in order to determine their mating type, either *MAT1-1* or *MAT1-2*, and subsequently sequenced, as described by Ramirez-Prado et al. (2008). The result is obtained by the migration band. A smaller band migration indicates that is *MAT1-1*, further band migration indicates that the strain is *MAT1-2* (Figure 4.23).

The resulting PCR showed that the tested strains were heterothallic with 24 strains assigned as *MAT1-1* and 13 strains as *MAT1-2*.

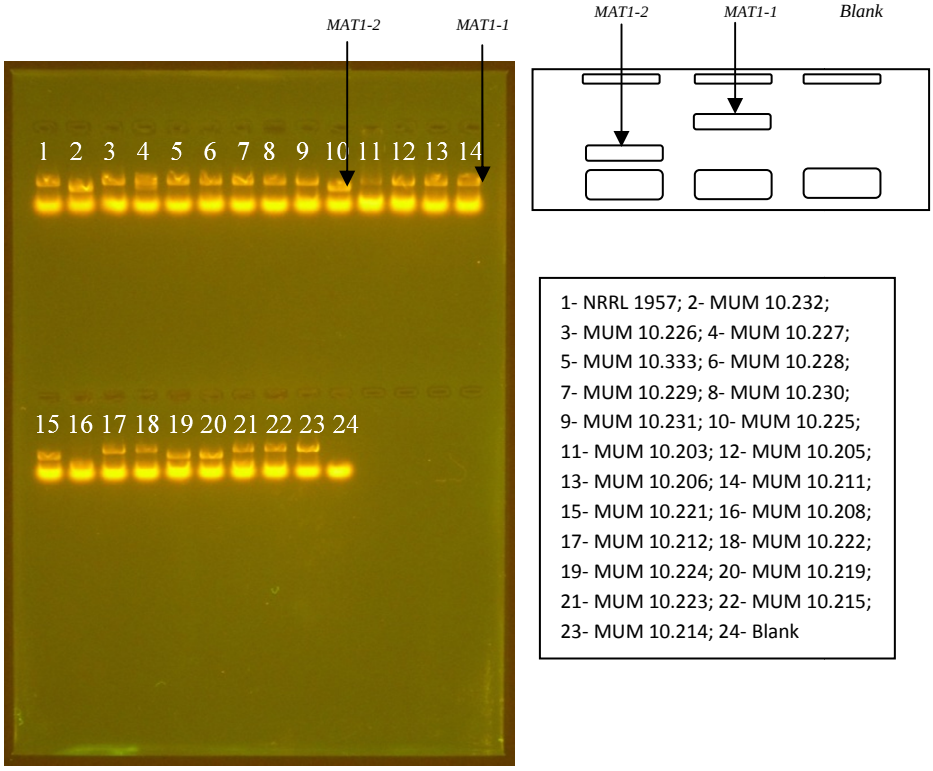


Figure 4.23 – Determination of mating-type genes in *Aspergillus* section *Flavi* strains isolated from Portuguese almonds and maize. The smaller band migration indicates that is *MAT1-1*, the further band migration indicates that the strain is *MAT1-2*. The mating type determination of the rest of the strains can be seen in Figure A.3.1.

The *MAT1-1* sequence set contained 375 aligned sequence positions (317 exonic positions, 58 intronic positions). The *MAT1-2* aligned sequence set contained 238 sequence positions (182 exonic positions, 56 intronic positions). *In silico* translation of the DNA sequences showed amino acid differences in some cases (Tables 4.6 and 4.7). In the case of *MAT1-1*, all species showed intra-specific single nucleotide polymorphisms (SNPs). In the

alignment (Table 4.6) *A. mottae* was used as the reference sequence because it is ancestral to *A. flavus* and *A. parasiticus*. *A. flavus* isolates had four synapomorphic amino acid differences from *A. mottae*. *A. parasiticus, A. transmontanensis* and *A. minisclerotigenes* had one or more synapomorphic amino acid differences from *A. mottae* and other species. *A. minisclerotigenes* NRRL 29002 from Australia had a single apomorphic difference from all other isolates of the species. *Aspergillus tamarii, A. caelatus, and A. pseudotamarii*, each represented by the type isolate shared seven synapomorphic differences from the *A. flavus/A. parasiticus* clade isolates and also had several apomorphic differences from each other. Similarly, *A. nomius* differed from each of the preceeding clades about equally.

The *MAT1-2* amino acid sequences were quite similar, with *A. sergii*, *A. parasiticus*, *A. minisclerotigenes* and *A. transmontanensis* having identical amino acid sequences (Table 4.7). The only *A. flavus MAT1-2* sequence and *A. bombycis MAT1-2* sequence differed from the above and each other at one amino acid site. *A. lanosus, A. alliaceus* and *A. avenaceus* display 5–7 amino acid differences from the sequences of the other species.

It is interesting that an *A. minisclerotigenes* isolate from Australia differs at one amino acid site from all other isolates of the species in the *MAT1-1* mating-type gene. These isolates appear to be components of a single gene pool and are regarded here as conspecific. By the results presented the known range of *A. minisclerotigenes* was extended to Europe in addition to South America and Australia (Pildain et al. 2008).

Nucleotide sequences comparison of the mating-type gene reveals that there are larger variations between species than within species; most isolates in this study showed species-specific SNPs. In some cases the SNPs were insufficient to change the amino acid residues. This is the case for all *MAT1-1* isolates of *A. parasiticus*, which had three SNPs but without amino acid replacement (Table 4.6). The small changes in the nucleotide sequence of the *MAT* gene might indicate the normal variation within species. Bigger changes, however, might indicate the loss of the *MAT* gene function (Turgeon, 1998).

Table 4.6 - Amino acid sequence alignment of a 317 bp fragment of MAT1-1 amplification. Regions with dots indicate identical amino acids residues; regions with amino acids are not conserved.

Species	Strains	MAT1-1 amino acid sequence
A. mottae	MUM 10.231	PEQLEELLKYLQDAKSQENTQSSYPKENLQSCLEFKADKNNGSTTPASANPRSSASRGKRTSDAKRRPLNSFIAFRSYYSVMFP-DLTQKAKSGILRFLWQNDPFKA
A. mottae	MUM 10.233	.....
A. flavus	MUM 10.206	.....F.....N.....V.....A.....
A. flavus	MUM 10.232	.....F.....N.....V.....A.....
A. flavus	NRRL 1957	.....F.....N.....V.....A.....
A. flavus	NRRL_28987	.....F.....N.....V.....A.....
A. minisclerotigenes	MUM 10.227	.....N.....A.....
A. minisclerotigenes	MUM 10.228	.....N.....A.....
A. minisclerotigenes	MUM 10.229	.....N.....A.....
A. minisclerotigenes	MUM 10.230	.....N.....A.....
A. minisclerotigenes	NRRL 29002	....K.....N.....A.....
A. parasiticus	MUM 10.212	.....N.....A.....I.....
A. parasiticus	MUM 10.215	.....N.....A.....I.....
A. parasiticus	MUM 10.224	.....N.....A.....I.....
A. parasiticus	MUM 10.225	.....N.....A.....I.....
A. transmontanensis	MUM 10.205	.....N.....S.....A.....
A. transmontanensis	MUM 10.211	.....N.....S.....A.....
A. transmontanensis	MUM 10.214	.....N.....S.....A.....
A. transmontanensis	MUM 10.221	.....N.....S.....A.....
A. transmontanensis	MUM 10.222	.....N.....S.....A.....
A. caelatus	NRRL 25528	.....A.G.P.P...N..I.....H.....S...T.....A.....
A. pseudotamarii	NRRL 25517	.....A.G.P.....N.....H.....S...T.....A.....
A. tamarii	NRRL 20818	.....A.G.P.....N....P...S.....H...I.....S...T.....V.....
A. nomius	NRRL 13137	.....V.A...S.....N.KF.....SH.....S.T...V.....A.....

Table 4.7 - Amino acid sequence alignment of a 238 bp fragment of MAT1-2 amplification

Species	Strains	Mating type 1-2 protein sequence
A. minisclerotigenes	MUM 10.226	AYPDFTNNEISIILGKQWKAESEEVKMQFRNMAEELKKKHAEDHPDYHYTPRKP
A. minisclerotigenes	MUM 10.203	.....
A. flavus	NRRL 28986	.....K.....
A. parasiticus	NRRL 502	.....
A. transmontanensis	MUM 10.223	.....
A. sergii	MUM 10.208	.....
A. sergii	MUM 10.219	.....
A. bombycis	NRRL 26010	.....D.....
A. lanosus	NRRL 3648	.....M.....A.V...S.....R.....Y.....
P. alliaceus	NRRL 20602	.....M.....A.V...S.....R.....---
P. alliaceus	NRRL 4181	.....M.....A.V...S.....R.....
A. alliaceus	NRRL 5108	...N.....M.....A.V...S.....R.....
A. avenaceus	NRRL 517	...E.....S.T..I.V...G..D.....

4.3.2.3 Mycotoxigenic profile

In *Aspergillus* section *Flavi* strains isolated from maize, cyclopiazonic acid (CPA) and aflatoxins (AF) were produced by 74% and 43% of the isolates, respectively (Table 4.8). Three hundred and one CPA producer strains and 179 AF producer strains were isolated. Beira Litoral exhibits the highest incidence of CPA producing strains (78%) and a mean incidence of aflatoxigenic strains (37%); Ribatejo has a mean incidence of CPA producing strains (72%) and the lowest incidence of AF producers (35%); while Alto Alentejo has the lowest incidence of CPA producing strains (70%) and the highest incidence of aflatoxigenic strains (45%).

Table 4.8 - Mycotoxin production of *Aspergillus* section *Flavi* isolated in three Portuguese Regions.

Region	CPA		Aflatoxins	
	Negative	Positive	Negative	Positive
Beira Litoral	41	125	98	68
Ribatejo	58	121	89	80
Alto Alentejo	27	55	51	31
Total	116	301	238	179

The three *A. parasiticus* strains produce the four AF, and do not produce CPA. *A. flavus* isolates show big mycotoxigenic diversity, where 18% do not produce any mycotoxin, 38.4% produce only CPA, 8.4% produce only AF and 35.2% produce both AFBs and CPA (Table 4.9). One *A. flavus* isolate, MUM 10.232, produces both AFGs and AFBs in high quantities and more weakly CPA, showing, therefore, a different mycotoxin profile when comparing with the other isolates of this species. In a study of 70 isolates of maize by Giorni et al. (2007), 61% of the strains produced detectable amounts of CPA, being slightly lower to the ones obtained in our study (72% in total), but a higher percentage of the isolates produced AF (70%).

Table 4.9- Mycotoxigenic profile of *A. flavus* isolates.

		Aflatoxins		
		Not producer	Producer	Total
CPA	Not producer	69	32	101
	Producer	147	135	282
	Total	216	167	383

From the remaining species in the section isolated from maize, two other species were found to include aflatoxin producing strains and three species CPA producing strains.

Five of the six strains identified as *A. minisclerotigenes* are producers of AFBs, AFGs and CPA as described by Pildain et al. (2008). One isolate, however, does not produce AFGs (MUM 10.228), which suggests that further studies should be taken in this isolate.

Fourteen strains of *A. tamarii* produce CPA (61%) where the rest of the isolates do not produce at detectable amounts, being this value slightly lower than the one obtained by Klich and Pitt (1988) where 77% of *A. tamarii* were CPA producers.

The isolates of *A. mottae* both produce large quantities of aflatoxins but one isolate (MUM 10.231) has the ability to produce detectable amounts of CPA while the other (MUM 10.233) does not.

As indicated earlier, aflatoxin producing stains may be rapidly screened by growing in CAM medium. All the 417 strains from the *Aspergillus* section *Flavi* isolated from maize were also screened for aflatoxin production using CAM. The results of aflatoxin production by fluorescence in CAM indicate a good correlation with the HPLC results (Table A.1), with an agreement of 373 in 417.

Simultaneous detection of aflatoxins and cyclopiazonic acid

AF and CPA analyses can be routinely used for identification purposes within the section. Two separate chromatographic runs with distinct columns and detectors for the detection of each toxin were required as described above. A straightforward HPLC procedure for the simultaneous detection of these compounds in fungal cultures was developed in the present work using a methanol/water mobile phase, post-column photochemical derivatization and fluorescence detection. Recently, Maragos (2009a,b) reported the photolysis of CPA to fluorescent products, being these products reported in methanol and aqueous acetonitrile (excitation max 372 nm; emission max 462 nm). However, in their methodology, a NH2 chromatography column was used. This column enables the separation of aflatoxins from CPA, but not each aflatoxin from each other (all four main AFs elute together). To accomplish a better separation within AFs, the same approach was tested using a C18 column.

The new methodology for the simultaneous detection of AFs and CPA, allowed the separation of AFBs from AFGs, as it may be seen from Figure 4.24 B, where retention times of 11, 12 and 18 minutes were obtained for AFBs, AFGs and CPA, respectively. Also in the case of fungal extracts, this separation is observed (Figure 4.24 A).

Not all the strains used in the development of this methodology were isolated from maize. Other strains were used as positive and negative controls, some of them were type strains and others were already characterized strains isolated from different commodities in our laboratory. Table 4.10 presents the mycotoxigenic profile of tested strains according to the traditional methodology – separate analysis of AFs and of CPA – and to the new methodology – simultaneous analysis of AFB, AFG and CPA.

The species belonging to this section have different mycotoxins profiles which help in their identification. According to the new methodology, all the *A. parasiticus* and the *A. nomius* were aflatoxigenic and AFG producers. The *A. transmontanensis* strains did not produce detectable amounts of AFB; none of the *A. tamarii* was aflatoxigenic; and six (54%) of the *A. flavus* were AFB producers. One of these *A. flavus* strains was also AFG producer (Figure 4.24A). CPA production was detected from all the *A. tamarii* and seven (63%) of the *A. flavus* strains. None of the *A. parasiticus* and *A. nomius* strains were CPA producers (Table 4.10).

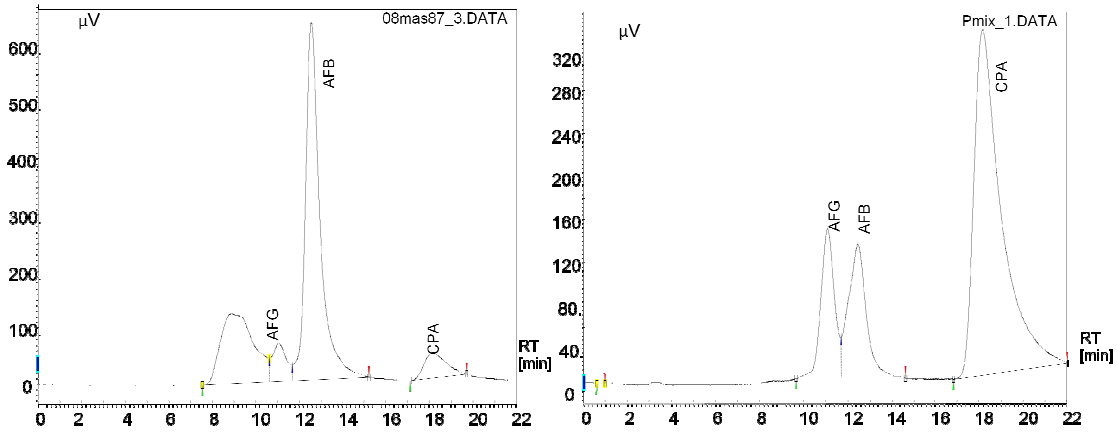


Figure 4.24- A-Fungi extract of 08MAsp87 with two peaks at 11 and 12 minutes for AFGs and AFBs, respectively, and one peak at 18 minutes for CPA; B-Standard of AFGs, AFBs (retention time of 11 and 12 minutes, respectively) and CPA (retention time around 18 minutes).

Table 4.10 - Mycotoxigenic profile (AFB1, AFB2, AFG1 and AFG2, and CPA) of tested *Aspergillus* section *Flavi* strains.

Strain	Species	Fluorescence in CAM	Traditional methodology					New methodology				
								NH_2 Column		C18 Column		
			Aflatoxins				CPA	Aflatoxins[b]	CPA	Aflatoxins		CPA
			B1	B2	G1	G2				Bs	Gs	
01UAsp 55	*A. flavus*	-	-	-	-	-	-	np	-	-	-	-
05BrUAsp 01	*A. flavus*	-	-	-	-	-	-	np	-	-	-	-
07AAsp37	*A. flavus*	Positive	+	+/-	-	-	+	np	+	+	-	+
08AAsp 35	*A. flavus*	-	-	-	-	-	-	np	-	-	-	-
08AAsp 42	*A. flavus*	Positive	+	+/-	-	-	+	np	+	+	-	+
08AAsp116*	*A. flavus*	Positive	+	+/-	-	-	+	np	+	+	-	+
07BNAsp01	*A. flavus*	Positive	+	+	-	-	+	np	-	+	-	+
08MAsp87	*A. flavus*	Positive	+	+	+/-	-/+	+	np	+	+	+/-	+
08MAsp153	*A. flavus*	-	-	-	-	-	-	np	-	-	-	-
08MAsp181	*A. flavus*	-	-	-	-	-	+	np	+	-	-	+
MUM 92.01	*A. flavus*	Positive	+/-	-	-	-	+	np	+	+	-	+
08AAsp 36	*A. parasiticus*	Positive	+	+	+	+	-	np	-	+	+	-
08AAsp 38	*A. parasiticus*	Positive	+	+	+	+	-	np	-	+	+	-
08AAsp 67**	*A. transmontanensis*	Positive	+	+	+	+	-	np	-	-	+	-
08AAsp117	*A. parasiticus*	Positive	+	+	+	+	-	np	-	+	+	-
08AAsp158	*A. parasiticus*	Positive	+	+	-/+	-/+	-	np	-	+	+	-
08MAsp86	*A. parasiticus*	Positive	+	+	+	+	-	np	-	+	+	-
MUM 92.02	*A. parasiticus*	Positive	+	+	+	+	-	np	-	+	+	-
08BNAsp02	*A. tamarii*	-	-	-	-	-	++	np	+	-	-	+
MUM 09.03	*A. tamarii*	-	-	-	-	-	+	np	+	-	-	+
MUM 09.04	*A. tamarii*	-	-	-	-	-	+	np	+	-	-	+
MUM 09.02	*A. nomius*	Positive	+	+	+	+	-	np	-	+	+	-
MUM 09.01	*A. nomius*	-	+	-/+	+	-/+	-	np	-	+	+	-

*08AAsp116=MUM 10.206

**08AAsp67=MUM 10.205

Strains of *A. flavus* producing only detectable AFBs were observed where only one peak corresponding to AFBs was obtained. Strains producing all aflatoxins were also obtained (Figure 4.24A) and the results were consistent when compared with standards (Figure 4.24B). The influence of detection limits need to be appreciated when interpreting these results, in that compound may be produced but below these limits.

When comparing both methodologies, in such one strain (08AAsp67) results did not agree, being AFBs not detected by the new methodology. It is interesting to mention that the fluorescence in CAM medium was green, instead of the more common blue one (data not shown), making possible to speculate that, at least, we faced, an atypical strain. In fact, this strain has been described as belonging to the new species *Aspergillus transmontanensis* (Figure 4.22, page 90).

The results showed that the separation of the four aflatoxins remained suboptimal. The detection of both toxins was undertaken at excitation and emission wavelengths that are not optimal for aflatoxins. Also the use of the photochemical reactor increases the detection limit of CPA making it more difficult to detect.

4.3.3 Identification and characterization of *Aspergillus* section *Nigri* isolates

4.3.3.1 Phenotypic identification

From the ninety five samples of maize grains obtained in the three Portuguese regions, *Aspergillus* section *Nigri* isolates species were found in seventy three samples (77%). From these, two hundred and seventy strains were isolated (Table 4.11). Morphological analysis revealed that all were biseriate and within the *Aspergillus niger* aggregate (Figure 4.25). Other biseriate species, such as *A. carbonarius* or *A. ibericus,* were not isolated. The incidence of *Aspergillus niger* aggregate differed among regions being present in 59%, 80% and 96% of the samples from Beira Litoral, Ribatejo and Alto Alentejo, respectively.

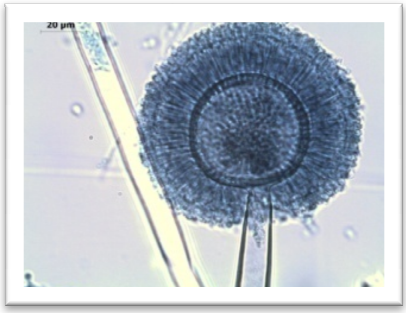


Figure 4.25 – Microscopic observation of *A.niger* (09MAS91).

Uniseriate species were not found in these maize samples, even though it had been previously isolated from Nigerian and Argentinean maize (Abarca et al., 1994; Magnoli et al, 2006). Also in other study, with Portuguese wine grapes, uniseriate strains were rarely isolated (two isolates in 770 isolated strains) (Serra et al., 2005), and only in a grape growing area located northern from the maize growing areas sampled in our study.

Aspergillus section *Nigri* species are common soil inhabitants contaminating ripening crops in Mediterranean, tropical and subtropical regions (Magan and Aldred, 2005) being more resistant to higher solar exposure and higher temperatures. Therefore, the higher incidence of isolates in the Ribatejo and in Alto Alentejo (Table 4.11) may be explained by the fact that both these regions have Mediterranean climates, very hot and dry during summer time, frequently achieving temperatures around 40 °C. Beira Litoral, on the other hand, has Atlantic influences with more moderate temperatures and higher precipitation, being more common other fungal species. The black aspergilla presence in Portuguese regions with Mediterranean climate had already been assessed in a survey of ochratoxigenic fungi in Portuguese wine grapes (Serra et al., 2003; Serra et al., 2005) where these species have been isolated from 22% of grape samples. Although the sampling sites were not the same, it was also possible to observe a higher incidence in Alto Alentejo (72−100%) than in Ribatejo (18−48%) (Serra et al., 2003). In these studies, temperature and relative humidity were found to determine the relative incidence of *Aspergillus* section *Nigri* in grapes.

Table 4.11 - Number of *Aspergillus* section *Nigri* isolates and samples from three Portuguese Regions

Region	Number of samples with isolations per total of samples	Number of *Aspergillus niger* aggregate isolates
Beira Litoral	19/32	32
Ribatejo	32/40	111
Alto Alentejo	22/23	127
Total	73/95	270

4.3.3.2 Mycotoxigenic profile

In *Aspergillus niger* aggregate strains isolated from maize, OTA and FB2 were produced by 14% and 39% of the isolates, respectively.

The percentages of producing strains differed for the two mycotoxins (table 4.12). Thirty seven OTA producer strains and 105 FB2 producer strains were isolated. Beira Litoral exhibits the lowest incidence of ochratoxigenic strains (6%) and the highest incidence of FB2 strains (50%); Ribatejo has the highest incidence of ochratoxigenic strains (18%) and a smaller incidence of FB2 producers (37%); while Alto Alentejo has a mean incidence of OTA (12%) and FB2 (38%) producing strains.

To evaluate if there is an association between the production of OTA and FB2, the *Phi* association value was calculated (Table 4.12). For the overall results the *Phi* – association value is fair (*Phi* < 0.1), and not significant ($p > 0.05$), meaning that their production is independent from each other.

Table 4.12 - Mycotoxin production of *Aspergillus niger* aggregate isolated in three Portuguese Regions

Region	OTA		FB2		*Phi*	*p-value*
	Negative	Positive	Negative	Positive		
Beira Litoral	30	2	16	16	0.015	0.932
Ribatejo	91	20	70	41	-0.037	0.688
Alto Alentejo	112	15	79	48	0.077	0.378
Total	233	37	165	105	0.010	0.865

As expected the OTA production by these strains is relatively low in all regions (Table 4.13), with production values ranging between 1 to 20 µg/Kg (0.4 ng/mL−8.5 ng/mL). The FB2 production is much higher and ranged between 1 and 5382 µg/Kg. Regarding OTA, even though Beira Litoral exhibits the lowest amount of producing isolates, the production mean for OTA is the highest when comparing with Ribatejo and Alto Alentejo. The same does

not apply to FB2 being Ribatejo the region with the highest production mean. The region with higher amount of isolates, Alto Alentejo, is the one with lower production means.

Table 4.13 - Mean values of OTA and FB2 production (µg/Kg) by *Aspergillus niger* aggregate isolated in three Portuguese Regions

Region	OTA (µg/Kg)	Mean levels (µg/Kg)	FB2 (µg/Kg)	Mean levels (µg/Kg)
Beira Litoral	6–20	13	1–2323	477
Ribatejo	1–18	6	1–3536	524
Alto Alentejo	1–18	6	1–5382	366

FB2 production varied between strains where almost 50% produce below 100 µg/Kg, 34 % produce between 100 and 1000 µg/Kg, and 16% produce at levels above 1000 µg/Kg (Table 4.14).

Table 4.14 - Number of *A. niger* aggregate strains producing OTA and FB2 and its distribution in three Portuguese Regions and among levels

Region	nº strains tested	nº OTA producing strains (0.8–18 µg/Kg)	nº of strains that produce FB2		
			<100 µg/Kg	[100-1000 µg/Kg]	>1000 µg/Kg
Beira Litoral	32	2	5	9	2
Ribatejo	111	20	19	15	7
Alto Alentejo	127	15	28	12	8

The production of OTA within this section varies according to different authors: from 3% to 28%, being extreme value up to 80% reported in the literature, as reviewed by Palumbo et al. (2011). In the previous Portuguese study concerning the production of OTA from strains isolated from grapes, percentages of OTA producing strains of *A. niger* aggregate were lower (4%). However, OTA producing strains were not equally distributed among all regions being the incidence in Southern regions higher. Magnoli et al. (2006) reported 6% of ochratoxigenic *A. niger* strains in previously disinfected Argentinean maize kernels, which lies within the value obtained from Portuguese grapes and maize.

The number of fumonigenic *A. niger* aggregate strains are significantly higher relatively to the findings in grapes by Abrunhosa et al. (2011b) where only 6% produce above this limit but similar to the ones obtain by Susca et al. (2010a). However, the values are much lower than in other studies in grapes and coffee beans (Logrieco et al, 2009; Mogensen et al., 2010;

Noonim et al., 2009) where 60% to 70% of the isolates were found to produce FB2 although in these cases a very low number of strains were tested.

It is clear that *A. niger* aggregate species can produce both OTA and FB2; however, it is not clear if there is an association between the productions of these two mycotoxins. OTA production values (0.4 ng/mL−8.5 ng/mL) are lower to the ones reported by Magnoli et al. (2006) which ranged between 2 ng/mL−24.5 ng/mL. The FB2 production is much higher and ranged between 1 μg/Kg and 5382 μg/Kg being lower, however, than the ones obtained by Mogensen et al. (2010) that ranged between 229 μg/Kg−6476 μg/Kg and by Susca et al. (2010a) that ranged between 100 μg/Kg−293000 μg/Kg.

Fusarium spp. are still regarded as the main producers of FB2 in grain commodities. As mentioned before (Chapter 2), these are predominantly considered as field fungi and except under extreme conditions, the concentrations of their fumonisins is not expected to increase during grain storage. However, *Aspergillus* have the ability to grow and produce mycotoxins during grain storage, at lower water availability conditions. Therefore, the requirements for FB2 production by *Aspergillus* and *Fusarium* strains are not the same. Although the optimum temperature and aW differ, the levels produced under these optimum conditions are of the same order of magnitude.

4.4 Conclusions

In conclusion, the isolated *Aspergillus* strains belong to five sections: *Flavi*, *Nigri*, *Wentii*, *Circumdati* and *Candidus*.

Within the *Aspergillus* section *Flavi*, the polyphasic approach revealed the presence of five distinct species: *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus tamarii*, *Aspergillus minisclerotigenes* and *Aspergillus mottae* (a not previously described species).

From the molecular study of eight isolates obtained in maize, one was identified as *A. flavus* and five as *A. minisclerotigenes*. Examination of morphological characters combined with the analysis of AF and CPA production and molecular data revealed that the other two *Aspergillus* section *Flavi* strains isolated did not match previously described species of the section. Therefore one new species was proposed, *Aspergillus mottae*.

These isolates were compared with type strains and other isolates from almonds. During this study two new species from the almond isolates have also been described,

Aspergillus transmontanensis and *Aspergillus sergii* (Soares et al., 2012). Both these species are closely related to *A. parasiticus*, however, exhibit characteristics more closely related to *A. flavus*. *A. transmontanensis* is predominately biseriate and produces AFBs and AFGs. *A. sergii* is predominantly uniseriate and produces both AFs and CPA.

A new method for the simultaneous detection of AFs and CPA was also developed. The sensitivity of this new methodology is lower than the methodologies used in separate for aflatoxins and CPA which may explain why weak producers of toxins were not detected (false negative results). This disadvantage needs to be weighed against the considerable merit of the simultaneous nature of the protocol.

Aspergillus section *Nigri* isolates are the second more common *Aspergillus* in maize. All the isolates belong to the *Aspergillus niger* aggregate, with very small levels of OTA production. All strains were screened for FB2 production and 39% showed to be producers. The fact that *Aspergillus* section *Nigri* are associated to commodities not previously associated with *Fusarium* and, consequently, with fumonisins, may extend the concern about fumonisins to a wider range of commodities.

Also, with the increasing concerns about climate changes, and with the rise of the global temperatures, the occurrence and production of FB2 by *Aspergillus niger* aggregate can have a wider impact.

CHAPTER FIVE: MYCOTOXIN DETECTION OF MAIZE SAMPLES

5.1- INTRODUCTION

5.1.1 Separation and detection

It is not possible to entirely prevent the production of mycotoxins, but eliminating the conditions necessary for fungal growth helps preventing the toxin formation.

Mycotoxigenic fungi grow better under certain environmental conditions but the presence of these fungi does not imply the occurrence of mycotoxins. In fact, the conditions in which these fungi produce their mycotoxins are very specific and independent of those required for fungal growth. On the other hand, the visible absence of moulds does not mean that mycotoxins are absent since these may remain in the product long after the producing fungus disappearance (Martí, 2006). The need of proper sampling procedures is, therefore, a pre-requisite for obtaining reliable results because of the heterogeneous distribution of mycotoxins in grains and other commodities.

According to Whitaker (2003), a sampling plan for mycotoxigenic analysis is defined by a mycotoxin test procedure and an accept/reject limit. This author states that a mycotoxin test procedure with several stages process generally consists of three steps: sampling, where is specified how the sample will be selected or taken from the bulk lot and the size of the sample; sample preparation, where the sample is ground in a mill to reduce particle size and a subsample is removed from the grinded sample; and analytical steps where the mycotoxin is extracted through solvents from the grinded subsample and quantified using approved procedures.

Due to the fact that some mycotoxins are toxic even in low concentrations, sensitive and reliable methods for their detection are required. Having these compounds varied structures it is not possible to use one standard technique to detect all mycotoxins.

5.1.2 Methods for the analysis of mycotoxins

A successful detection method should be robust, sensitive and have a high degree of flexibility, over a wide range of compounds, but which can be very specific when required. All techniques should be reproducible to a high level, and the results gained must be relevant and easy to analyse.

Conventional analytical methods for mycotoxin detection include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Most of these methods employ solid phase column cleanup of extracts and immunoaffinity techniques, to remove interferences and improve the measurement of mycotoxins and/or to concentrate the analyte.

The use of rapid screening methods has been gaining importance, due to the fact that are well suited to rapid, routine diagnostic application in mycotoxins detection. It includes rapid methods such as: enzyme linked immunosorbent assay (ELISA), flow through membrane based immunoassay or fluorescence polarization method.

Thin Layer Chromatography (TLC)

Screening methods are mostly based on thin layer chromatography (TLC), which is a very effective and simple technique. It is a cheaper alternative to other analytical methods and is unquestionably the method of choice in many parts of the world, especially in developing countries that are the main exporters to Europe of food and food products, which are potentially contaminated with aflatoxins (e.g. figs, peanuts, pistachios, and spices). This method is used specially for surveillance purposes and control of regulatory limits (Gilbert and Anklam 2002).

TLC uses a stationary phase, usually alumina or silica, which is highly polar (standard) or non-polar (reverse phase). The mobile phase is a solvent. The sample is applied and then "run" the plate by allowing a solvent (or combination of solvents) to move up the plate by capillary action. Depending on the polarity of the components of the mixture, different compounds will travel different distances up the plate. More polar compounds will "stick" to the polar silica gel and travel short distances on the plate. Non-polar substances will spend more time in the mobile solvent phase and travel larger distances on the plate. The measure of the distance a compound travels is called the R_f value. This number, between zero and one, is defined as the distance the compound moved from the baseline (where it was originally spotted) divided by the distance the solvent front moved from the baseline. In fungal cultures, the standard procedure for mycotoxin detection consists in applying an agar plug to the plate and the same procedure described above is used.

High Performance Liquid Chromatography (HPLC)

HPLC coupled with UV, diode array (DAD) or fluorescence detector (FL) is currently the most widely used technique for the analysis of major mycotoxins occurring in cereals. Aflatoxins, ochratoxin A, fumonisins and zearalenone are routinely analyzed by HPLC-FL, DON by HPLC-UV (DAD) and CPA by HPLC-UV with good accuracy and precision. Being HPLC-FL highly sensitive, selective and repeatable, specific labelling reagents have been developed for the derivatization of non-fluorescent mycotoxins to form fluorescent derivatives (González-Sapienza and Venâncio, 2011).

Either pre-column derivatization with trifluoroacetic acid (TFA), or post-column derivatization with Kobra Cell® (i.e. electrochemical bromination cell) or photochemical reactor (i.e. UV irradiation), can be used to enhance fluorescence of aflatoxins B1 and G1, whereas pre-column derivatization with either OPA or NDA reagents is required for the detection of fumonisins B1, B2 and B3. Several HPLC methods for identifying various mycotoxins in a number of cereals and cereal-based products have been validated by collaborative studies, and their performance characteristics, such as accuracy, repeatability, reproducibility, detection and quantification limits were established (Pascal, 2009).

Gas Chromatography (GC)

Gas chromatographic methods based on flame ionization (FID), electron capture (ECD) and mass spectrometry (MS) detection are the most widely used methods for quantitative simultaneous determination of trichothecenes (mainly type A) in cereals and cereal-based products (Krska et al., 2007). These methods require a preliminary clean-up of extracts, generally by MycoSep® columns, and pre-column derivatization of the purified extract with specific reagents. Mass spectrometry (MS), or tandem mass spectrometry (MS/MS), offers an advantage in confirming the identity of chromatographic peak. The main problems associated to GC analysis include increased trichothecene responses (up to 120%), non-linearity of calibration curves, drifting responses, carry-over or memory effects from previous samples, and high variation in terms of reproducibility and repeatability (Petterson and Langseth, 2002).

Liquid chromatography coupled with mass spectrometry (LC-MS)

LC coupled with atmospheric pressure ionization mass spectrometry has been used for many years, mainly as a technique for mycotoxin confirmation. At the present time, LC-MS and LC-MS/MS are the most promising techniques for the simultaneous screening, identifying and measuring of a large number of mycotoxins. This technique has been applied with different levels of success to the simultaneous determination of several mycotoxins (up to 39) in various food matrices , although major problems related to the extraction and cleanup steps have not been adequately undertaken and solved (Lattanzio et al., 2007).

Rapid Screening Methods

Most rapid screening methods rely on antibodies to detect mycotoxins (immunological assays), and differ according to how this antibody is used in the assay.

Enzyme-linked immunosorbent assays (ELISA) for mycotoxin assay have been available for more than a decade. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin (Zheng et al., 2006). Most commercially available kits rely on a competitive, heterogeneous ELISA format in which the toxin from the sample competes with a labelled toxin (such as toxin-enzyme conjugate) for a limited number of antibody-binding sites. In such assays, the presence of toxin is therefore measured by the absence of a response (i.e. colour) (Pittet, 2005). However, ELISA methods are not useful in providing a definitive confirmation of the toxins and an accurate quantitative determination (Muscarella et al., 2009), since they have not been validated at sufficiently low levels and are limited in the range of matrices tested (Gilbert and Anklam, 2002).

Membrane-based, flow-through assay is an adaption of the ELISA format and is based in an anti mycotoxin antibody coated on a membrane surface. The mycotoxin is extracted from a ground sample and a portion of the extract is then added to the membrane, followed by an addition of mycotoxin-enzyme conjugate. Mycotoxin and mycotoxin-enzyme conjugate compete for the limited antibody binding sites. After a washing step, the enzyme substrate is added and reacts with the mycotoxin-coupled enzyme and colour develops (Zheng et al., 2006). The advantage of this method is that allows the visual determination of the toxin (González-Sapienza and Venâncio, 2011). For a negative sample there will be a visible colour spot in the centre of the membrane and for a positive sample there will be no colour. This

method has been used for the detection of OTA in roasted coffee (Sibanda et al., 2002), being also useful for multi-mycotoxins essays (González-Sapienza and Venâncio, 2011).

Fluorescence polarization immunoassay is based on the competition between mycotoxin and a mycotoxin-fluorescent tracer for a mycotoxin specific antibody. Binding the antibody to the tracer increases polarization; in the presence of free mycotoxin, lesser antibody is bound to the tracer, reducing polarization. This way, this method measures the polarization value of the molecule and which is inversely proportional to the mycotoxin concentration (Pittet, 2005; Zheng et al., 2006). This method has two important differences from ELISA: the detection does not involve an enzyme reaction, and separation of the bound and free compounds is not required. As a result, fluorescence polarization assays do not require a wash step and do not require waiting for an enzyme reaction for colour development (González-Sapienza and Venâncio, 2011).

5.2 MATERIALS AND METHODS

5.2.1 Mycotoxin detection of maize samples

Aflatoxins/OTA and Fumonisins extraction methodology

Several assays with different solvent mixtures and shaking times were tested allowing the development of a simultaneous extraction method for aflatoxins, ochratoxin A and fumonisins based in protocols provided by Vicam.

Three different conditions were tested:

A- Stirring in an Erlenmeyer flask with methanol:water (80:20)

B- Shaking in centrifuge tubes and sonicating with acetonitrile: methanol: water (25:25:50)

C- Shaking in centrifuge tubes and sonicating with methanol: water (80:20)

Immunoaffinity columns

AflaOchra™ HPLC and Fumonistest™ WB immunoaffinity columns were obtained from Vicam (Labtech Int. Ltd., Sussex). These columns (single use only) have at least 80% recovery.

Solutions and reagents

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

PBS

0.2 g of potassium chloride (KCl)

0.2 g of potassium dihydrogen phosphate (KH_2PO_4)

1.2 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4)

8.0 g of sodium chloride (NaCl) (Fisher Scientific)

990 mL of distilled water

The pH was adjusted to 7.0±0.1 with 6N HCl and the volume made to 1 L with distilled water.

Extraction Solution (80:20 v/v)

800 mL of methanol (Fisher scientific)

200 mL of distilled water

Extraction Solution (25:25:50 v/v)

250 mL of acetronitrile (Fisher scientific)

250 mL of methanol (Fisher scientific)

500 mL of distilled water

6N Chloridric acid (100 mL)

47.2 mL of 37% HCl (Fisher Scientific)

52.8 mL of distilled water

Sample extraction: Methodology A

Maize samples were ground (through a 1 mm screen) and mixed thoroughly. Sub-samples of 25 g were weighed and placed into a 250 mL Erlenmeyer with 2.5 g of NaCl. Extraction solvent (100 mL) was added and the suspension stirred for 30 minutes in a magnetic stirrer. The solution was sonicated for 15 minutes and poured into a Whatman No. 4, 12 cm fluted filter paper, avoiding the transfer of solid material onto the filter. The filtrate (10 mL) was diluted into 40 mL of PBS and filtered through a Whatman glass microfiber filter.

Sample extraction: Methodology B and C

Maize samples were ground (through a 1 mm screen) and mixed thoroughly. Sub-samples of 25 g were weighed along with 1.25 g of NaCl and placed into 250 mL centrifuge tubes. Extraction solvent (50 mL) was added to the tubes shake for 20 minutes in an orbital shaker. The tubes were sonicated for 15 minutes and centrifuged for another 15 min at 2500 x *g*. Supernatant was poured into a Whatman No. 4, 12 cm fluted filter paper, avoiding the transfer of solid material onto the filter. The same extraction process was repeated and the extract was filtered through the same filter paper, combining the two filtrates. The filtrate (10 mL) was diluted into 40 mL of PBS and filtered through a Whatman glass microfiber filter.

Column Chromatography:

AflaOchra

From the resulting solution, 10 ml (10 ml = 0.5 g sample equivalent) was pipeted and passed completely through AflaOchra™HPLC affinity column at a rate of about 1-2 drops/second. PBS solution (10 mL) was added to wash the column at a rate of 1-2 drops/second until air comes through the column. A 4 mL vial was put under the column and 2 ml HPLC grade methanol passed through the column at a rate of 1 drop/second or slower to elute AflaOchra™HPLC column. The collected methanol elute was divided in two 2 mL vials for quantification of AF and OTA.

FumoniTest™ *WB*

From the resulting solution, 10 ml (10 ml = 0.5 g sample equivalent) was pipeted and passed completely through FumoniTest™ *WB* affinity column at a rate of about 1-2 drops/second. PBS solution (10 mL) was added to wash the column at a rate of 1-2 drops/second until air comes through the column. A 4 mL vial was put under FumoniTest™ *WB* column and 1.5 ml HPLC grade methanol passed through the column at a rate of 1 drop/second or slower to elute FumoniTest™ *WB* column. The collected methanol eluate was dried down under a stream of nitrogen at 60 °C.

Method validation

Different validation tests were used. For the extraction methodology A, replicate samples with 4 µg/Kg and 8 µg/Kg of AF and 3.5 µg/Kg and 7 µg/Kg of OTA were tested. In methodologies B and C analysis of replicate spiked samples with 40 µg/Kg of AF; 7 µg/Kg of OTA and 200 µg/Kg of FB1 and FB2 were performed. Spiked samples were allowed to equilibrate for 24 hours prior to extraction. In addition, a matrix blank was also analyzed to determine any residual mycotoxin levels. Overall, two batches of duplicate spiked samples and one blank sample were analysed.

Reference sample of naturally contaminated maize with 3.6 µg/Kg of AF and 4.5 µg/Kg of OTA (batch MTC-9994, R-Biopharm, Rhône LTD) was also used.

CPA extraction methodology

Solutions and reagents

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

Solution of 2% of sodium bicarbonate (2 %$NaHCO_3$)

20 g of $NaHCO_3$

100 mL of distilled water

Extraction solution (70:30 v/v)

700 mL of methanol

30 mL of 2 %$NaHCO_3$

Solution of 10% of Potassium chloride (10%KCl)

100g of KCl

1000 mL of distilled water

Sample extraction

Maize samples were ground (through a 1 mm screen) and mixed thoroughly. Sub-samples of 25 g were weighed and placed into 250 mL Erlenmeyer flask. It was added 50 mL of extraction solvent and shaked for one hour on gyratory shaker. The resulting solution was filtered through Whatman No. 4 filter paper and pipeted 25 mL of the filtered extract into a 250 mL separatory funnel. It was added 50 mL of *n*-hexane (to defat the extract) and mixed gently to avoid the formation of an emulsion. After the separation of the two layers the lower aqueous layer was carefully transferred to another separation funnel and 25 mL of 10% KCl in water was added. The ***n***-hexane layer was discarded.

The solution was acidified with 1 mL of 6N HCl and 25 mL of chloroform was added.

The solution was gently mixed and the lower organic layer was collected in a 250 mL Erlenmeyer. The extraction process was repeated with additional 25 mL chloroform, and the two chloroform extracts were combined in the same Erlenmeyer. A small portion of anhydrous

sodium sulfate (Na_2SO_4) was added and left stand for 1 hour. The extract was filtered and collected into a 200 mL rotary evaporator flask and evaporated to dryness at 40 °C in a rotary evaporator. The dry residue was dissolved with 2 mL mobile phase, mixed in vortex for 30 seconds and sonicated for 15 minutes. Finally, it was filtered through 0.45 µm MilliporeTM membrane filter.

Method validation

This method was initially validated by analysis of replicate spiked samples with 200 µg/Kg of CPA. Spiked samples were allowed to equilibrate for 24 hours prior to extraction. In addition, a matrix blank was also analyzed to determine any residual mycotoxin levels. Overall, two batches of duplicate spiked samples and one blank sample were analysed.

5.3 Results

5.3.1 Extraction methodology validation

Grains highly contaminated by aflatoxin and fumonisin are unevenly distributed in a grain lot and may be concentrated in a very small percentage of the product. The sampling scheme proposed by Whitaker (2003) could not be followed due mainly to the size of the bulk sample that were in the order of the tons, and the way the sample could be taken from the silos. This way, the level of uncertainty is high but it was not possible to calculate it.

Three extraction methodologies were tested and the one with better recovery values for the simultaneous extraction of AF, OTA and FB was used for sample analyses.

Sample extraction: Methodology A

This method was only used for the extraction of AF and OTA. For both values of AF of 4 µg/Kg and 8 µg/Kg, validation tests revealed low recovery values with no reproducibility (Table 5.1). Recovering results for OTA were good, being around 61% and 76% (Table 5.2). The need of a good extraction method that would allow the simultaneous extraction of both mycotoxins and additionally of fumonisins as well, influenced the decision of not using this method.

Table 5.1- Recovery values of AF extraction method A, for each AF.

	AFB1		AFB2		AFG1		AFG2	
	4 µg/Kg	8 µg/Kg	4 µg/Kg	8 µg/Kg	4 µg/Kg	8 µg/Kg	4 µg/Kg	8 µg/Kg
1st day Recovery%								
Replicate 1	32.2	30.5	43.8	21.6	33.5	20.5	30.5	31.25
Replicate2	39	31.6	39.5	31.4	45.8	23.1	67.5	42
2nd day Recovery%								
Replicate 1	35	37.5	56	39.1	23.1	30	45%	48.5
Replicate2	25.5	44.9	23.3	50.5	21.2	35	29.5	57.25
SD	6	7	14	12	11	7	18	11

SD Standard deviation
Recommended range of recovery (%) 70-110 (CE, N.o 401/2006)

Table 5.2- Recovery values of OTA extraction method A.

	OTA	
	3.5 µg/Kg	7 µg/Kg
1st day Recovery%		
Replicate 1	66	74
Replicate2	70.3	75.7
2nd day Recovery%		
Replicate 1	68	70
Replicate2	61	73.4
SD	4	3

SD Standard deviation

Recommended range of recovery (%) 70-110 (CE, N.o 401/2006)

Sample extraction: Methodologies B and C

Two methods were evaluated and the recovery values were compared for three mycotoxins: AF, OTA and FB. As previously referred, in both methodologies replicate spiked samples with 40 µg/Kg of AF; 7 µg/Kg of OTA and 200 µg/Kg of FB1 and FB2 were analysed. Validation tests revealed better recovery values in method C for OTA and AF (Figure 5.1), and similar recovery values for FB (Table 5.3). FB1 recovery values are abnormal which suggests that even though the samples were homogenised, the mycotoxin distribution in the blank and in the spiked samples was not equal, and the already contaminated blank sample had values of FB1 superior to the ones in the spiked sample. Aflatoxins recovery values are slightly below what is recommended for AFB1, AFB2 and AFG1.

Table 5.3- Comparison of recovery values of simultaneous mycotoxin extraction methods.

	AFB1		AFB2		AFG1		AFG2		OTA		FB1		FB2	
	B*	C*	B	C	B	C	B	C	B	C	B	C	B	C
1st day Recovery%														
Replicate 1	43	66	50	71	53	68	62	82	70	73	177	156	98	96
Replicate2	40	69	53	70	56	72	66	80	69	70	131	180	100	102
2nd day Recovery%														
Replicate 1	36	71	49	72	54	79	62	83	70	75	140	120	110	114
Replicate2	45	73	55	74	54	77	65	81	71	79	230	300	97	108
Mean (%)	41	70	52	72	54	74	64	81	70	74	169	189	101	105
SD	4	3	3	2	1	5	2	1	1	4	45	78	6	8

B* Extraction Methodology B

C* Extraction Methodology C

SD Standard deviation

Recommended range of recovery (%) 80-110 for AF, 70-110 for OTA, 60-120 for FB1 and FB2 (CE, N.o 401/2006).

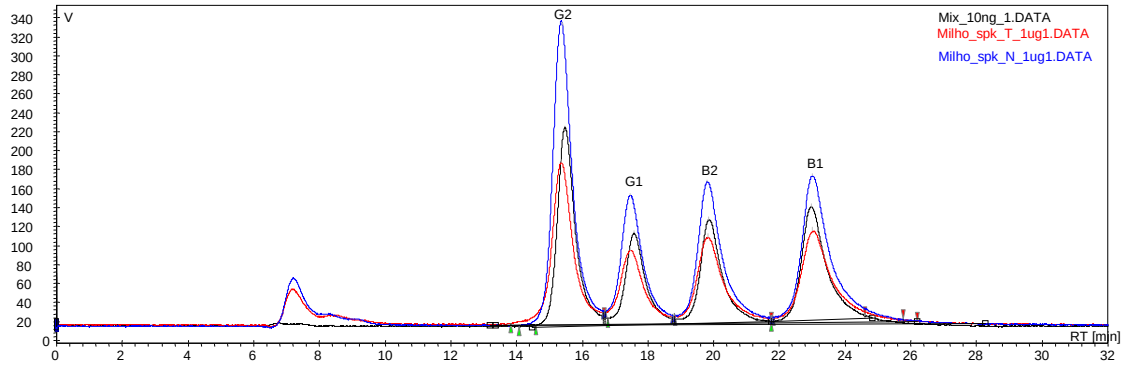


Figure 5.1 – Chromatograms of two AF spiked maize samples in the two different methodologies. In pink, chromatogram of the methodology C; in green, chromatogram of methodology B; in blue, standard of the four aflatoxins AFB1, AFB2, AFG1 and AFG2.

Recovery values of the reference material using extraction methodology C, were 77% for OTA, above to the spiking recovery values. However, only 51% of AFB1 and AFB2 were obtained. Recovery values of AF were even lower.

CPA extraction methodology

CPA recovery values are not yet legislated. Nevertheless, the recovering values are high, validating the extraction methodology (Table 5.4).

Table 5.4- Recovery values of CPA extraction

	CPA
	200 µg/Kg
1st day	
Recovery%	
Replicate 1	80
Replicate2	84
2nd day	
Recovery%	
Replicate 1	83
Replicate2	82
SD	2

In conclusion, validation tests revealed recovery values between 70- 82 % for aflatoxins (AF), 83% for cyclopiazonic acid (CPA) , 70% for ochratoxin A (OTA), 110 % for fumonisin B2

(FB2) and 189% for fumonisin B1 (FB1) (Table 5.5). Calibration parameters of instrumentation can be assessed in Table A.3.2.

Table 5.5 – Recovery and detection limits of aflatoxins (AFB1, AFB2, AFG1, AFG2), cyclopiazonic acid (CPA), ochratoxin A (OTA) and fumonisins B1 (FB1) and B2 (FB2) in ground maize

Mycotoxins	contamination value (µg/Kg)	Recovery (%)	Detection limits
AFB1	40	70%	0.1 µg/Kg
AFB2	40	72%	0.1 µg/Kg
AFG1	40	74%	0.1 µg/Kg
AFG2	40	82%	0.1 µg/Kg
CPA	200	83%	2 µg/Kg
OTA	7	77%	0.07 µg/Kg
FB1	200	189%	84 µg/Kg
FB2	200	105%	75 µg/Kg

5.3.2 Mycotoxin detection in maize samples

Mycotoxins were detected in 83% the samples, with prevalence for FB1. Hence, 83% percent of the samples were positive for FB1, 46% were positive for FB2 and only 5% were positive for AF (Table 5.6).

Beira Litoral was the region with more positive samples for AF, with three positive samples (9%), having also 25 samples positive for FB1 (78%) and seven samples positive for FB2 (22%).

Table 5.6 - Mycotoxin detection of maize obtained in three Portuguese Regions

Region	AFG2		AFG1		AFB2		AFB1		OTA		FB1		FB2	
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Beira Litoral	32	0	29	2	32	0	29	3	32	0	7	25	25	7
Ribatejo	39	1	38	1	40	1	39	2	40	0	6	34	17	23
A. Alentejo	23	0	23	0	23	0	23	0	23	0	3	20	9	14
Total	94	1	90	3	95	1	90	5	95	0	16	79	51	44

Ribatejo had two positive samples for AF (5%), one had only detectable values for AFB1 and other had detectable values for all AF (Figure 5.2), 85% of the samples were positive for FB1 and 58% for FB2 (Figure 5.3).

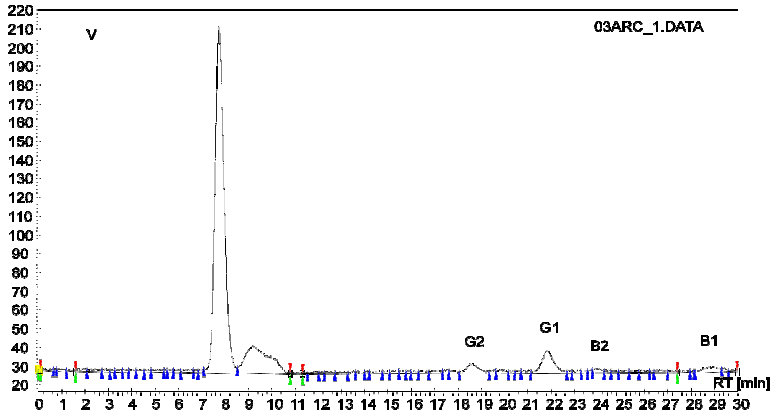


Figure 5.2 – Chromatogram of aflatoxins analyses of a storage maize sample taken in the region of Ribatejo.

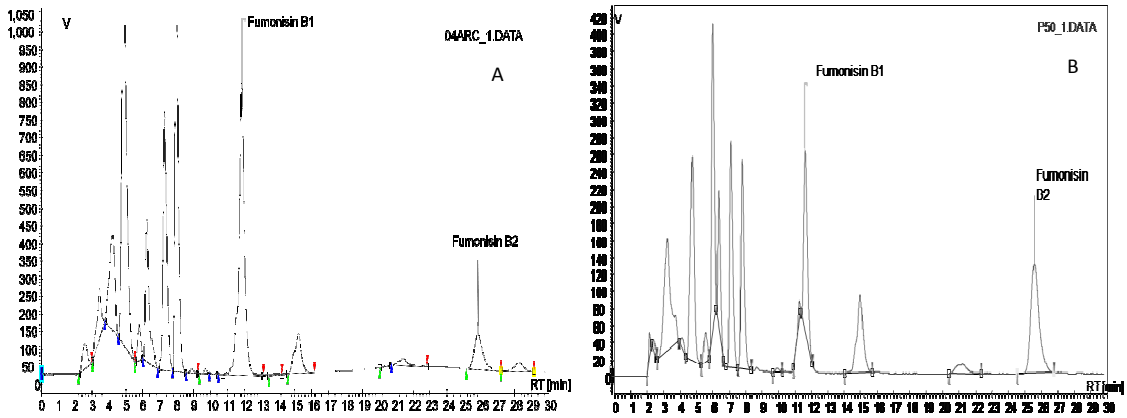


Figure 5.3 – A: Chromatogram of fumonisin analyses of a storage maize sample taken in the region of Ribatejo. B: Chromatogram of fumonisins B1 and B2 standard.

Alto Alentejo had no positive samples of AF, 87% were positive for FB1 and 61% of the samples were positive for FB2.

As previously referred AF were detected in 5% of the samples with levels for total AF ranging from 0.1-0.5 µg/Kg. These values are considerably lower than the ones obtained by Bankole and Mabekoje (2004) that reported the incidence of contaminated maize in Nigeria in 18.4% of their samples ranging between 3-138 µg/Kg. A similar value was obtained by Janardhana et al. (1999) where 17.5% of samples taken in the region of Karnataka, in India, were positive for AFB1 with values ranging 23-26.8 µg/Kg. These studies were conducted in countries where the natural incidence of aflatoxins in food and feed is well known. Practically,

all tropical countries encounter the problem of aflatoxin contamination, due to the fact that its occurrence is climate related.

Fumonisins B1 were detected in 83% of the samples and FB2 in 46%.The majority of FB2 positive samples (39%) had values below 100 µg/Kg and only a small percentage (7%) had values between [100-1000 µg/Kg]. For FB1, about half of the samples had values below 100 µg/Kg, 17% had values between [100-1000 µg/Kg] and 16% had values above 1000 µg/Kg (Table 5.7). Bankole and Mabekoje (2004) reported the fumonisin detection in 78.6% of maize samples. FB1 was also the predominant toxin detected in terms of concentration (70–1780 mg/kg) as well as for the number of positive samples. FB2 was detected in 68% of samples, being slightly superior to the percentage obtained in our study, ranging from 53-230 mg/Kg. All the contamination levels are, however, considerably superior to the ones detected in the Portuguese maize.

Fusarium species are predominantly considered as field fungi and fumonisins are formed in maize only before harvest or during the early stage of drying. This might explain why the majority of the samples were contaminated with fumonisins and only a small portion had aflatoxins. This suggests that the post-harvest process is adequate.

Table 5.7 - Number of samples with detected FB1 and FB2 and its distribution in three Portuguese Regions and among levels

Region	nº samples tested	<100 µg kg^{-1}		[100-1000 µg kg^{-1}]		>1000 µg kg^{-1}	
		FB1	FB2	FB1	FB2	FB1	FB2
Beira Litoral	32	17	5	5	2	3	-
Ribatejo	40	20	21	7	2	7	-
Alto Alentejo	23	11	11	4	3	5	-
Total	95	48	37	16	7	15	-

No samples showed the presence of OTA. Even though a great number of *Aspergillus* section *Nigri* strains were isolated, the presence of this mycotoxin was not detected, suggesting good post-harvest practices. This is a similar result obtained by Magnoli et al. (2006) where no positive samples were detected. In a study about the occurrence of OTA in maize samples taken in Bangladesh, 40% of the samples taken in markets were positive, which suggested inappropriate storage conditions (Dawlatana et al., 2008).

Scudamore and Hetmanski (1992) reported that no ochratoxin A was found in 50 samples of different maize products. However, in a later study about the multi-mycotoxin contamination of maize products (Scudamore at al., 1998), two samples of maize gluten contained detectable levels of ochratoxin A, at a level of 2 µg/kg.

In our study, no samples had detectable amounts of CPA which is consistent with our results.

5.4 Conclusions

In conclusion, the detected values of both fumonisins and aflatoxins in the samples were not of much concern. It was below the values imposed by the EU legislation, where a maximum level of the sum of FB1 and FB2 in unprocessed maize is imposed in 2000 µg/kg and of AF is 10 µg/kg.

Cyclopiazonic acid may co-occur with aflatoxins as it can be formed by the same mould, *Aspergillus flavus*. The occurrence of both *Aspergillus tamarii* and *Aspergillus mottae*, also producers of CPA, is too low to influence the occurrence of this mycotoxin, even if given the right conditions. Given that aflatoxins were found in very few samples, it seems unlikely that it would be present in these samples.

This way, we can conclude that the post-harvest management was adequate and maize was stored in proper conditions, with low water availability, preventing fungal development and mycotoxin production.

CHAPTER 6: GENERAL CONCLUSIONS AND PERSPECTIVES

The general conclusions taken in this thesis are:

1. Climate changes and the different agroecological conditions were not the main factor that influenced the harvest campaign timeline. In recent years, cereal production in Portugal has dropped due to both adverse weather conditions and the overall negative pressure, forcing low prices, on the cereals market. Despite this, maize production has maintained its levels of productivity;

2. What concerns *Aspergillus* species isolations at each region, small differences were observed. The regions with Mediterranean climates had a higher percentage of *Aspergillus* section *Nigri* isolates in comparison with the region with transitional between Atlantic and Mediterranean climate;

3. There were no major differences between the isolates obtain in the different types of dryers in the region of Beira Litoral;

4. The most common member of *Aspergillus* section *Flavi* from the more than 400 isolates collected from maize samples obtained from three agroecological zones in Portugal was the *A. flavus* L-strain (85%), followed by *A. tamarii* (6%) and *A. parasiticus* (1%);

5. A new species belonging to *Aspergillus* section *Flavi* was described, *Aspergillus mottae*, after a polyphasic characterization of the isolates;

6. Even though *Aspergillus* section *Flavi* isolates, especially of *A. flavus*, were present in high numbers in the maize kernels, very few samples had detectable amounts of aflatoxins (5%). We can conclude that the post-harvest management was adequate and maize was stored in proper conditions, with low water availability, preventing fungal development and mycotoxin production;

7. A new method for simultaneous detection of AF and CPA from culture media was developed;

8. *Aspergillus* section *Nigri* isolates are the second more common *Aspergillus* in maize. All the isolates belong to the *Aspergillus nige*r aggregate, with very small levels of OTA production;

9. The presence of fumonisins (FB1 and FB2) in the majority of the samples (83 and 46%, respectively) suggests that there was pre-harvest contamination with *Fusarium* species. There is no evidence that the presence of FB2 is due to the presence of *Aspergillus niger* aggregate

isolates. In fact, no OTA was found in any samples, which suggests that these isolates did not have the right conditions to produce OTA and FB2, either;

10. Overall, the two Producer's Society and the Agricultural Cooperative showed to have good practices, minimizing the occurrence of mycotoxins.

Perspectives:

1. Widen this study to the rest of the Portuguese regions in order to have a national survey about the occurrence of mycotoxins in maize. This would provide valuable information about the storage conditions and the handling of this product;

2. Further isolations from maize samples obtained in other regions of Portugal should be made in order to assert if the *Aspergillus mottae* species is present in more regions, and to obtain more isolates;

3. Repeating and widen maize sampling will also be important to assert if climate changes are affecting the incidence of fungi and their toxins.

4. It would be important to study field samples to fully understand the pre-harvest contamination of maize, and to assert if the aflatoxins contaminations detected in some samples were pre or post-harvested;

5. It is possible that the simultaneous detection of AF and CPA methodology could be used for other mycotoxins detection (e.g. aflatrem). In addition, the method may have utility for CPA detection in *Penicillium* and in food commodities without having to change columns and conditions between runs.

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APPENDIX 1

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates.

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS01	Ribatejo	yellow green	b	r/fr	+	orange	+	-	-	++	+++	+	*Aspergillus flavus*
08MAS02	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS03	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS04	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
08MAS05	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS06	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS07	Ribatejo	yellow green	b/u	fr	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS15	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	+++	++	*Aspergillus flavus*
08MAS16	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
08MAS17	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS18	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS23	Ribatejo	yellow green	b	s	-	orange	+	-	-	+++	+++	+++	*Aspergillus flavus*
08MAS24	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+++	+++	++	*Aspergillus flavus*
08MAS25	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS26	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS27	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+	++	+++	*Aspergillus flavus*
08MAS28	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS29	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS30	Alto Alentejo	dark green	u	r	-	orange	+	++	+++	+	++	-	*Aspergillus parasiticus*
08MAS31	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	++	+++	-	*Aspergillus flavus*
08MAS32	Alto Alentejo	yellow green	b	fr	-	orange	+	-	-	+	+	+++	*Aspergillus flavus*
08MAS33	Alto Alentejo	yellow green	b/u	fr	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS34	Alto Alentejo	yellow green	b	s	-	orange	+	-	-	+	+++	+	*Aspergillus flavus*
08MAS35	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS36	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS37	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS38	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS39	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	+	+++	-	*Aspergillus flavus*
08MAS55	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS73	Alto Alentejo	olive brown	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS79	Alto Alentejo	olive brown	b/u	r	-	brown	-	-	-	-	-	++	*Aspergillus tamarii*
08MAS81	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS82	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS83	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS84	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS85	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS86	Ribatejo	dark green	u	r	+	orange	+	+++	+++	+	+++	-	*Aspergillus parasiticus*
08MAS87	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS88	Ribatejo	yellow green	b	s	-	orange	-	-	-	+	++	+++	*Aspergillus flavus*
08MAS89	Ribatejo	yellow green	b	s	-	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS90	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS91	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS92	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS93	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS94	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS95	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS96	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS97	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	++	*Aspergillus flavus*
08MAS98	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS153	Ribatejo	yellow green	b	s	+	orange	-	-	+	-	+	-	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS156	Ribatejo	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS159	Ribatejo	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS161	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS162	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS163	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS164	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS168	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS169	Beira Litoral	dark green	u	r	+	orange	+	+++	+++	+++	+++	-	*Aspergillus parasiticus*
08MAS170	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS171	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS172	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS173	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS174	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS175	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS176	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	++	++	*Aspergillus flavus*
08MAS177	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS178	Beira Litoral	yellow green	b	s	+	orange	+	-	+	-	+	+++	*Aspergillus flavus*
08MAS179	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS180	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+++	+	*Aspergillus flavus*
08MAS181	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS182	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+++	-	*Aspergillus flavus*
08MAS183	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS184	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS185	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS186	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS187	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+/-	+	+++	*Aspergillus flavus*
08MAS188	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS189	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+	-	*Aspergillus flavus*
08MAS190	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+++	+++	++	*Aspergillus flavus*
08MAS191	Beira Litoral	yellow green	b	s	+	orange	+	-	+	+	+++	+	*Aspergillus flavus*
08MAS192	Beira Litoral	yellow green	b	s	-	orange	-	-	-	+	+++	-	*Aspergillus flavus*
08MAS193	Beira Litoral	yellow green	b	s	+	orange	+	-	+	+	+++	+	*Aspergillus flavus*
08MAS194	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS195	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS196	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS197	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS198	Beira Litoral	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS199	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS200	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS201	Alto Alentejo	yellow green	b/u	s	+	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
08MAS202	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS203	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+++	+++	+++	*Aspergillus flavus*
08MAS204	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS205	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS206	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+	++	+++	*Aspergillus flavus*
08MAS207	Alto Alentejo	yellow green	b	s	-	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS208	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS209	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS210	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS211	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS212	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS213	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS214	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS215	Alto Alentejo	yellow green	b	s	-	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS216	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS217	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS218	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS219	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS220	Alto Alentejo	yellow green	b/u	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS221	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS222	Alto Alentejo	yellow green	b/u	s	-	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS223	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS224	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS225	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS226	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	-	++	++	*Aspergillus flavus*
08MAS227	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS228	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS229	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS230	Alto Alentejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS231	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS246	Alto Alentejo	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS267	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS268	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS281	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS283	Alto Alentejo	brown	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS303	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS304	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS325	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS326	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS328	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS365	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS377	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS379	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS383	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS384	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS385	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS386	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS387	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS389	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS393	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS398	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS400	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS401	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS408	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+	+++	-	*Aspergillus flavus*
08MAS409	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS410	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS411	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS412	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS413	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS414	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS415	Beira Litoral	olive green	b	s	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS416	Beira Litoral	yellow green	b	s	+	orange	+	-	-	+++	+++	+++	*Aspergillus flavus*
08MAS417	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS418	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
08MAS419	Beira Litoral	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS420	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
08MAS421	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS422	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS423	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS427	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS430	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS431	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS432	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS433	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS434	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS435	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
08MAS436	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS437	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
08MAS438	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS439	Beira Litoral	yellow green	b	s	+	orange	+	-	+	+	+++	-	*Aspergillus flavus*
08MAS440	Beira Litoral	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS441	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS442	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS443	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS444	Beira Litoral	yellow green	b/u	s	-	orange	+	-/+	++	-	+	++	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS445	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS446	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS447	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS448	Beira Litoral	yellow green	b/u	s	-	orange	+	-	-	+++	+++	+	*Aspergillus flavus*
08MAS449	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS450	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS451	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS452	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS453	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS454	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS455	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS456	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS457	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS458	Alto Alentejo	yellow green	b/u	s	-	orange	+	-	-	++	+++	++	*Aspergillus flavus*
08MAS459	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS460	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS461	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS462	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	+	+	*Aspergillus flavus*
08MAS463	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS464	Beira Litoral	yellow green	b/u	s	-	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS465	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS466	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS467	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+	+++	++	*Aspergillus flavus*
08MAS477	Alto Alentejo	dark green	u	r	-	orange	+	+++	+++	+++	+++	-	*Aspergillus parasiticus*
08MAS491	Alto Alentejo	olive green	b	s	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS501	Beira Litoral	olive green	b	s	-	brown	-	-	-	-	-	++	*Aspergillus tamarii*
08MAS504	Beira Litoral	olive green	b	s	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS506	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS507	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS508	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS509	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS510	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+	++	+++	*Aspergillus flavus*
08MAS513	Beira Litoral	yellow green	b	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
08MAS514	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS515	Beira Litoral	yellow green	b	s	+	orange	+	-	-	++	+++	-	*Aspergillus flavus*
08MAS516	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS517	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS518	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS519	Beira Litoral	yellow green	b	s	+	orange	-	-	+	-	++	-	*Aspergillus flavus*
08MAS520	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	-	*Aspergillus flavus*
08MAS521	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS522	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS523	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS524	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+	+++	+	*Aspergillus flavus*
08MAS525	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS526	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS527	Beira Litoral	yellow green	b	s	-	orange	+	-	-	+	++	+++	*Aspergillus flavus*
08MAS528	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS529	Beira Litoral	yellow green	b/u	s	+	orange	+	-	-	-	+	+	*Aspergillus flavus*
08MAS530	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS531	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS532	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS533	Beira Litoral	yellow green	b	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS534	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS535	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS536	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS537	Beira Litoral	yellow green	b/u	s	-	orange	+	-	-	-	++	++	*Aspergillus flavus*
08MAS538	Beira Litoral	yellow green	b/u	s	-	orange	+	-	-	-	+	++	*Aspergillus flavus*
08MAS539	Alto Alentejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS540	Beira Litoral	yellow green	b/u	s	-	orange	+	+	++	+	++	-	*Aspergillus flavus*
08MAS541	Beira Litoral	yellow green	b/u	s	-	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS542	Beira Litoral	yellow green	b	s	-	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
08MAS543	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS544	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS545	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS546	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS547	Beira Litoral	yellow green	b	s	-	orange	+	-	-	-	+++	+	*Aspergillus flavus*
08MAS548	Beira Litoral	yellow green	b	s	+	orange	+	+	+++	+	+++	+++	*Aspergillus flavus*
08MAS549	Beira Litoral	yellow green	b	s	-	orange	+	-	-	-	++	+++	*Aspergillus flavus*
08MAS550	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS551	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS552	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS553	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
08MAS554	Beira Litoral	yellow green	b/u	s	+	orange	-	-	-	-	+	+	*Aspergillus flavus*
08MAS555	Beira Litoral	yellow green	b/u	s	+	orange	+	-	-	+	+++	++	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
08MAS556	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS557	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	++	+	*Aspergillus flavus*
08MAS558	Beira Litoral	yellow green	b	s	+	orange	+	-	+	-	++	++	*Aspergillus flavus*
08MAS559	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
08MAS560	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	+	*Aspergillus flavus*
08MAS561	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS562	Beira Litoral	yellow green	b	s	+	orange	+	-	+	-	+	+	*Aspergillus flavus*
08MAS563	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS564	Beira Litoral	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
08MAS565	Beira Litoral	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
08MAS566	Beira Litoral	yellow green	b	s	+	orange	+	+	++	+	++	-	*Aspergillus minisclerotigenes*
08MAS567	Alto Alentejo	yellow green	b	s	+	orange	+	++	+++	+++	+++	+	*Aspergillus flavus*
08MAS568	Alto Alentejo	yellow green	b/u	s	+	orange	+	+	+++	+	+++	-	*Aspergillus flavus*
08MAS569	Alto Alentejo	yellow green	b	s	+	orange	+	+++	+++	+++	+++	+++	*Aspergillus minisclerotigenes*
08MAS570	Alto Alentejo	yellow green	b	s	+	orange	+	++	+++	++	+++	++	*Aspergillus minisclerotigenes*
08MAS571	Alto Alentejo	yellow green	b	s	+	yellow	+	+++	+++	+++	+++	-	*Aspergillus mottae*
08MAS572	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+++	+++	+	*Aspergillus minisclerotigenes*
08MAS573	Alto Alentejo	yellow green	b	s	+	orange	+	-	-	+++	+++	+	*Aspergillus minisclerotigenes*
08MAS574	Alto Alentejo	yellow green	b	s	+	orange	+	+	+++	++	+++	+	*Aspergillus minisclerotigenes*
08MAS575	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
08MAS581	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS630	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	-	*Aspergillus tamarii*
08MAS634	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS637	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*
08MAS659	Beira Litoral	olive green	b/u	r	-	brown	-	-	-	-	-	+	*Aspergillus tamarii*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS01	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	+++	+++	-	*Aspergillus flavus*
09MAS02	Ribatejo	yellow green	b/u	s	+	orange	+	+	+++	+++	+++	+++	*Aspergillus flavus*
09MAS03	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS04	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	++	+++	-	*Aspergillus flavus*
09MAS05	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	-	+	-	*Aspergillus flavus*
09MAS06	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS07	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	-	*Aspergillus flavus*
09MAS08	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	-	*Aspergillus flavus*
09MAS09	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS12	Ribatejo	yellow green	b	s	+	orange	+	-	-	++	+++	+	*Aspergillus flavus*
09MAS13	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	+	*Aspergillus flavus*
09MAS14	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS15	Ribatejo	yellow green	b	s	+	orange	-	-	-	+	++	-	*Aspergillus flavus*
09MAS16	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS17	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS18	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS19	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS21	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS22	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS23	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	-	*Aspergillus flavus*
09MAS24	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS25	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS26	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS27	Ribatejo	yellow green	b	s	+	orange	-	-	+	-	+	-	*Aspergillus flavus*
09MAS28	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS29	Ribatejo	yellow green	b	s	-	orange	+	-	-	+++	+++	-	*Aspergillus flavus*
09MAS30	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS31	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	+	*Aspergillus flavus*
09MAS33	Ribatejo	yellow green	b	s	+	orange	+	-	-	+	+++	-	*Aspergillus flavus*
09MAS34	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	-	*Aspergillus flavus*
09MAS35	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS36	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
09MAS37	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	++	*Aspergillus flavus*
09MAS38	Ribatejo	yellow green	b	s	+	orange	+	-	-	+++	+++	++	*Aspergillus flavus*
09MAS39	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS40	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS41	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS42	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	++	+	*Aspergillus flavus*
09MAS43	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	+++	+	*Aspergillus flavus*
09MAS46	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	+	-	*Aspergillus flavus*
09MAS47	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
09MAS48	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS49	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS50	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+	*Aspergillus flavus*
09MAS51	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS52	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS53	Ribatejo	olive green	b/u	r	-	dark brown	-	-	-	-	-	+	*Aspergillus tamarii*
09MAS63	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS64	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
09MAS65	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	++	+++	+	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS66	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	++	+++	*Aspergillus flavus*
09MAS67	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS68	Ribatejo	olive green	b/u	r	-	dark brown	-	-	-	-	-	+	*Aspergillus tamarii*
09MAS69	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	-	+	+	*Aspergillus flavus*
09MAS70	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS71	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	+++	+++	++	*Aspergillus flavus*
09MAS72	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS73	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS74	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS75	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS76	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS80	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS81	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
09MAS82	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS83	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
09MAS84	Ribatejo	yellow green	b/u	s	+	orange	-	-	-	+	-	++	*Aspergillus flavus*
09MAS85	Ribatejo	yellow green	b/u	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS86	Ribatejo	yellow green	b/u	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS87	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS92	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS93	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
09MAS94	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS95	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS96	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS104	Ribatejo	olive green	b/u	r	-	dark brown	-	-	-	-	-	+	*Aspergillus tamarii*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS105	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	++	*Aspergillus flavus*
09MAS106	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	-	*Aspergillus flavus*
09MAS107	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS108	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	+++	+++	*Aspergillus flavus*
09MAS116	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
09MAS117	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS118	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS119	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS120	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS121	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS122	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS123	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS124	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS125	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
09MAS126	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	++	+++	*Aspergillus flavus*
09MAS127	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS128	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS129	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	++	+++	*Aspergillus flavus*
09MAS130	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS131	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
09MAS132	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS133	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS134	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS137	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS138	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS139	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS140	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS141	Ribatejo	yellow green	b	s	-	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS142	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS143	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS144	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS145	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS146	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS157	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS159	Alto Alentejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS187	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS188	Alto Alentejo	yellow green	b	s	-	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS200	Ribatejo	yellow green	b/u	s	+	orange	+	+	+++	+	+++	++	*Aspergillus mottae*
09MAS201	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	++	+	*Aspergillus flavus*
09MAS213	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+	+	*Aspergillus flavus*
09MAS214	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS215	Ribatejo	yellow green	b	s	+	orange	+	-	+	-	+++	+	*Aspergillus flavus*
09MAS236	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS237	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS250	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+++	+	*Aspergillus flavus*
09MAS252	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	++	*Aspergillus flavus*
09MAS253	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	+++	+++	*Aspergillus flavus*
09MAS254	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS273	Ribatejo	yellow green	b	s	-	orange	+	-	-	+++	+++	+++	*Aspergillus flavus*
09MAS274	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*

Table A.1. – Characterization of *Aspergillus* section *Flavi* isolates (continued).

		Morphologic observation				Media		HPLC					
Strains Code	**Region**	Colour (15 days CYA)	Seriation*	Conidia**	Sclerotia	AFPA	CAM***	AFG2	AFG1	AFB2	AFB1	CPA	**Species**
09MAS275	Ribatejo	yellow green	b	s	-	orange	+	-	-	-	++	+++	*Aspergillus flavus*
09MAS276	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS277	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS278	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*
09MAS279	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS280	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS281	Ribatejo	yellow green	b	s	-	orange	+	-	-	+	++	+++	*Aspergillus flavus*
09MAS282	Ribatejo	yellow green	b	s	-	orange	+	-	-	-	+	+++	*Aspergillus flavus*
09MAS283	Ribatejo	yellow green	b	s	+	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
09MAS284	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	+	+++	*Aspergillus flavus*
09MAS285	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS286	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS287	Ribatejo	yellow green	b	s	-	orange	-	-	-	-	-	++	*Aspergillus flavus*
09MAS288	Ribatejo	yellow green	b	s	-	orange	+	-	-	++	+++	+++	*Aspergillus flavus*
09MAS289	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	+++	*Aspergillus flavus*
09MAS290	Ribatejo	yellow green	b	s	+	orange	+	-	-	-	++	+++	*Aspergillus flavus*
09MAS291	Ribatejo	yellow green	b	s	+	orange	-	-	-	-	-	-	*Aspergillus flavus*

* Seriation u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate

** Conidia s: smooth; r- rough;

***CAM -: not detected; + : detected

APPENDIX 2

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates.

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS10	Ribatejo	black	b	s	beige	-	0	324,7	*Aspergillus section Nigri*
08MAS11	Ribatejo	black	b	s	beige	-	0	324,7	*Aspergillus section Nigri*
08MAS12	Ribatejo	black	b	sr	beige	-	0	454,14	*Aspergillus section Nigri*
08MAS13	Ribatejo	black	b	s	beige	-	0	0,6	*Aspergillus section Nigri*
08MAS14	Ribatejo	black	b	s	beige	-	0	483,15	*Aspergillus section Nigri*
08MAS19	Beira Litoral	black	b	s	beige	-	0	215	*Aspergillus section Nigri*
08MAS20	Beira Litoral	black	b	s	beige	-	0	0,4	*Aspergillus section Nigri*
08MAS21	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS22	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS40	Alto Alentejo	black	b	s	beige	-	0	45	*Aspergillus section Nigri*
08MAS41	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS42	Alto Alentejo	black	b	s	beige	-	0	44,71	*Aspergillus section Nigri*
08MAS43	Alto Alentejo	black	b	s	beige	-	0	31,78	*Aspergillus section Nigri*
08MAS44	Alto Alentejo	black	b	s	beige	-	0	38,8	*Aspergillus section Nigri*
08MAS45	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS46	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS47	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS48	Alto Alentejo	black	b	s	yellow	-	0	96,72	*Aspergillus section Nigri*
08MAS49	Alto Alentejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS50	Alto Alentejo	black	b		beige	-	0	0	*Aspergillus section Nigri*
08MAS52	Alto Alentejo	black	b	s	brown	-	0	0	*Aspergillus section Nigri*
08MAS53	Alto Alentejo	black	b		beige	-	0	0	*Aspergillus section Nigri*
08MAS54	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS56	Alto Alentejo	black	b	s	beige	-	0,38	0	*Aspergillus section Nigri*
08MAS57	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS58	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS59	Alto Alentejo	black	b	s	beige	-	0	3,6	*Aspergillus section Nigri*
08MAS60	Alto Alentejo	black	b	s	beige	-	0	450,2	*Aspergillus section Nigri*
08MAS61	Alto Alentejo	black	b	s	beige	-	0	10,92	*Aspergillus section Nigri*
08MAS62	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS63	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS64	Alto Alentejo	black	b	sr	brown yellow	-	0	0	*Aspergillus section Nigri*
08MAS65	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS66	Alto Alentejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS67	Alto Alentejo	black	b	s	beige	-	0,4	1,89	*Aspergillus section Nigri*
08MAS68	Alto Alentejo	black	b	s	beige brown	-	0	20,27	*Aspergillus section Nigri*
08MAS69	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS99	Ribatejo	black	b	s	brown	-	0	0	*Aspergillus section Nigri*
08MAS100	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS102	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS103	Ribatejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS105	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS106	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS107	Ribatejo	black	b	s	beige	+	1,87	21,19	*Aspergillus section Nigri*
08MAS108	Ribatejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS109	Ribatejo	black	b	s	beige	-	0	1,13	*Aspergillus section Nigri*
08MAS110	Ribatejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS111	Ribatejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS113	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS114	Ribatejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS115	Ribatejo	black	b	s	beige	-	0	259,33	*Aspergillus section Nigri*
08MAS116	Ribatejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS117	Ribatejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS118	Ribatejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS119	Ribatejo	black	b	sr	beige	+	7,48	0	*Aspergillus section Nigri*
08MAS120	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS121	Ribatejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS122	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS123	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS124	Ribatejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS125	Ribatejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS126	Ribatejo	black	b	sr	beige brown centre	-	0,8	0	*Aspergillus section Nigri*
08MAS128	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS130	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS134	Ribatejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS135	Ribatejo	black	b	sr	beige	-	0	3,2	*Aspergillus section Nigri*
08MAS137	Ribatejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS138	Ribatejo	black	b	s	brown yellow	-	0	0	*Aspergillus section Nigri*
08MAS140	Ribatejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS141	Ribatejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS143	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS144	Ribatejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS145	Ribatejo	black	b	s	brown	-	0	4,98	*Aspergillus section Nigri*
08MAS146	Ribatejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS147	Ribatejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS148	Ribatejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS149	Ribatejo	black	b	s	beige yellow	-	0		*Aspergillus section Nigri*
08MAS150	Ribatejo	black	b	s	beige yellow	-	0	6,46	*Aspergillus section Nigri*
08MAS151	Ribatejo	black	b	s	brown	-	0	0	*Aspergillus section Nigri*
08MAS160	Ribatejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS232	Alto Alentejo	black	b	s	beige	-	0,34	64	*Aspergillus section Nigri*
08MAS233	Alto Alentejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS234	Alto Alentejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS235	Alto Alentejo	black	b	s	beige brown	-	0	4,98	*Aspergillus section Nigri*
08MAS236	Alto Alentejo	black	b	s	beige brown centre	+	3,17	0	*Aspergillus section Nigri*
08MAS237	Alto Alentejo	black	b	sr	beige brown	-	0	0	*Aspergillus section Nigri*
08MAS238	Alto Alentejo	black	b	sr	beige	+	2,8	0	*Aspergillus section Nigri*
08MAS239	Alto Alentejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS241	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS242	Alto Alentejo	black	b	sr	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS243	Alto Alentejo	black	b	s	brown	-	0	88,79	*Aspergillus section Nigri*
08MAS244	Alto Alentejo	black	b	s	brown	-	0	0	*Aspergillus section Nigri*
08MAS245	Alto Alentejo	black	b	sr	brown yellow	-	0	0	*Aspergillus section Nigri*
08MAS247	Alto Alentejo	black	b	sr	brown	-	0	0	*Aspergillus section Nigri*
08MAS248	Alto Alentejo	black	b	sr	beige	-	0	7,77	*Aspergillus section Nigri*
08MAS249	Alto Alentejo	black	b	s	beige brown	-	0	0	*Aspergillus section Nigri*
08MAS250	Alto Alentejo	black	b	s	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS251	Alto Alentejo	black	b	s	beige yellow	-	0	3,96	*Aspergillus section Nigri*
08MAS253	Alto Alentejo	black	b	s	brown	-	0	0	*Aspergillus section Nigri*
08MAS254	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS255	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS256	Alto Alentejo	black	b	sr	beige brown centre	-	0	3,18	*Aspergillus section Nigri*
08MAS257	Alto Alentejo	black	b	s	brown	-	0	6,06	*Aspergillus section Nigri*
08MAS258	Alto Alentejo	black	b	s	beige brown centre	+	5,4	515	*Aspergillus section Nigri*
08MAS259	Alto Alentejo	black	b	s	beige brown centre	-	0	4,13	*Aspergillus section Nigri*
08MAS261	Alto Alentejo	black	b	s	beige brown centre	-	0	2,24	*Aspergillus section Nigri*
08MAS262	Alto Alentejo	black	b	s	beige yellow	-	0	7,4	*Aspergillus section Nigri*
08MAS264	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS266	Alto Alentejo	black	b	s	beige yellow	-	0	1902,47	*Aspergillus section Nigri*
08MAS269	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS270	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS271	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS273	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS274	Alto Alentejo	black	b	sr	beige brown	-	0	0	*Aspergillus section Nigri*
08MAS275	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS276	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS277	Alto Alentejo	black	b	s	beige	+	7,58	68,4	*Aspergillus section Nigri*
08MAS278	Alto Alentejo	black	b	s	beige	-	0	26,3	*Aspergillus section Nigri*
08MAS279	Alto Alentejo	black	b	sr	beige	+	3,84	0	*Aspergillus section Nigri*
08MAS280	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS284	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS285	Alto Alentejo	black	b	s	beige yellow	-	0	12,16	*Aspergillus section Nigri*
08MAS286	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS287	Alto Alentejo	black	b	sr	brown	+	2,17	100,47	*Aspergillus section Nigri*
08MAS288	Alto Alentejo	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS289	Alto Alentejo	black	b	r	beige brown centre	-	0	0	*Aspergillus section Nigri*
08MAS290	Alto Alentejo	black	b	s	beige	-	0	396,93	*Aspergillus section Nigri*
08MAS291	Alto Alentejo	black	b	s	brown	-	0	46,52	*Aspergillus section Nigri*
08MAS294	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS296	Alto Alentejo	black	b	s	beige	-	0	396,93	*Aspergillus section Nigri*
08MAS297	Alto Alentejo	black	b	s	beige	-	0	7,52	*Aspergillus section Nigri*
08MAS298	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS299	Alto Alentejo	black	b	s	brown	-	0,65	53,45	*Aspergillus section Nigri*
08MAS300	Alto Alentejo	black	b	s	beige	-	0	52,56	*Aspergillus section Nigri*
08MAS301	Alto Alentejo	black	b	s	beige	+	4,6	0	*Aspergillus section Nigri*
08MAS305	Alto Alentejo	black	b	sr	brown yellow	-	0	0	*Aspergillus section Nigri*
08MAS306	Alto Alentejo	black	b	sr	brown claro	-	0	0	*Aspergillus section Nigri*
08MAS309	Alto Alentejo	black	b	s	brown	-	0	73,73	*Aspergillus section Nigri*
08MAS310	Alto Alentejo	black	b	s	beige	+	5	0	*Aspergillus section Nigri*
08MAS311	Alto Alentejo	black	b	s	beige brown	-	0	5,67	*Aspergillus section Nigri*
08MAS312	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS313	Alto Alentejo	black	b	s	beige yellow	-	0	4,59	*Aspergillus section Nigri*
08MAS314	Alto Alentejo	black	b	s	brown	-	0	5,3	*Aspergillus section Nigri*
08MAS315	Alto Alentejo	black	b	s	beige yellow	-	0	292,93	*Aspergillus section Nigri*
08MAS316	Alto Alentejo	black	b	sr	beige	-	0	0	*Aspergillus section Nigri*
08MAS317	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS318	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS319	Alto Alentejo	black	b	r	brown	-	0	29,31	*Aspergillus section Nigri*
08MAS320	Alto Alentejo	black	b	r	beige yellow	-	0	14,29	*Aspergillus section Nigri*
08MAS321	Beira Litoral	black	b	sr	beige brown centre	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS322	Beira Litoral	black	b	s	beige	+	0	92,87	*Aspergillus section Nigri*
08MAS324	Beira Litoral	black	b	s	amarelo	-	0	821,3	*Aspergillus section Nigri*
08MAS394	Beira Litoral	black	b	r	beige	-	0	0	*Aspergillus section Nigri*
08MAS395	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS397	Beira Litoral	black	b	s	beige	-	0	207	*Aspergillus section Nigri*
08MAS406	Beira Litoral	black	b	s	beige	-	0	3	*Aspergillus section Nigri*
08MAS407	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS424	Beira Litoral	black	b	s	beige	-	0	128,8	*Aspergillus section Nigri*
08MAS429	Beira Litoral	black	b	s	brown yellow	-	0	75,45	*Aspergillus section Nigri*
08MAS468	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS469	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS470	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS471	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS472	Beira Litoral	black	b	s	beige	+	2,7	0	*Aspergillus section Nigri*
08MAS473	Beira Litoral	black	b	s	beige	+	8,5	8,37	*Aspergillus section Nigri*
08MAS474	Beira Litoral	black	b	r	beige	-	0	0	*Aspergillus section Nigri*
08MAS475	Beira Litoral	black	b	s	beige	-	0	560,6	*Aspergillus section Nigri*
08MAS476	Beira Litoral	black	b	s	beige	-	0	202,8	*Aspergillus section Nigri*
08MAS478	Alto Alentejo	black	b	s	beige	-	0	12,5	*Aspergillus section Nigri*
08MAS479	Alto Alentejo	black	b	r	beige	-	0	5,96	*Aspergillus section Nigri*
08MAS480	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS481	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*
08MAS482	Alto Alentejo	black	b	s	brown yellow	-	0	0	*Aspergillus section Nigri*
08MAS483	Alto Alentejo	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS484	Alto Alentejo	black	b	s	beige yellow	-	0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
08MAS485	Alto Alentejo	black	b	sr	beige	-	0	29,78	*Aspergillus section Nigri*
08MAS486	Alto Alentejo	black	b	sr	Beige	-	0	0	*Aspergillus section Nigri*
08MAS487	Alto Alentejo	black	b	sr	Beige	-	0	37,21	*Aspergillus section Nigri*
08MAS511	Beira Litoral	black	b	sr	Beige	-	0	0	*Aspergillus section Nigri*
08MAS512	Beira Litoral	black	b	sr	Beige	-	0	0	*Aspergillus section Nigri*
08MAS576	Beira Litoral	black	b	s	Beige	-	0	140,64	*Aspergillus section Nigri*
08MAS577	Beira Litoral	black	b	s	beige brown centre	-	0	40,64	*Aspergillus section Nigri*
08MAS578	Beira Litoral	black	b	s	beige brown centre	-	0	4,46	*Aspergillus section Nigri*
08MAS579	Alto Alentejo	black	b	s	beige brown centre	-	0	21,11	*Aspergillus section Nigri*
08MAS580	Beira Litoral	black	b	r	beige brown centre	-	0	2,67	*Aspergillus section Nigri*
08MAS588	Beira Litoral	black	b	s	beige	-	0	191,19	*Aspergillus section Nigri*
08MAS594	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS596	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS599	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS600	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS602	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS603	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
08MAS656	Beira Litoral	black	b	s	beige	-	0	0	*Aspergillus section Nigri*
09MAS20	Ribatejo	black	b	s	beige	-	0	71,9	*Aspergillus section Nigri*
09MAS32	Ribatejo	black	b	s	beige		0	36,72	*Aspergillus section Nigri*
09MAS44	Ribatejo	black	b	s	beige		0	1,99	*Aspergillus section Nigri*
09MAS45	Ribatejo	black	b	s	beige		0,81	0	*Aspergillus section Nigri*
09MAS54	Ribatejo	black	b	s	beige		0	27,42	*Aspergillus section Nigri*
09MAS55	Ribatejo	black	b	s	beige		2,77	0	*Aspergillus section Nigri*
09MAS56	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
09MAS57	Ribatejo	black	b	s	beige		0	325,29	*Aspergillus section Nigri*
09MAS58	Ribatejo	black	b	s	beige		2,8	0	*Aspergillus section Nigri*
09MAS59	Ribatejo	black	b	s	beige		0,73	127,21	*Aspergillus section Nigri*
09MAS60	Ribatejo	black	b	s	beige		0	65,3	*Aspergillus section Nigri*
09MAS61	Ribatejo	black	b	s	beige		1,2	0	*Aspergillus section Nigri*
09MAS62	Ribatejo	black	b	r	beige		0	0	*Aspergillus section Nigri*
09MAS77	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS78	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS79	Ribatejo	black	b	sr	beige		3,1	268,63	*Aspergillus section Nigri*
09MAS88	Ribatejo	black	b	s	beige		0	2,09	*Aspergillus section Nigri*
09MAS89	Ribatejo	black	b	s	beige		0	424,44	*Aspergillus section Nigri*
09MAS90	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS91	Ribatejo	black	b	s	beige		0	22,38	*Aspergillus section Nigri*
09MAS97	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS98	Ribatejo	black	b	s	beige		0	25,45	*Aspergillus section Nigri*
09MAS99	Ribatejo	black	b	sr	beige		0	26,51	*Aspergillus section Nigri*
09MAS100	Ribatejo	black	b	s	beige		0	493,51	*Aspergillus section Nigri*
09MAS101	Ribatejo	black	b	s	beige		0	87,388	*Aspergillus section Nigri*
09MAS102	Ribatejo	black	b	s	beige		0	280,02	*Aspergillus section Nigri*
09MAS103	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS109	Ribatejo	black	b	s	beige		0	2,63	*Aspergillus section Nigri*
09MAS110	Ribatejo	black	b	s	beige		0	28,5	*Aspergillus section Nigri*
09MAS111	Ribatejo	black	b	s	beige		0	119,31	*Aspergillus section Nigri*
09MAS112	Ribatejo	black	b	s	beige		0	10,13	*Aspergillus section Nigri*
09MAS113	Ribatejo	black	b	s	beige		0	235,16	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
09MAS114	Ribatejo	black	b	s	beige		5,4	0	*Aspergillus section Nigri*
09MAS135	Ribatejo	black	b	s	beige		4,2	175,54	*Aspergillus section Nigri*
09MAS136	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS147	Ribatejo	black	b	sr	beige		0	5,24	*Aspergillus section Nigri*
09MAS148	Ribatejo	black	b	sr	beige		0	0	*Aspergillus section Nigri*
09MAS149	Ribatejo	black	b	s	beige		1,2	9,14	*Aspergillus section Nigri*
09MAS150	Ribatejo	black	b	s	beige		0	837,79	*Aspergillus section Nigri*
09MAS151	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS152	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS153	Alto Alentejo	black	b	s	brown		0	0	*Aspergillus section Nigri*
09MAS154	Alto Alentejo	black	b	s	brown		0,4	0	*Aspergillus section Nigri*
09MAS155	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS156	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS158	Alto Alentejo	black	b	s	beige		0	603,18	*Aspergillus section Nigri*
09MAS160	Alto Alentejo	black			beige		0	0	*Aspergillus section Nigri*
09MAS161*	Alto Alentejo	black	b	s	beige		0	0,46	*Aspergillus section Nigri*
09MAS162*	Alto Alentejo	black	b	s	beige		6	0	*Aspergillus section Nigri*
09MAS184	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS185	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS186	Alto Alentejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS199	Ribatejo	black	b	sr	beige		0	503,43	*Aspergillus section Nigri*
09MAS202	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS204	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS216	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS217	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*

Table A.2 – Characterization of *Aspergillus* section *Nigri* isolates (continued).

		Morphological observation			Media		HPLC		
Strain code	**Region**	Colour (7 days CYA)	Seriation*	Conidia**	AFPA	CAM***	OTA	FB2	**Gender/section**
09MAS218	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS219	Ribatejo	black	b	s	beige		0	13,35	*Aspergillus section Nigri*
09MAS220	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS221	Ribatejo	black	b	s	beige		1,4	0	*Aspergillus section Nigri*
09MAS222	Ribatejo	black	b	s	beige		2,2	0	*Aspergillus section Nigri*
09MAS223	Ribatejo	black	b	s	beige		0	89,88	*Aspergillus section Nigri*
09MAS224	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS225	Ribatejo	black	b	s	beige		1,6	25,78	*Aspergillus section Nigri*
09MAS238	Ribatejo	black	b	s	beige		2,3	0	*Aspergillus section Nigri*
09MAS239	Ribatejo	black	b	s	beige		4	0	*Aspergillus section Nigri*
09MAS240	Ribatejo	black	b	s	brown		0	0	*Aspergillus section Nigri*
09MAS241	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS243	Ribatejo	black	b	s	beige		5,4	0	*Aspergillus section Nigri*
09MAS244	Ribatejo	black	b	sr	beige		3,25	0	*Aspergillus section Nigri*
09MAS256	Ribatejo	black	b	s	beige		0	1250	*Aspergillus section Nigri*
09MAS257	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS258	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS259	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*
09MAS260	Ribatejo	black	b	s	beige		0	0	*Aspergillus section Nigri*

* Seriation- u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate

** Conidia- s: smooth; r- rough; sr- slightly rough

***CAM -: not detected; + : detected

APPENDIX 3

Table A.3.1 - Accession numbers of the deposited sequences at Gen Bank.

Code	ID	Mating type	MCM7	Beta-tubulin	Calmodulin	RNA-Polymerase	TSR1	Species
MUM 10.206	JF412783	HM803049	HM803079	HM803091	HM803019	HM802977	HM802992	*A. flavus*
MUM 10.232	JF412782	HM803048	HM803080	HM803096	HM803018	HM802975	HM802990	*A. flavus*
MUM 10.226	JF412776	HM802956	HM803061	HM803088	HM803016	HM802971	HM802993	*A.minisclerotigenes*
MUM 10.227	JF412777	HM803043	HM803068	HM803094	HM803032	HM802970	HM802995	*A.minisclerotigenes*
MUM 10.228	JF412778	HM803038	HM803062	HM803098	HM803025	HM802976	HM803000	*A.minisclerotigenes*
MUM 10.229	JF412779	HM803052	HM803078	HM803095	HM803031	HM802989	HM802998	*A.minisclerotigenes*
MUM 10.230	JF412780	HM803045	HM803063	HM803097	HM803014	HM802987	HM802994	*A.minisclerotigenes*
MUM 10.203	JF412781	HM802963	HM803067	HM803083	HM803026	HM802973	HM802999	*A.minisclerotigenes*
MUM 10.225	JF412784	HM803057	HM803074	HM803085	HM803027	HM802984	HM803001	*A. parasiticus*
MUM 10.212	JF412785	HM803036	HM803069	HM803092	HM803012	HM802969	HM803011	*A. parasiticus*
MUM 10.224	JF412786	HM803058	HM803075	HM803100	HM803033	HM802986	HM803007	*A. parasiticus*
MUM 10.215	JF412787	HM803037	HM803073	HM803081	HM803022	HM802974	HM803008	*A. parasiticus*
MUM 10.208	JF412770	HM802957	HM803076	HM803099	HM803017	HM802978	HM803004	*A. sergii*
MUM 10.219^{T}	JF412769	HM802967	HM803071	HM803082	HM803029	HM802985	HM803005	*A. sergii*
MUM 10.205	JF412771	HM803035	HM803070	HM803087	HM803021	HM802979	HM803002	*A. transmontanensis*
MUM 10.211	JF412772	HM803054	HM803060	HM803102	HM803023	HM802968	HM803003	*A. transmontanensis*
MUM 10.221	JF446612	HM803056	HM803072	HM803093	HM803028	HM802972	HM802996	*A. transmontanensis*
MUM 10.222	JF412773	HM803047	HM803064	HM803089	HM803030	HM802981	HM803009	*A. transmontanensis*
MUM 10.214^{T}	JF412774	HM803050	HM803065	HM803101	HM803020	HM802980	HM802997	*A. transmontanensis*
MUM 10.223	JF446613	HM802958	HM803077	HM803084	HM803024	HM802983	HM803010	*A. transmontanensis*
MUM 10.233	JF412768	HM803040	HM803066	HM803090	HM803013	HM802982	HM802991	*A. mottae*
MUM 10.231^{T}	JF412767	HM803042	HM803059	HM803086	HM803015	HM802988	HM803006	*A. mottae*
CBS 117610	EF409241			EF203158	EF202049			*A. arachidicola*
CBS 117611				EF203160	EF202052			*A. arachidicola*
CBS 117612				EF203159	EF202051			*A. arachidicola*
CBS 117615				EF203161	EF202050			*A. arachidicola*
NRRL 4868	JN185451			JN185450				*A. chungii*
NRRL 506	AF459735			JN185446	JN185447			*A. effuses*
CBS 110.55	FJ491463			EF203135	EF202056			*A. fasciculatus*
NRRL 1957	AF027863			AF255064	AF255041			*A. flavus*
NRRL 4818	EF661563			EF661489	EF661512			*A. flavus* var. *columnaris*
NRRL 3751	EF661554			EF661488	EF661511			*A. kambarensis*
NRRL 447	EF661560			EF661483	EF661506			*A. oryzae*
NRRL 502	AY373859			AY373859	AF255040			*A. parasiticus*
CBS 822.72	FJ491470			EF203163	EF202046			*A. toxicarius*

CBS=Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
MUM=Micoteca da Univerdade do Minho
NRRL=Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

Table A.3.2- Calibration parameters of instrumentation.

Mycotoxin		Calibration Curve	r^2	LOD (ng/mL)
Aflatoxins	B1	y = 5E-05x + 0,471	0.996	1
	B2	y = 2E-05x + 0,109	0.996	1
	G1	y = 0,000x - 0,163	0.999	1
	G2	y = 7E-05x + 0,299	0.98	1
Cyclopiazonic acid		y = 0,337x + 0,365	0,98	1977
Ochratoxin A		y = 4564x + 389.4	0.999	0.73
Fumonisins	B1	$y = 2E\text{-}07x^2 – 0.069x + 8941$	0.999	84
	B1	y = 210.8x - 2792	0.997	89
	B2	y = 221.3x + 911.5	0.999	75

Table A.3.3- Isolation codes and its correspondence of the deposit code at Micoteca da Universidade do Minho.

MUM	**Isolation**
MUM 10. 232	08MAS567
MUM 10. 226	08MAS569
MUM 10.227	08MAS570
MUM 10.333	08MAS571
MUM 10.228	08MAS572
MUM 10.229	08MAS573
MUM 10.230	08MAS574
MUM 10.231	09MAS200
MUM 10.225	08AAsp36
MUM 10.203	08AAsp37
MUM 10.205	08AAsp67
MUM 10.206	08AAsp116
MUM 10.211	09AAsp146
MUM 10.221	09AAsp152
MUM 10.208	08AAsp183
MUM 10.212	09AAsp187
MUM 10.222	09AAsp201
MUM 10.224	09AAsp235
MUM 10.219	09AAsp494
MUM 10.223	09AAsp298
MUM 10.215	09AAsp266
MUM 10.214	09AAsp260

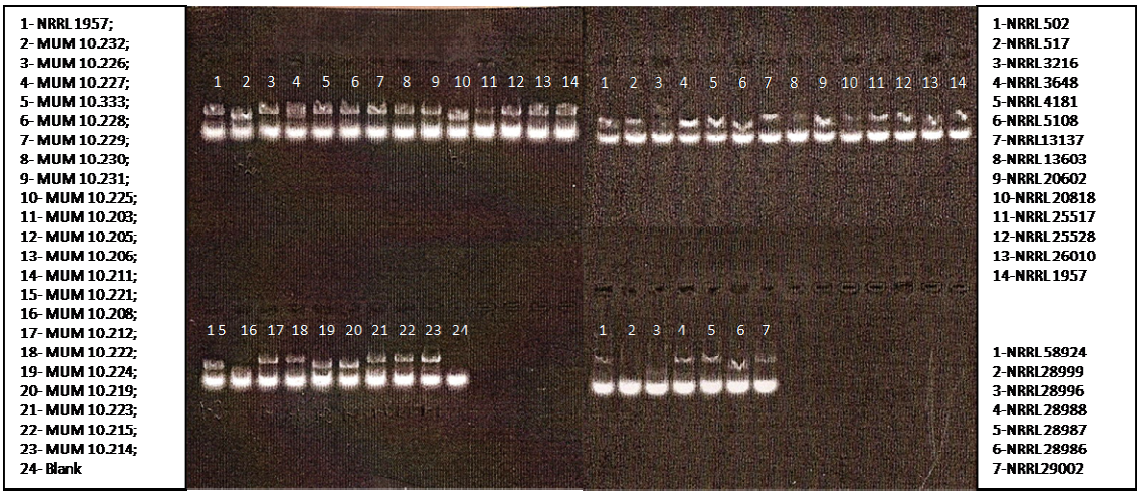

Figure A.3.1 – Determination of mating-type genes in *Aspergillus* section *Flavi* strains used in the study.