UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXV

S.S.D. AGR/12 PATOLOGIA VEGETALE

New insights on *Aspergillus flavus* population in maize crops to boost the application of biocontrol with atoxigenic strains in Europe

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General Introduction

Introduction

Maize Description: Maize (*Zea mays* L.), a revolutionary crop, was domesticated from teosinte in southern Mexico about 9000 years ago and in 1492 it was introduced for the first time in Europe by Cristoforo Colombo from Spain. In the 16th century the maize growing areas extended to North-Eastern Europe (Rebourg et al., 2003; Yi et al., 2021).

Maize or corn is an annual monocotyledon crop belonging to the family of Poaceae (Gramineae) (Fig.1), its development is divided into two physiological stages, vegetative and reproductive. Single stalk is the main growth organ of maize with a height that can vary from one to five meters. Maize is a monoic plant with reproductive organs, female (ear) and male (tassel), located in the same plant but separated in space. Grains, or kernels as commonly called, are caryopsis, and composed by three major parts: pericarp/hull, endosperm (nutriment storage organ), and embryo or germ (Coe, 2001) The root system is characterized by one single primary root as well as seminal root that are variable in number and shoot borne roots which are developed in the consecutive shoot nodes (FAO, 1992).

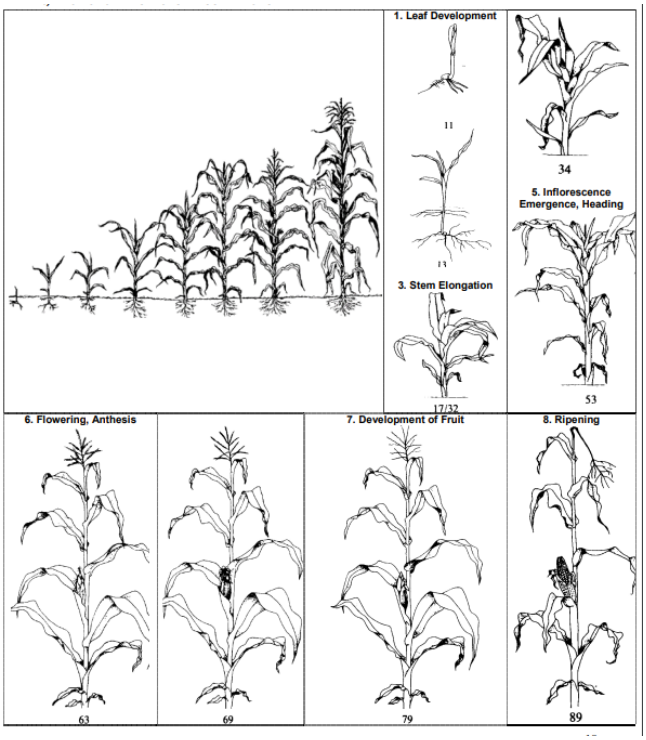

Figure 1. BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical) growth stages: maize (U. Meier, 2018)

Geographical distribution: Maize growing areas are widespread in the world and range from the tropics to the north temperate region and from low to mid-altitude to highlands. This led to high adaptability in different environmental conditions and enhance the genetic diversity of maize (Orozco-Ramírez et al., 2017; Diaw et al., 2021). According to FAOStat (2021) maize cultivation in America is the highest followed by Asian countries, 36.1% and 34.1%, respectively, of agricultural land. Africa and Europe represent 20.9% and 8.9% of total maize growing areas, and over the last decades an expansion (+46%) of maize growing areas has been recorded particularly in Ukraine, Argentina, China, and Indonesia.

Maize Production: Nowadays, in the agri-food system, maize plays a major role for food security for many low-income countries in Latin America and Sub-Saharan Africa due to population growth. However, in high-income countries, maize is used primarily as livestock feed (Fig. 2) (Erenstein, 2010). Maize is the second most produced and consumed crop in the world (Erenstein et al., 2022). Compared to other cereals, maize is considered as a multi-purpose crop; it is used for human consumption, livestock feed, biofuel production as well as oil and starch extraction and other substances for industrial purposes (Ramirez-Cabral, et al. 2017;Santpoort, 2020).

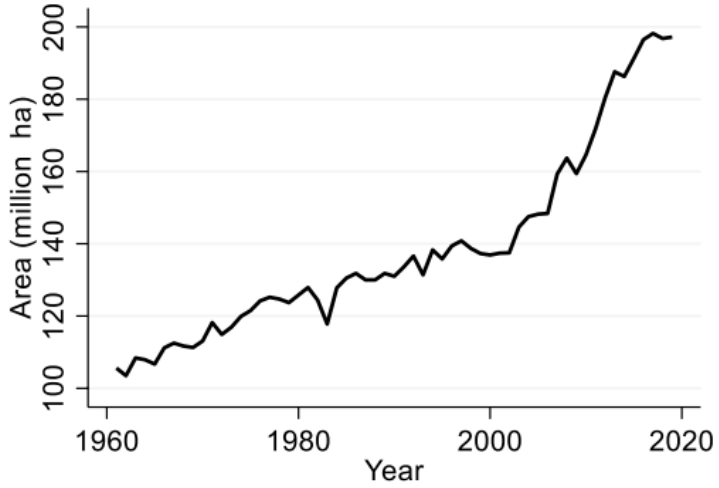

Figure 2. Dynamics of key maize indicators 1961–2020: maize area (M ha) (FAOStat, 2021)

The global distribution of maize production in 2021/2022 shows the United States as the largest producer with a volume amounting to about 384 million metric tons (31.5%). China and Brazil rounded off the top maize producing countries with 22.4% and 9.5% respectively, with a minor

role of European Union (EU) countries (5.8%) and Argentina (4.4%) (USDA, 2022). In Italy, maize is among the main grown cereals and is cultivated mainly in the Northern part (Po valley) with 578,417 ha. The production in 2022 reached 578.4 million tonnes (Eurostat, 2022; Istat, 2022)

Mycotoxins: Mycotoxins are stable secondary metabolites, that occur in a wide variety of agricultural commodities before harvest, at harvest or under post-harvest conditions, as well as during the food and feed chain processing and might be accumulated in dairy products such as cheese and milk when animals are fed with contaminated feed (Magan and Aldred 2007;Moretti et al., 2017).*Fusarium, Aspergillus* and *Penicillium* are the most important mycotoxin-producing genera and are widely distributed across the world (Sweeney and Dobson, 1998; Eskola et al. 2020). In recent years, with the increase of the cereal consumption as well as the international trade, mycotoxin became a main food safety issue due to its harmful risk for human and animal health; they cause acute and chronic mycotoxicosis (Bennett and Klich, 2003). Maize is a crop highly susceptible to mycotoxins.

Aflatoxins: In the early 1960s aflatoxins were discovered after the turkey "X" disease outbreak in England that caused the death of 100,000 turkey poults due to contaminated Brazilian peanut used as animal feed (Blount, 1961). In Italy, aflatoxins were detected in the early 2000s and since 2012 have spread in the southern part of European countries (Battilani et al., 2016).

Chemically, aflatoxins are difuranocoumarin derivatives synthetised through the polyketide pathway (Fig.1) and characterized by a polycyclic structure derived from a coumarin nucleus attached to a bifuran system. Furthermore, the solubility of aflatoxins is high in moderately polar solvent like chloroform, methanol and dimethylsulfoxide and low in water (Nakai et al., 2008; Turner et al., 2009).

Aflatoxin B_1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) are the main four aflatoxins, that are classified based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography (TLC) (Bennett and Klich, 2003). Additionally, two other compounds, aflatoxins M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are not related to cereals, but can be detected in milk derived from mammals fed on contaminated diets by AFB1 and AFB2 (Bennett and Klich, 2003; Wu et al., 2009). AFB1 is classified by International Agency for Research on Cancer as a Group 1 human carcinogen and is associated with hepatocellular carcinoma (HCC). Aflatoxins have been proven as genotoxic by their action

on the DNA and can cause a serious health threats including growth impairment, immune system suppression, hepatocellular carcinoma, acute toxicity, and death (K. C. Ehrlich et al., 2005; IARC, 2002).

Causal Agent: Aflatoxins are produced mainly by *Aspergillus* section *Flavi* (phylum: Ascomycota, order: Eurotiales). *Aspergillus flavus* Link and *A. parasiticus* Speare are the most important filamentous fungi commonly associated with aflatoxin production worldwide in different crops such as maize, peanuts, tree nuts, cottonseed, and other crops economically important (Cotty et al., 1994; Grubisha and Cotty, 2015)

Originally, *A. flavus* was identified as aflatoxin producer of B and G types (Codner et al., 1963). Recently, the strains of *A. flavus* were recognised as the only producing AFB1 and AFB2, unlike the strains of *A. parasiticus* have been validated as the main producer of B and G types (Varga et al., 2009; Amaike and Keller, 2011).

A. flavus is a facultative and opportunistic fungus in humans and animals, living as saprophyte using a wide range of organic substrates in the soil with worldwide spread; it is an ubiquitous fungus and can be present in air, soil, and water (Klich, 2007; Uka et al., 2020). Hence, the geographical study on the distribution of *A. flavus* showed a great predominance in Southeast Asia, the Middle East, Africa and Latin America; this fungus is well adapted to hot temperature and arid climate conditions.

Aflatoxins Pathway Gene Cluster: Ehrlich et al. (2005) identified 25 genes clustered within a 70-kb DNA region involved in aflatoxin biosynthesis. In addition, the aflatoxin gene cluster contains three pairs of sister genes (norB and norA; hypB1 and hypB2, and omtA and omtB). Among these genes are large ones of about 5 to 7 kb each, encoding the fatty acid synthase (FAS) alpha (5.8 kb) and beta (5.1 kb) subunits (FAS α and FASβ) and polyketide synthase (PKS; 6.6 kb), Excluding these three large genes, the average size of the other 22 genes is about 2 kb. The major genes involved from conversion steps to aflatoxins and their functions are in details below (Figure 3) (Yu et al., 2004).

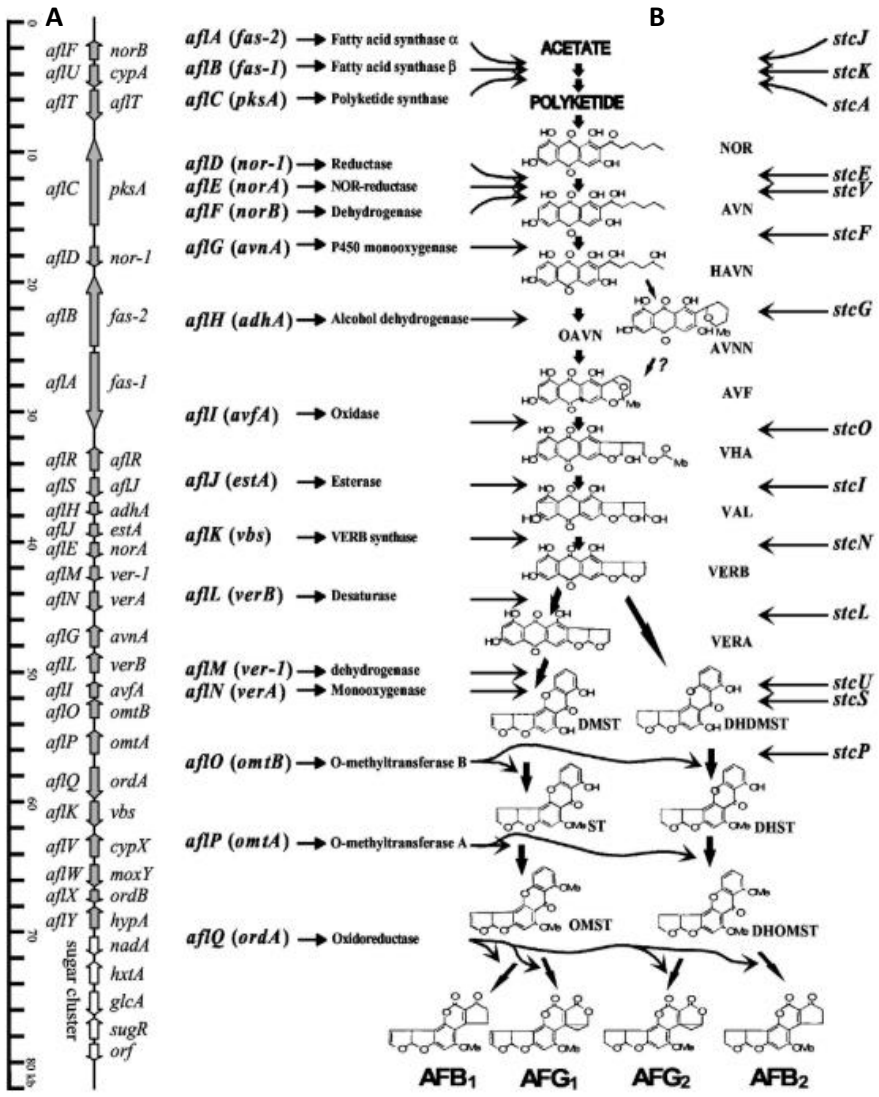

Figure 3. Clustered genes (A) and the aflatoxin biosynthetic pathway (B) (Yu et al. 2004)

Aflatoxin Regulations: several measures and regulations have been taken into consideration by the National and International authorities in over 100 countries to protect humans and animals' health from the possible health risk (Van Egmond et al., 2007). For instance, a legal maximum level (MLs) has been set by the EU for aflatoxins in food and feed. Particularly, the ML of AFB1 on maize for human consumption is 5 µg/kg, and 10 µg/kg considering the sum of aflatoxins (AFB1, AFB2, AFG1, and AFG2) according to commission regulation (EC) No 1881/2006 (European Commission (EC), 2006).

Ecology: In 2003, for the first time there was a significant occurrence of *A. flavus* in Italy due to the dry and hot summer during maize growing season that was leading to water stress in maize crops (Battilani et *al.*, 2005). The optimal growth of *A. flavus* is reported to be between a temperature of 19-35°C (Northolt and van Egmond, 1981). However, the temperature between 25 and 35°C induced a high production of aflatoxin with the optimum observed at 28 - 30°C (OBrian et al., 2007; Schmidt-Heydt et al., 2009). Temperature is the environmental factor that plays a crucial role in sporulation and aflatoxin production. Regarding the water activity (a_w), the study of Giorni et al. (2016) showed that a positive correlation between a_w and aflatoxin production exists when the $a_w \geq 0.95$, while aflatoxin production increases with decreasing a_w when it is lower than 95%. Additionally, the dynamic of the a_w through the growing season based on Degree Days (DD) was well described by the logistic regression by (Battilani et al., 2013).

Epidemiology: Soil is considered as reservoir for primary inoculum (Fig. 4), and plays an important role in the life cycle of *A. flavus* (Horn, 2003). Hence, contaminated plant residues contribute in maintaining the saprophytic stage of sclerotia, mycelium or conidia as overwinter bodies for the following season and when the conditions are favourable sclerotia germinate to ensure the primary infection after sporulation and conidia dispersal (Fig. 4) (Scheidegger and Payne, 2003).

The dispersion of conidia is carried by insects or wind to arrive on maize silks on which the fungus starts to colonize tissues (Fig. 4), followed by colonization of glumes, kernel and rarely the cob (Widstrom, 1996). Besides the external factors, tissue wounds caused by insects or birds are considered as an entry site for the fungus and ensuring the secondary infection (Payne, 1998; Cotty et al., 2008).

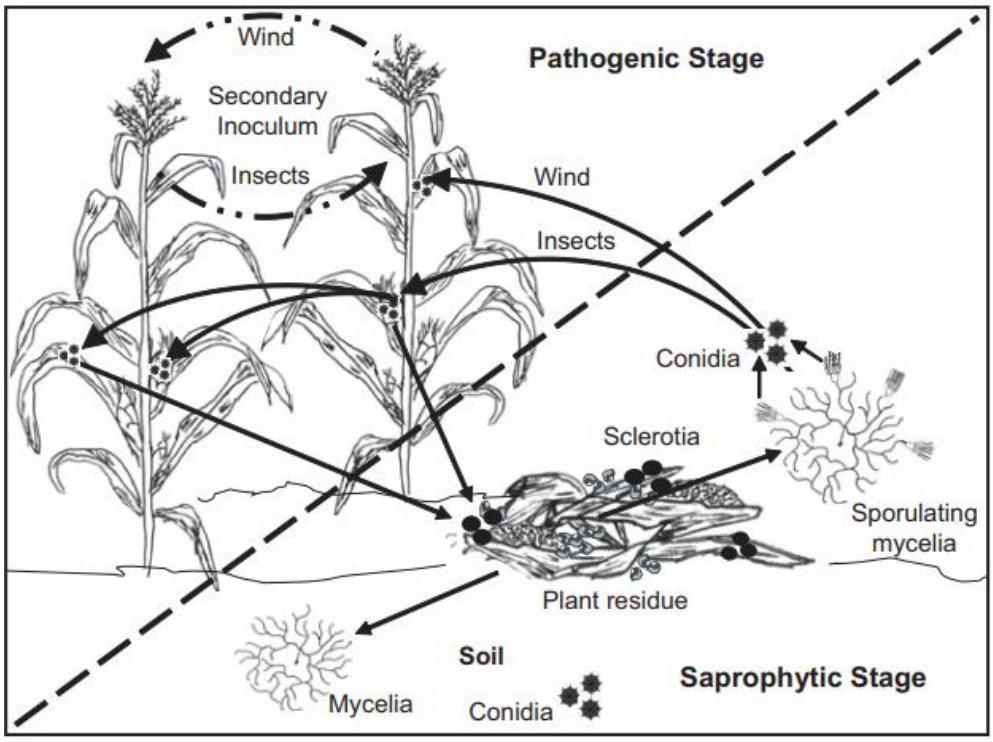

Figure 4. Schematic diagram showing the saprophytic and pathogenic stages during the life cycle of *A. flavus* (Abbas et al., 2009)

Morphological description: Traditionally, the classification of *Aspergillus* section *Flavi*, is based on morphological features, for instance the colonies of *A. flavus* are characterised by ivy green while for *A. parasiticus* cress green. Microscopic observations reveal that *A. flavus* and *A.parasiticus* can be differentiated by the lengths of conidiophore that are 500µm and 200 µm, respectively. Additionally, conidiophore for *A. flavus* has thinner walled and less roughened conidia than those of *A.parasiticus* (Raper and Fennel, 1965); Giorni et al., 2007). Sclerotia size is also a feature to describe the diversity in *A. flavus*, two morphotypes have been distinguished in this species based on sclerotia size as well as their aflatoxins production. The L strain (>400 µm in diameter) produce fewer, larger sclerotia. In contrast the S strain (generally <400 µm) produces numerous sclerotia and aflatoxin production is greater than in the L strain (Cotty, 1989; Cotty et al., 1994; Bock et al., 2004). *A. flavus* group S_{BG} (producing small size sclerotia and aflatoxins B and G) has been reported in Argentina and described as *A. minisclerotigenes* (Pildain et al., 2008). The S-type *A. flavus* is an important causal agent of aflatoxin contamination in several areas worldwide (Jaime-Garcia and Cotty, 2006; Probst et al., 2007). However, S_{BG} has a more limited distribution but is suspected to be an important causal agent of contamination and associated with

drier agroecological zones in Bènin also in semi-arid and sub-humid parts of West Africa (Cardwell and Cotty 2002; Probst et al., 2014).

***Aspergillus flavus* Reproduction:** *A. flavus* is well known as anamorph with dominance of asexual reproduction that only produce asexual spores and sclerotia as fruiting body to overwinter during the unfavourable season. However, the sexual form was reported *in vitro* and classified as *Petromyces flavus* (Horn et al., 2009). Additionally, the occurrence of the sexual recombination in sclerotia may happen in the field only in case sclerotia are incubated in the laboratory for 4 months which are allowed to be in contact with natural population of *A. flavus* (Horn et al., 2016). The frequency and the occurrence of sexual recombination within the natural population of *A. flavus* still not well known (Ojiambo et al. 2018; Moore 2021)

Vegetative Compatibility Group (VCG): the dominance of asexual reproduction limits the gene flow within the strains belonging to different Vegetative Compatibility Groups (VCGs) of *A. flavus*, due to the vegetative incompatibility which is widespread among the filamentous fungi (Papa, 1986) regulated by unlinked *vic* loci (Leslie, 1993). VCGs are a self-versus-non-self-recognition that have been described in different fungi. Thus, VCG form a stable vegetative heterokaryon through hyphal fusion between two isolates with *vic* loci, however hyphal fusion between two different VCGs does not occur and leads to programmed cell death in the zone of contact (Leslie, 1993; Glass and Kaneko, 2003; Glass and Dementhon, 2006).

Vegetative Compatibility Analysis: A VCG may contain few to many *A. flavus* members and these are believed to descend from the same clonal lineage (Papa, 1986). Genetic diversity of *A. flavus* population still not clear, therefore the study of Grubisha and Cotty, 2009 confirmed that isolates within a VCG are closely related and distinct from other VCGs.

Vegetative Compatibility Analysis (VCAs) allows assigning isolates to their corresponding. VCGs are defined through a complementation test between nitrate non-utilizing auxotrophs (*nit* mutants). Three different classes of mutant might be recovered e.g. NiaD (nitrate non-utilizing), nirA (nitrite and nitrate non-utilizing), and *cnx* (hypoxanthine and nitrate non-utilizing) (Bayman and Cotty, 1991; Papa, 1986).

Based on the result of VCA retrieved from previous works on *A. flavus* population, the frequency to obtain niaD is greater than cnx and nirA mutants (Pildain et al., 2004; Barros et al., 2006; Mauro

et al., 2013). cnx mutants are preferred to be part of a tester pair iof a VCG due to its strongest reactions with compatible niaD or nirA isolates (Bayman and Cotty, 1991). The variation in proportion of mutants type might be explained by the physical size of the gene involved in nitrate assimilation or the difference mutation susceptibility of some loci (Klittich and Leslie, 1988).

VCA is a tuseful to identify and study the diversity of *A. flavus* populations in different agro-ecosystem worldwide as well as in other fungal genera like *Fusarium*, *Neurospora*, and *Verticillium* (Bayman and Cotty, 1991; Leslie, 1993). Ortega-Beltran et al., (2018) identified 136 distinct VCGs as a result of a 3-year study of the population of *A. flavus* L morphotype associated with maize production in Sonora, Mexico. Mauro et al., (2013) identified 48 VCGs through the complementation test for *A. flavus* recovered from maize kernels from 5 maize growing areas of northern Italy between 2003 and 2010. However, Sweany et al., (2011) were able to identify 16 VCGs from 669 isolates of *A. flavus* from ears and soil in 11 Louisiana corn fields. Habibi and Banihashemi, (2008) identified 16 VCGs from 44 *A. flavus* isolates from sesame collected in Iran. Barros et al., (2006) identified 56 VCGs from 100 *A. flavus* isolates collected from soil in an Argentinian peanut-growing area. Forty-one, 9 and 15 VCGs were individuated from *A. flavus, A. parasiticus*, and *A. tamarii*, respectively (Horn and Greene, 1995). Bayman and Cotty, 1991 identified 13 VCGs from 61 isolates of *A. flavus* from soil as well as from cotton seeds in an Arizona cotton field and Papa, (1986) assigned 32 isolates from Georgia maize kernels in 22 VCGs. In addition, all the previously mentioned studies and the genetic studies showed no evidence of gene flow between VCGs, including VCGs of opposite mating-type. The data indicates that VCGs diverged before domestication of agricultural hosts (> 10,000 year before present) (Grubisha and Cotty, 2009).

All the members of a VCG share epidemiologic and physiologic features, including aflatoxin-producing ability and in some VCGs aflatoxins producer or not producer might coccure (Leslie, 1993; Mehl and Cotty, 2010; Mehl and Cotty, 2013). Identification and characterisation of *A. flavus* members of multiple VCGs, including investigatimg their aflatoxin producing abilities, that have been conducted over the years have led to develop new strategies to mitigate toxigenic strain occurrences and reduce the negative effect on food and feed safety by selecting candidates of the VCGs members that are unable to produce aflatoxins to be used as biocontrol agents (Cotty, 1989).

In addition, the displacement of one fungus by another was discovered when *A. flavus* has been co-inoculated with *A. niger* that caused a notable reduction of aflatoxin contamination (Wicklow et al., 1980). Ehrlich et al, (1985) found that the co-existence of *A. flavus* and *P. oxalicum* had a negative effect on aflatoxin production not only the concentration of aflatoxin was reduced but a metabolite of *P.oxalicum* was also reduced.

Discover of atoxigenic strains of *A.flavus* as a bicontrol agent: The use of atoxigenic strain of *A. flavus* as a biocontrol agent to mitigate aflatoxins concentration began in the late 1980s, when an atoxigenic *A. flavus* isolate from Arizona cottonseed inoculated into wounded cotton bolls in the greenhouse caused a notable reduction in aflatoxin concentration (Brown et al., 1991); this was confirmed by a field experiment when the atoxigenic strain of *A. flavus* was applied either simultaneously with, or one day prior to, a toxigenic strain during the preharvest period on maize. The application of an atoxigenic strain reduced from 80 to 90% of preharvest aflatoxin in kernels, which revealed its potential to be used as a biocontrol agent for aflatoxin mitigation (Brown et al., 1991).

Molecular analysis of aflatoxin biosynthesis cluster of the active ingredient AF36 showed a single mutation in the *pksA* (*aflC*) gene which induce a premature stop codon and make it inactive and inhibits the synthesis of any metabolite that interfere in the aflatoxin production (Ehrlich and Cotty, 2004). The study of Chang et al. (2005) showed a complete deletion of some or all the genes in the aflatoxin cluster in 38 atoxigenic strains of *A. flavus* collected from southern United States. Furthermore, Mauro et al. (2013) detected six deletion patterns of genes in the aflatoxin biosynthesis gene cluster on strains isolated form maize kernels from Northern Italy and 10 atoxigenic strains had the entire cluster deleted. Similar result have been observed in Serbia where the entire cluster was missing in an atoxigenic strain of *A. flavus* (Savic et al., 2020).

Biocontrol mechanism: Although the use of atoxigenic strains as a biocontrol agent had a positive impact to displace the native toxigenic strains, the mechanisms are not well understood (Geromy, 2021). Early studies of Cotty and Bayman, (1993) suggested competitive exclusion as a mechanism to displace the native aflatoxin producers through a physical degradation that led to reduction in aflatoxins concentration which differs from fungistatic, and anti-fungicidal mechanisms.

Competitive exclusion is the main components of the biocontrol strategies which guarantee an optimal efficacy and long-term modification to fungal population structures of *A. flavus* through displacement of toxigenic strains (Atehnkeng et al., 2008; Kinyungu et al. 2019).

Moreover, mechanism through physical contact or thigmoregulation has been suggested by (Huang et al., 2011), showing that aflatoxin inhibition required touching or close physical interaction between atoxigenic and toxigenic strains, and neither nutrient competition nor signal molecule released by the atoxigenic strain explain that inhibition. This hypothesis was tested using different filter insert pore sizes. The complete inhibition (>50%) occurred when 74 µm of pore size was used. This means that inhibition occurs when the toxigenic and atoxigenic isolates can contact each other or grow within the same compartment. Hence, in nature, toxigenic and atoxigenic strains can grow together in one corn kernel and toxin inhibition will occur if they come in contact. Similar results were also obtained by Rao et al., (2020)

Recently, the role of chemical compound secreted by the atoxigenic strain has been involved as a possible chemical mechanism to control aflatoxin production by the toxigenic strains. In fact, the extrolites which are uncharacterized metabolites, and the Volatile Organic Compound (VOCs) were investigated by Moore et al., (2019). Further investigation, demonstrated that aflatoxin production was greatly reduced as well as cyclopiazonic acid (CPA) in toxigenic strains used in the study of Moore et al.(2021) and due to the presence of VOCs, their Chemosensing might be one of the factors interfering in aflatoxin production by the atoxigenic strains. Non-aflatoxigenic VOCs (3-octanone, trans-2-methyl-2- butenal and 2,3- dihydrofuran and decane) significantly reduced aflatoxins in all aflatoxigenic strains.

Besides and all the previous study demonstrated many possible ways of how the aflatoxins production overcome by the atoxigenic *A. flavus*, better understanding the different mechanisms by which the atoxigenic candidates for a biocontrol use to mitigate aflatoxins contamination, should be taken into consideration to improve the effectiveness of biocontrol strategy for long-term use.

The history of using atoxigenic strains of *A. flavus*: The use of the native atoxigenic strains of *A. flavus* was reported for the first time in Yuma valley of Arizona where the isolate AF36 was recovered from cotton seed field belonging to VCG YV36 (Grubisha and Cotty, 2015). The results validated AF36 as a biocontrol candidate because of its potential as a biocompetitor with the ability

to reduce the aflatoxin production by toxigenic strains (Cotty 1989; Cotty, 1990). Furthermore, at the same period NRRL 21882 was another atoxigenic biocompetitive agent of *A. flavus* recovered from Georgia peanut, also showed a significant potential to reduce aflatoxin in laboratory tests (Dorner et al., 1992). Field trials of AF36 and NRLL21882 were conducted throughout the 1990's and by the early 2002's Environmental Protection Agency (EPA) approved their use as pre-harvest biopesticide (USEPA, 2003, 2004).

AF36 prevail® is the first biocontrol product developed by using the native atoxigenic strain of *A. flavus* AF36 authorised to be distributed and commercialised in the US. AF36 prevail® is now used in different economic crops such as cotton, maize, pistachio, almond and fig (Ortega-Beltran et al., 2019; Moral et al., 2020). Another successful biocontrol product named Afla-Guard® formulated with the NRLL21882 strain as an active ingredient and registered for use in peanut and maize grow in the US. Th application of Afla-Guard® also resulted in major reduction of aflatoxins during the storage period (Dorner and Lamb, 2006)

Biocontrol technologies have been improved over the years, moving from using single strain to multiple native strains belonging to different VCGs thought to increase the efficacy. Four strains biocontrol has been implemented in different African countries, Aflasafe™ manufactured and commercialised by the International Institute of Tropical Agriculture (IITA) in Ibadan Nigeria and approved by the authorities in charge of biopesticides in African nations on maize and groundnut (Bandyopadhyay et al., 2016). Aflasafe products belongs to the new biocontrol generation by using four distinctives atoxigenic VCGs native to a given agroecosystem (Probst and Bandyopadhyay 2011). Field trials conducted in hundreds of farms in diverse environments for many years show a reduction of 75 to 100% of aflatoxin concentration (Atehnkeng et al., 2008; Bandyopadhyay et al., 2016). Similar, FouSure™ is the first biocontrol product formulated with 4 native atoxigenic strains of *A. flvaus* in Texas and has received the USEPA Experimental Use Permit for use on maize (Shenge et al., 2017). Nowadays, the field trials of Alfasafe™ are ongoing by developing and registering new active ingredient to be use as an active ingrideint under the trade name Aflasafe to extend the geographical area of biocontrol product to cover the need of farmers and protect human health in many different African nations (Atehnkeng et al., 2014 ; Agbetiameh et al., 2020). Using multiple atoxigenic strains in one product is a challenging technology that might provide a long-term stability and efficiency (Mehl et al., 2012).

All the previous successful works mentioned above were encouraging for developing other atoxigenic biocontrol products through population characterization. At the European level, significant efforts were devoted to developing new strategies to reduce aflatoxin concentration particularly after the outbreak in 2003 in Italy with a significant risk in maize. Giorni et al. (2007), started to investigate *Aspergillus* section *Flavi* fungi in six northern Italian regions to obtain useful information regarding the relative role of the key species, ability to produce sclerotia and production of the main toxic secondary metabolites, aflatoxin and CPA. In addition the study of Mauro et al., (2013), demonstrated that 46% *A. flavus* were unable to produce detectable aflatoxin and belonging to 25 atoxigenic VCGs. The incidence of VCGs IT4, IT6 and IT18 are dominant in the soil and they are widely distributed. VCGs IT4, IT6 and IT18 are well adapted in the Italian maize production area and the selected strains are potentially good candidate for aflatoxin biocontrol.

Moreover, field trials have been conducted and demonstrated reduction of AFB1 greater than 90% in two successive years of survey only with one atoxigenic isolate, A2085, that was deposited under provisions of the Budapest Treaty in the Belgian Co-Ordinated Collections of Micro-Organisms (BCCM/MUCL; accession MUCL54911). AF-X1™ is the name of the commercial product authorized to be applied in maize in Italy; further work to obtain the full approval at European level is still ongoing (Mauro et al., 2018).

Recently, a new biocontrol product has been developed and reported a great reduction of aflatoxins in maize in Serbia by using for the first time the native atoxigenic of *A. flavus* named Mytoolbox Af01 and characterized by a partial-cluster deletion from *aflT* to *aflN*. Field trials managed between 2016 and 2017 in irrigated and non-irrigated maize field showed a high efficacy of the product to reduce aflatoxin contamination up to 73% (Savic et al., 2020).

Biocontrol Development: Use of the native atoxigenic strains of *A. flavus* to target agroecosystem area is a primordial criterion to start the process of biocontrol product development because are well adapted to the environment, cropping system and climate and soil condition. In addition, native atoxigenic candidates have a great ability to compete against other microorganisms for local resources which allow to have a fast approval registration unlike exotic fungi (Mehl et al., 2012). This process composes by the following steps such as morphological identification of the isolates

(Fig. 5), test their inability to produce toxins particularly aflatoxins, and assessing their potential to outcompete aflatoxin producer under controlled and field conditions (Moral et al., 2020).

Another criterion of atoxigenic candidates' selection is their ability to move from the soil to the grain and other harvested crop in the neighboring treated field (Fig. 5) (Agbetiameh et al., 2019). Field trials allowed to select the most efficient candidates and evaluate their ability to reduce aflatoxin contamination. Monitoring the efficacy of the biocontrol active ingredient should be done in multiple fields and in multiple agroecological areas in several years (Senghor et al., 2019). Evaluating a biocontrol product in limited number of fields and locations may not reveal the true value of the product (Ortega-Beltran and Bandyopadhyay, 2019; Weaver et al., 2019).

Biocontrol registration: Registration process by specific authority must always be done before manufacturing, and after submitting the required parameters on the efficacy, safety, quality and social benefits (Bandyopadhyay et al., 2016). Aflatoxin biocontrol products like other agricultural input, should be produced in large quantity for commercial field use. All the commercialized biocontrol products to mitigate aflatoxins are grain based. Using grain-based formulation provide many advantages to the farmers including easy in the application and slow release of active ingredients in the environment for a long period, sustaining competitive displacement of toxigenic strains (Bandyopadhyay et al., 2021).

Biocontrol manufacturing: In the beginning the product was manufactured by using laboratory scale method by USDA-ARS and ACRPC (Cotty, 2007). This method required several workers to produce around 300 kg of product per week. The process involved sterilization of sorghum grains in an autoclave, soaking the autoclaved grains in a spore suspension, incubating the inoculated grains for 18 h at 31°C, rapid drying of the grains before sporulation begins, and bagging the product (Atehnkeng et al., 2014).

Recently, manufacturing strategies of atoxigenic based biocontrol have been optimized, by using dry spores formulation developed by Ortega-Beltran et al, (2021), and the result of 2 years of survey showed a reduction in aflatoxins contamination below 4 ppb in groundnut and maize in Gambia and Senegal with more than 770 fields tested.

Figure 5 Illustration of the process to select atoxigenic isolates of *Aspergillus flavus* to develop aflatoxin biocontrol products (Moral et al., 2020)

Challenges and future perspectives of biocontrol use in aflatoxin mitigation: Many years of trials of biocontrol to manage aflatoxin contamination revealed this is the most effective tool in different crops worldwide and the advantages largely outweigh the eventual disadvantages. The positive impact is that the atoxigenic strains of *A. flavus* can benefit treated and other plants for several years (Khan et al., 2021). Ecological wise the deployment of atoxigenic genotype is stable in the environment, the carry-over studies demonstrated the persistence if the applied atoxigenic genotype as a biocontrol in the field over the year after its application (Atehnkeng et al., 2022; Weaver andAbbas, 2019). Also the movement of the active ingredient from the treated field to untreated fields through wind or insect dispersal (Horn, 2003; Weaver and Abbas, 2019) thus can provide a protection from aflatoxin contamination in the crop for the following season as well as the neighboring fields as demonstrated in large scale fields trial in Nigeria (Ola et al., 2022).

Despites all the advantages, the use of atoxigenic strains of *A. flavus* as a biocontrol have been faced many challenges such as sociological, economic, regulatory, institutional, policy related and technical in nature (Bandyopadhyay et al., 2022). Furthermore, climate change is being the main threat for food and feed security and safety in many regions all over the world, and aflatoxins are

playing a key role due to the hot and dry weather driven by the climate change (Camardo Leggieri 2015; Battilani et al., 2016).

Perhaps, still more work has to be done to incorporate the biocontrol technologies for the aflatoxin management particularly in the areas where the climate change has a notable negative effect (Bandyopadhyay et al., 2016). Monitoring the variation of the meteorological data in aflatoxin management may aid to select atoxigenic strains most adapted to the hot and dry weather and resistent to climate change events and cropping cycle (Cotty and Jaime-Garcia, 2007). Moreover, the perspective of Moral et al. (2020) suggested developing a simulation model that predict future of toxigenic and atoxigenic of *A. flavus* population scenarios to assist farmers and decision makers through Decision Support System (DSS). A mechanistic predictive model is available (Battilani et al., 2013), it should be improved in account of the interaction between toxigenic and atoxigenic strains.

Objectives:

Aflatoxins are the most toxic metabolites naturally occurring worldwide in different important crops and commodities. *Aspergillus flavus* is the main aflatoxin producer in particular AFB1 which is classified in group one, carcinogenic to humans and animals, by IARC. Since 2003, aflatoxins has become serious concern for many European countries, in particular the Southern part of Europe.

The use of atoxigenic strains as a biocontrol tool showed a high effectiveness to reduce AFs contamination. AF-X1 is the first atoxigenic based biocontrol product using *A. flavus* strain MUCL54911, belonging to the VCG IT006, endemic to Italy and is available for farmers and available to be distributed in the Italian maize commercial fields with a temporary authorisation.

Investigating the carryover data of the atoxigenic based product AF-X1 and its multiyear influence on the fungal community's resident in the Italian maize growing areas, and the Southern European population structure of *Aspergillus flavus* and how widely distributed is the VCG IT006 of AF-X1, together have long been a question of great interest in the development and registration of the biocontrol product for aflatoxins mitigation.

The current PhD research project was conducted in partnership with Corteva Agriscience to provide new insights on AF-X1 that support the advocacy and registration process in Southern Europe.

The main objectives of this Research project were:

A. Evaluate the long-term persistence of the active ingredient of AF-X1 in the northern Italian maize growing areas and the multiyear influence of commercial applications of AF-X1 on the structure of *A. flavus* and other fungal communities' resident in the soil; **(Chapter2)**
B. Characterize *A. flavus* population structure and genetic diversity in southern European countries, Greece, Spain and Serbia, and evaluate the distribution of the VCG IT006 to extend the geographical application of AF-X1; **(Chapter 3)**
C. Study the long-term efficacy of the pre-harvest treatment of AF-X1 in preventing AF production in the Italian maize during the storage period; **(Chapter 4)**

Thesis Outlines

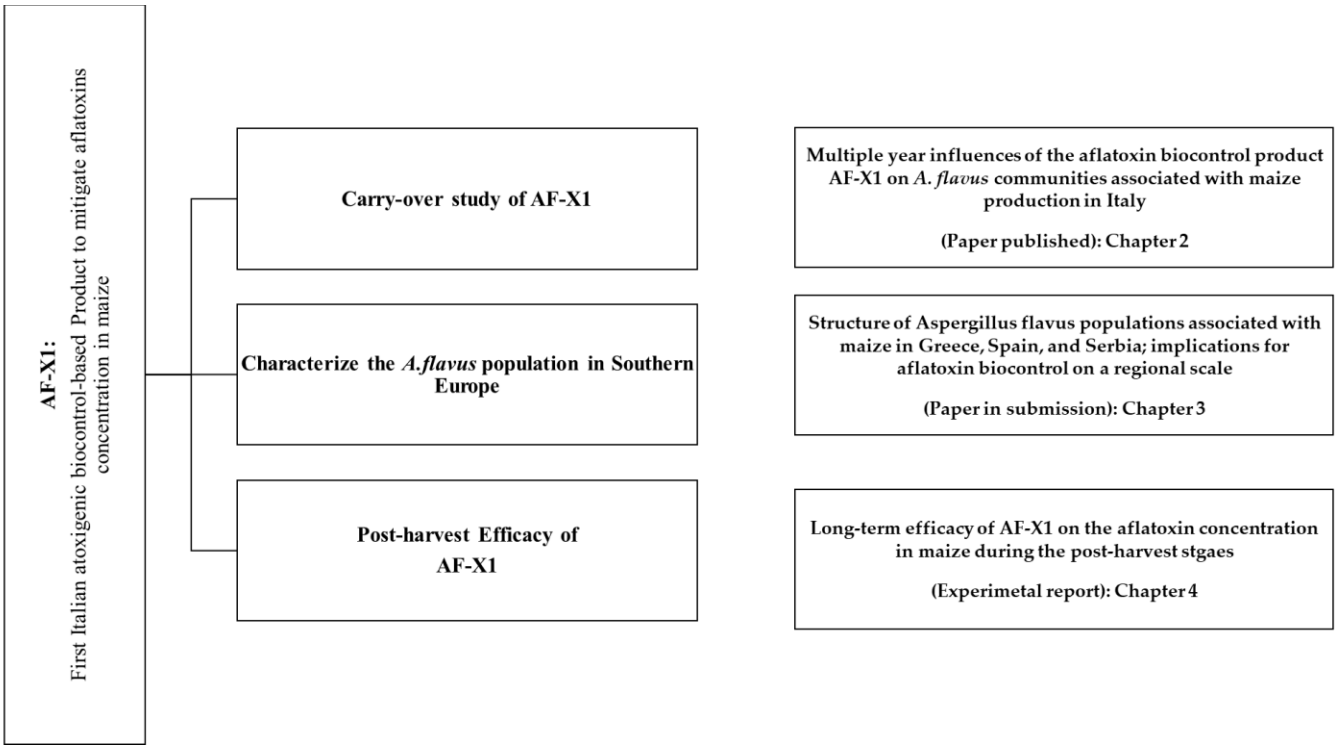

Figure 6: the main outlines of the current PhD research project: the flow shows the structure of the thesis dissertation with different chapters.

Reference

1. Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A., and Weaver, M. A. (2009). Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Reviews, 28(2–3), 142–153. https://doi.org/10.1080/15569540903081590
2. Agbetiameh, D, Ortega-Beltran, A., Awuah, R., Atehnkeng, J., Elzein, A., Cotty, P., and Bandyopadhyay, R. (2020). Field efficacy of two atoxigenic biocontrol products for mitigation of aflatoxin contamination in maize and groundnut in Ghana. Biological Control, 150(June), 104351. https://doi.org/10.1016/j.biocontrol.2020.104351
3. Agbetiameh, Daniel, Ortega-Beltran, A., Awuah, R. T., Atehnkeng, J., Islam, M. S., Callicott, K. a., Cotty, P. J., and Bandyopadhyay, R. (2019). Potential of Atoxigenic *Aspergillus flavus* Vegetative Compatibility Groups Associated With Maize and Groundnut in Ghana as Biocontrol Agents for Aflatoxin Management. Frontiers in Microbiology, 10(September). https://doi.org/10.3389/fmicb.2019.02069
4. Amaike, S., and Keller, N. P. (2011). *Aspergillus flavus* . Annual Review of Phytopathology, 49(1), 107–133. https://doi.org/10.1146/annurev-phyto-072910-095221
5. Atehnkeng, J., Ojiambo, P., Ortega-Beltran, A., Augusto, J., Cotty, P., and Bandyopadhyay, R. (2022). Impact of frequency of application on the long-term efficacy of the biocontrol product Aflasafe in reducing aflatoxin contamination in maize. Frontiers in Microbiology, November, 1–14. https://doi.org/10.3389/fmicb.2022.1049013
6. Atehnkeng, J., Ojiambo, P. S., Cotty, P. J., and Bandyopadhyay, R. (2014). Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link: FR vegetative compatibility groups in preventing aflatoxin contamination in maize (Zea mays L.). Biological Control, 72, 62–70. https://doi.org/10.1016/j.biocontrol.2014.02.009
7. Atehnkeng, J., Ojiambo, P. S., Ikotun, T., Sikora, R. a., Cotty, P. J., and Bandyopadhyay, R. (2008a). Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25(10), 1264–1271. https://doi.org/10.1080/02652030802112635

8. Atehnkeng, J., Ojiambo, P. S., Ikotun, T., Sikora, R. A., Cotty, P. J., and Bandyopadhyay, R. (2008b). Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25(10), 1264–1271. https://doi.org/10.1080/02652030802112635
9. Bandyopadhyay, R., Ortega-Beltran, a., Akande, a., Mutegi, C., Atehnkeng, J., Kaptoge, L., Senghor, a. L., Adhikari, B. N., and Cotty, P. J. (2016). Biological control of aflatoxins in Africa: Current status and potential challenges in the face of climate change. World Mycotoxin Journal, 9(5), 771–789. https://doi.org/10.3920/WMJ2016.2130
10. Bandyopadhyay, Ranajit, Ortega-Beltran, A., Konlambigue, M., Kaptoge, L., Falade, T. D. O., and Cotty, P. J. (2022). Development and scale-up of bioprotectants to keep staple foods safe from aflatoxin contamination in Africa. In J. Kohl, J. and Ravensverg, W (Ed.), Microbial bioprotectants for plant disease management (Issue November, pp. 587–628). https://doi.org/10.19103/as.2021.0093.16
11. Barros, G. G., Torres, A. M., Rodriguez, M. I., and Chulze, S. N. (2006). Genetic diversity within Aspergillus flavus strains isolated from peanut-cropped soils in Argentina. Soil Biology and Biochemistry, 38(1), 145–152. https://doi.org/10.1016/j.soilbio.2005.04.028
12. Battilani, P., Camardo Leggieri, M., Rossi, V., and Giorni, P. (2013). AFLA-maize, a mechanistic model for Aspergillus flavus infection and aflatoxin B1 contamination in maize. Computers and Electronics in Agriculture, 94, 38–46. https://doi.org/10.1016/j.compag.2013.03.005
13. Battilani, P., Toscano, P., Van Der Fels-Klerx, H. J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T., and Robinson, T. (2016). Aflatoxin B 1 contamination in maize in Europe increases due to climate change. Scientific Reports, 6(April), 1–7. https://doi.org/10.1038/srep24328
14. Battilani, P., Scandolara, A., Barbano, C., Pietri, A., Bertuzzi, T., Marocco, A., Berardo, N., Vannozzi, G.P., Baldini, M., Miele, S., Salera, E., Maggiore, T. (2005). Monitoraggio della contaminatinazione da micotossine nel mais. L'informatore agrario, 61, 47-49.
15. Bayman, P., and Cotty, P. J. (1991). Vegetative compatibility and genetic diversity in the Aspergillus flavus population of a single field. Canadian Journal of Botany, 69(8), 1707–1711. https://doi.org/10.1139/b91-216

16. Bayman, Paul, and Cotty, P. J. (1991). Improved Media for Selecting Nitrate-Nonutilizing Mutants in Aspergillus Flavus . Mycologia, 83(3), 311–316. https://doi.org/10.1080/00275514.1991.12026015
17. Bennett, J. W. K. M. (2003). Mycotoxins. Clin Microbiol Rev, 16, 497–516.
18. Bock, C. H., Mackey, B., and Cotty, P. J. (2004). Population dynamics of Aspergillus flavus in the air of an intensively cultivated region of south-west Arizona. Plant Pathology, 53(4), 422–433. https://doi.org/10.1111/j.0032-0862.2004.01015.x
19. Brown, R. L., Cotty, P. J., and Cleveland, T. E. (1991). Reduction in aflatoxin content of maize by atoxigenic strains of Aspergillus flavus. Journal of Food Protection, 54(8), 623–626. https://doi.org/10.4315/0362-028X-54.8.623
20. Chang, P., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the a X atoxin biosynthesis gene cluster and X anking regions in nona X atoxigenic Aspergillus X avus isolates. 42, 914–923. https://doi.org/10.1016/j.fgb.2005.07.004
21. Chang, P. K., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic Aspergillus flavus isolates. Fungal Genetics and Biology, 42(11), 914–923. https://doi.org/10.1016/j.fgb.2005.07.004
22. Codner, R. C., Sargeant, K., and Yeo, R. (1963). Production of aflatoxin by the culture of strains of Aspergillus flavus-oryzae on sterilized peanuts. Biotechnology and Bioengineering, 5(3), 185–192. https://doi.org/10.1002/bit.260050303
23. Cotty, P. J., Bayman, D. S., Egel, D. S., and Elias, K. S. (1994). AGRICULTURE , AFLATOXINS AND ASPERGILLUS. In K. Powell (Ed.), The Genus Aspergillus (pp. 1–2). Plenum Press.
24. Cotty, P. J., Probst, C., and Jaime-Garcia, R. (2008). Etiology and management of aflatoxin contamination. In and A. V. J. F. Leslie, R. Bandyopadhyay (Ed.), Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade (pp. 287–299). CAB International.
25. Cotty, P.J., Antilla, L. And Wakelyn, P.J. (2007). Competitive exclusion of aflatoxin producers: Farmer-driven research and development. Biological Control: A Global Perspective, 241–253.
26. Cotty and BAyman. (1993). Competitive Exculusion of a Toxigenic Strain of Aspergillus

flavus by and Atoxigenic Strain. Postharvest Pathology and Mycotoxins.

27. Cotty, P. (1989). Virulence and cultural characteristics of two Aspergillus flavus strains pathogenic on cotton. Phytopathology, 79, 808–814.
28. Cotty, P. J., and Jaime-Garcia, R. (2007). Influences of climate on aflatoxin producing fungi and aflatoxin contamination. International Journal of Food Microbiology, 119(1–2), 109–115. https://doi.org/10.1016/j.ijfoodmicro.2007.07.060
29. Cove, D. J. (1976). Chlorate toxicity in Aspergillus nidulans. Molecular and General Genetics MGG, 146(2), 147–159. https://doi.org/10.1007/bf00268083
30. Diaw, Y., Tollon-Cordet, C., Charcosset, A., Nicolas, S. D., Madur, D., Ronfort, J., David, J., and Gouesnard, B. (2021). Genetic diversity of maize landraces from the South-West of France. PLoS ONE, 16(2 February), 1–21. https://doi.org/10.1371/journal.pone.0238334
31. Dorner, J. W., and Lamb, M. C. (2006). Development and commercial use of afla-guard®, an aflatoxin biocontrol agent. Mycotoxin Research, 22(1), 33–38. https://doi.org/10.1007/BF02954555
32. Dorner, Joe W., Cole, R. J., and Blankenship, P. D. (1992). Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. Journal of Food Protection, 55(11), 888–892. https://doi.org/10.4315/0362-028X-55.11.888
33. Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of Aspergillus flavus used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. Applied Microbiology and Biotechnology, 65(4), 473–478. https://doi.org/10.1007/s00253-004-1670-y
34. Ehrlich, K. C., Yu, J., and Cotty, P. J. (2005). Aflatoxin biosynthesis gene clusters and flanking regions. Journal of Applied Microbiology, 99(3), 518–527. https://doi.org/10.1111/j.1365-2672.2005.02637.x
35. Ehrlich, K., Ciegler, A., Klich, M., and Lee, L. (1985). Fungal competition and mycotoxin production on corn. Experientia, 41(5), 691–693. https://doi.org/10.1007/BF02007725
36. Erenstein, O. (2010). The evolving maize sector in Asia: Challenges and opportunities. Journal of New Seeds, 11(1), 1–15. https://doi.org/10.1080/15228860903517770
37. Erenstein, O., Jaleta, M., Sonder, K., Mottaleb, K., and Prasanna, B. M. (2022). Global maize production, consumption and trade: trends and Rand D implications. Food Security,

0123456789. https://doi.org/10.1007/s12571-022-01288-7

38. Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., and Krska, R. (2020). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%. Critical Reviews in Food Science and Nutrition, 60(16), 2773–2789. https://doi.org/10.1080/10408398.2019.1658570
39. European Commission (EC). (2006). Commission Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union, L364, 5-24.
40. Eurostat. (2022). Eurostat. https://doi.org/https://ec.europa.eu/eurostat/databrowser/explore/all/all_themes
41. Food and Agriculture Organization of the United Nations (FAO). (1992). Maize in human nutrition.
42. Giorni, P., Bertuzzi, T., and Battilani, P. (2016). Aflatoxin in maize, a multifaceted answer of Aspergillus flavus governed by weather, host-plant and competitor fungi. Journal of Cereal Science, 70, 256–262. https://doi.org/10.1016/j.jcs.2016.07.004
43. Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., and Battilani, P. (2007). Studies on Aspergillus section Flavi isolated from maize in northern Italy. International Journal of Food Microbiology, 113(3), 330–338. https://doi.org/10.1016/j.ijfoodmicro.2006.09.007
44. Grubisha, L. C., and Cotty, P. J. (2009). Twenty-four microsatellite markers for the aflatoxin-producing fungus Aspergillus flavus. Molecular Ecology Resources, 9(1), 264–267. https://doi.org/10.1111/j.1755-0998.2008.02378.x
45. Grubisha, Lisa C., and Cotty, P. J. (2015). Genetic analysis of the Aspergillus flavus vegetative compatibility group to which a biological control agent that limits aflatoxin contamination in U.S. crops belongs. Applied and Environmental Microbiology, 81(17), 5889–5899. https://doi.org/10.1128/AEM.00738-15
46. Habibi, A., and Banihashemi, Z. (2008). Vegetative compatibility groups among isolates of Aspergillus flavus from sesame seed in Iran and mycotoxin production. Phytopathologia Mediterranea, 47(1), 28–34. https://doi.org/10.14601/Phytopathol_Mediterr-2540
47. Horn, B., and Greene, R. (1995). Vegetative Compatibility within Populations of Aspergillus flavus, A. parasiticus, and A. tamarii from a Peanut Field. Mycologia, 87(3), 324–332. https://doi.org/10.1080/00275514.1995.12026537
48. Horn, B. W. (2003). Ecology and population biology of aflatoxigenic fungi in soil. Journal

of Toxicology - Toxin Reviews, 22(2–3), 351–379. https://doi.org/10.1081/TXR-120024098

49. Horn, B. W., Gell, R. M., Singh, R., Sorensen, R. B., and Carbone, I. (2016). Sexual reproduction in aspergillus flavus sclerotia: Acquisition of novel alleles from soil populations and uniparental mitochondrial inheritance. PLoS ONE, 11(1), 1–22. https://doi.org/10.1371/journal.pone.0146169
50. Horn, B. W., Moore, G. G., and Carolina, N. (2009). Sexual reproduction in Aspergillus flavus. 101(3), 423–429. https://doi.org/10.3852/09-011
51. Huang, C., Jha, A., Sweany, R., DeRobertis, C., and Damann, K. E. (2011). Intraspecific aflatoxin inhibition in Aspergillus flavus is thigmoregulated, independent of vegetative compatibility group and is strain dependent. PLoS ONE, 6(8). https://doi.org/10.1371/journal.pone.0023470
52. IARC. (2002). No Title. IARC SUMMARIES AND EVALUATIONS. Aflatoxins, 82–171.
53. Instituto nazionale di statistica (Istat). (2022). No Title. https://doi.org/www.istat.it
54. Jaime-Garcia, R., and Cotty, P. J. (2006). Spatial distribution of Aspergillus flavus and its toxigenic strains on commercial cottonseed from south Texas and its relationship to aflatoxin contamination. Plant Pathology, 55(3), 358–366. https://doi.org/10.1111/j.1365-3059.2006.01358.x
55. Khan, R., Ghazali, F. M., Mahyudin, N. A., and Samsudin, N. I. P. (2021). Aflatoxin biosynthesis, genetic regulation, toxicity, and control strategies: A review. Journal of Fungi, 7(8). https://doi.org/10.3390/jof7080606
56. Klich, M. A. (2007). Aspergillus flavus: The major producer of aflatoxin. Molecular Plant Pathology, 8(6), 713–722. https://doi.org/10.1111/j.1364-3703.2007.00436.x
57. Klittich, C., and Leslie, J. F. (1988). Nitrate reduction mutants of fusarium moniliforme (gibberella fujikuroi). Genetics, 118(3), 417–423. https://doi.org/10.1093/genetics/118.3.417
58. Leslie, J. F. (1993). Fungal Vegetative Compatibility. Annual Review of Phytopathology, 31(1), 127–150. https://doi.org/10.1146/annurev.py.31.090193.001015
59. Mauro, A., Battilani, P., Callicott, K. A., Giorni, P., Pietri, A., and Cotty, P. J. (2013). International Journal of Food Microbiology Structure of an Aspergillus fl avus population

from maize kernels in northern Italy. International Journal of Food Microbiology, 162(1), 1–7. https://doi.org/10.1016/j.ijfoodmicro.2012.12.021

60. Mauro, A., Garcia-cela, E., Pietri, A., Cotty, P. J., and Battilani, P. (2018). Biological Control Products for Aflatoxin Prevention in Italy : Commercial Field Evaluation of Atoxigenic Aspergillus flavus Active Ingredients. Toxins, 1–14. https://doi.org/10.3390/toxins10010030
61. Mehl, H. L., and Cotty, P. J. (2010). Variation in competitive ability among isolates of aspergillus flavus from different vegetative compatibility groups during maize infection. Phytopathology, 100(2), 150–159. https://doi.org/10.1094/PHYTO-100-2-0150
62. Mehl, Hillary L., and Cotty, P. J. (2013). Nutrient environments influence competition among aspergillus flavus genotypes. Applied and Environmental Microbiology, 79(5), 1473–1480. https://doi.org/10.1128/AEM.02970-12
63. Mehl, Hillary L., Jaime, R., Callicott, K. a., Probst, C., Garber, N. P., Ortega-Beltran, A., Grubisha, L. C., and Cotty, P. J. (2012). Aspergillus flavus diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. Annals of the New York Academy of Sciences, 1273(1), 7–17. https://doi.org/10.1111/j.1749-6632.2012.06800.x
64. Meier, U. (2018). Growth stage of mono- and dicoyle-donous plants. BBCH Monograph. In Uwe Meier (Ed.), Growth stage of mono- and dicoyle-donous plants. BBCH Monograph. Julius Kuhn-Institute (JKI). https://doi.org/10.5073/20180906-074619
65. Moore, G. G., Lebar, M. D., and Carter-Wientjes, C. H. (2019). The role of extrolites secreted by nonaflatoxigenic Aspergillus flavus in biocontrol efficacy. Journal of Applied Microbiology, 126(4), 1257–1264. https://doi.org/10.1111/jam.14175
66. Moore, Geromy G. (2021). Practical considerations will ensure the continued success of pre-harvest biocontrol using non-aflatoxigenic Aspergillus flavus strains. Critical Reviews in Food Science and Nutrition, 0(0), 1–18. https://doi.org/10.1080/10408398.2021.1873731
67. Moore, Geromy G., Lebar, M. D., Carter-Wientjes, C. H., and Gilbert, M. K. (2021). The potential role of fungal volatile organic compounds in Aspergillus flavus biocontrol efficacy. Biological Control, 160(October 2020), 104686. https://doi.org/10.1016/j.biocontrol.2021.104686

68. Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., and Ortega-Beltran, A. (2020a). Present status and perspective on the future use of aflatoxin biocontrol products. Agronomy, 10(4), 1–19. https://doi.org/10.3390/agronomy10040491
69. Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., and Ortega-Beltran, A. (2020b). Present status and perspective on the future use of aflatoxin biocontrol products. Agronomy, 10(4). https://doi.org/10.3390/agronomy10040491
70. Moretti, A., Logrieco, A. F., and Susca, A. (2017). Mycotoxins: An underhand food problem. Methods in Molecular Biology, 1542(58), 3–12. https://doi.org/10.1007/978-1-4939-6707-0_1
71. Nakai, V. K., de Oliveira Rocha, L., Gonçalez, E., Fonseca, H., Ortega, E. M. M., and Corrêa, B. (2008). Distribution of fungi and aflatoxins in a stored peanut variety. Food Chemistry, 106(1), 285–290. https://doi.org/10.1016/j.foodchem.2007.05.087
72. Northolt, M., and van Egmond, H. (1981). No Title. In D. E. Pepin, G. A., Patterson, D. S. P., and Gray (Ed.), Fourth Meeting on Mycotoxins in Animal Disease (pp. 106–108). Ministry of Agriculture, Fisheries and Food.
73. Note, T. (2001). Indices of multilocus linkage disequilibrium. 101–102.
74. OBrian, G. R., Georgianna, D. R., Wilkinson, J. R., Yu, J., Abbas, H. K., Bhatnagar, D., Cleveland, T. E., Nierman, W., and Payne, G. A. (2007). The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. Mycologia, 99(2), 232–239. https://doi.org/10.1080/15572536.2007.11832583
75. Ojiambo, P. S., Battilani, P., Cary, J. W., Blum, B. H., and Carbone, I. (2018). Cultural and genetic approaches to manage aflatoxin contamination: Recent insights provide opportunities for improved control. Phytopathology, 108(9), 1024–1037. https://doi.org/10.1094/PHYTO-04-18-0134-RVW
76. Ola, O. T., Ogedengbe, O. O., Raji, T. M., Eze, B., Chama, M., Ilori, O. N., Awofisayo, M. A., Kaptoge, L., Bandyopadhyay, R., Ortega-Beltran, A., and Ndarubu, A. A. (2022). Aflatoxin biocontrol effectiveness in the real world—Private sector-led efforts to manage aflatoxins in Nigeria through biocontrol-centered strategies. Frontiers in Microbiology, 13(September), 1–10. https://doi.org/10.3389/fmicb.2022.977789

77. Orozco-Ramírez, Q., Perales, H., and Hijmans, R. J. (2017). Geographical distribution and diversity of maize (Zea mays L. subsp. mays) races in Mexico. Genetic Resources and Crop Evolution, 64(5), 855–865. https://doi.org/10.1007/s10722-016-0405-0
78. Ortega-Beltran, A., and Bandyopadhyay, R. (2019). Comments on "trial summary on the comparison of various non-aflatoxigenic strains of aspergillus flavus on mycotoxin levels and yield in maize" by M.S. molo, et al. Agron. J. 111:942–946 (2019). Agronomy Journal, 111(5), 2625–2629. https://doi.org/10.2134/agronj2019.04.0281
79. Ortega-Beltran, A., Kaptoge, L., Senghor, A. L., Aikore, M. O. S., Jarju, P., Momanyi, H., Konlambigue, M., Falade, T. D. O., and Bandyopadhyay, R. (2021). Can it be all more simple? Manufacturing aflatoxin biocontrol products using dry spores of atoxigenic isolates of Aspergillus flavus as active ingredients. Microbial Biotechnology, March. https://doi.org/10.1111/1751-7915.13802
80. Ortega-Beltran, A., Moral, J., Picot, A., Puckett, R. D., Cotty, P. J., and Michailides, T. J. (2019). Atoxigenic aspergillus flavus isolates endemic to almond, fig, and pistachio orchards in California with potential to reduce aflatoxin contamination in these crops. Plant Disease, 103(5), 905–912. https://doi.org/10.1094/PDIS-08-18-1333-RE
81. Ortega-Beltran, A., Moral, J., Puckett, R. D., Morgan, D. P., Cotty, P. J., and Michailides, T. J. (2018). Fungal communities associated with almond throughout crop development: Implications for aflatoxin biocontrol management in California. PLoS ONE, 13(6), 1–15. https://doi.org/10.1371/journal.pone.0199127
82. Papa, K. E. (1986). Heterokaryon Incompatibility in Aspergillus flavus. Mycologia, 78(1), 98. https://doi.org/10.2307/3793383
83. Payne, G. A. (1998). Process of contamination by aflatoxin producing fungi and their impacts on crops. In and D. B. K. K. Sinha (Ed.), Mycotoxins in Agriculture and Food Safety (pp. 279–306). Marcel Dekker Inc.
84. Pildain, M. Belén, Vaamonde, G., and Cabral, D. (2004). Analysis of population structure of Aspergillus flavus from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. International Journal of Food Microbiology, 93(1), 31–40. https://doi.org/10.1016/j.ijfoodmicro.2003.10.007
85. Pildain, María B., Frisvad, J. C., Vaamonde, G., Cabral, D., Varga, J., and Samson, R. A. (2008). Two novel aflatoxin-producing Aspergillus species from Argentinean peanuts.

International Journal of Systematic and Evolutionary Microbiology, 58(3), 725–735. https://doi.org/10.1099/ijs.0.65123-0

86. Probst, C., and Bandyopadhyay, R. Price L.E. Cotty, P. J. (2011). Identification of Atoxigenic Aspergillus flavus Isolates to Reduce Aflatoxin Contamination of Maize in Kenya. Plant Disease, 95(2), 212–218.
87. Probst, C., Bandyopadhyay, R., and Cotty, P. J. (2014). Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa. International Journal of Food Microbiology, 174, 113–122. https://doi.org/10.1016/j.ijfoodmicro.2013.12.010
88. Probst, Claudia, Njapau, H., and Cotty, P. J. (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. Applied and Environmental Microbiology, 73(8), 2762–2764. https://doi.org/10.1128/AEM.02370-06
89. Raksha Rao, K., Vipin, A. V., and Venkateswaran, G. (2020). Mechanism of inhibition of aflatoxin synthesis by non-aflatoxigenic strains of Aspergillus flavus. Microbial Pathogenesis, 147(May), 104280. https://doi.org/10.1016/j.micpath.2020.104280
90. Ramirez-Cabral, N. Y. Z., Kumar, L., and Shabani, F. (2017). Global alterations in areas of suitability for maize production from climate change and using a mechanistic species distribution model (CLIMEX). Scientific Reports, 7(1), 1–13. https://doi.org/10.1038/s41598-017-05804-0
91. Raper KB, F. DI. (1965). The genus Aspergillus. Baltimore (Williams and Wilkins. (ed.)).
92. Rebourg, C., Chastanet, M., Gouesnard, B., Welcker, C., Dubreuil, P., and Charcosset, A. (2003). Maize introduction into Europe: The history reviewed in the light of molecular data. Theoretical and Applied Genetics, 106(5), 895–903. https://doi.org/10.1007/s00122-002-1140-9
93. Santpoort, R. (2020). THE drivers of maize area expansion in sub-Saharan Africa. How policies to boost maize production overlook the interests of smallholder farmers. Land, 9(3). https://doi.org/10.3390/land9030068
94. Savic, Zagorka, Jaji, I., Stankov, A., and Vukoti, J. (2020). Biological Control of Aflatoxin in Maize Grown in Serbia. 1–11.
95. Scheidegger, K. A., and Payne, G. A. (2003). Unlocking the secrets behind secondary metabolism: A review of Aspergillus flavus from pathogenicity to functional genomics. Journal of Toxicology - Toxin Reviews, 22(2–3), 423–459. https://doi.org/10.1081/TXR-

120024100

96. Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., and Geisen, R. (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of Aspergillus flavus in relation to various combinations of water activity and temperature. International Journal of Food Microbiology, 135(3), 231–237. https://doi.org/10.1016/j.ijfoodmicro.2009.07.026
97. Senghor, L. A., Ortega-Beltran, A., Atehnkeng, J., Callicott, K. A., Cotty, P. J., and Bandyopadhyay, R. (2019). The Atoxigenic Biocontrol Product Aflasafe SN01 Is a Valuable Tool to Mitigate Aflatoxin Contamination of Both Maize and Groundnut Cultivated in Senegal. Plant Disease, August, PDIS-03-19-0575. https://doi.org/10.1094/pdis-03-19-0575-re
98. Shenge, Kenneth C., Hillary L. Mehl, and P. J. C. (2017). Interactions among active ingredients of a multi-isolate aflatoxin biocontrol product. APS Annual Meeting.
99. Sweany, R. R., Damann, K. E., and Kaller, M. D. (2011). Comparison of soil and corn kernel Aspergillus flavus populations: Evidence for niche specialization. Phytopathology, 101(8), 952–959. https://doi.org/10.1094/PHYTO-09-10-0243
100. Sweeney, M.J; Dobson, D. . (1998). Mycotoxin production by Aspergillus, Fusarium and Penicillium species. International Journla of Food Microbiology, 43, 141–158.
101. Turner, N. W., Subrahmanyam, S., and Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. Analytica Chimica Acta, 632(2), 168–180. https://doi.org/10.1016/j.aca.2008.11.010
102. Uka, V., Cary, J. W., Lebar, M. D., Puel, O., De Saeger, S., and Diana Di Mavungu, J. (2020). Chemical repertoire and biosynthetic machinery of the Aspergillus flavus secondary metabolome: A review. Comprehensive Reviews in Food Science and Food Safety, 19(6), 2797–2842. https://doi.org/10.1111/1541-4337.12638
103. USDA. (2022). U.S. Department of Agriculture. https://www.usda.gov/
104. USEPA. (2003). Biopesticide Registration Action Document Aspergillus flavus AF36. https://doi.org/www.epa.gov/oppbppd1/
105. USEPA. (2004). Biopesticide Registration Action Document Aspergillus flavus AF36,. https://doi.org/www.epa.gov/oppbppd1/
106. Van Egmond, H. P., Schothorst, R. C., and Jonker, M. A. (2007). Regulations

relating to mycotoxins in food : Perspectives in a global and European context. Analytical and Bioanalytical Chemistry, 389(1), 147–157. https://doi.org/10.1007/s00216-007-1317-9

107. Varga, J., Frisvad, J. C., and Samson, R. A. (2009). A reappraisal of fungi producing aflatoxins. World Mycotoxin Journal, 2, 263–277.

108. Weaver, M. A., and Abbas, H. K. (2019). Field Displacement of Aflatoxigenic Aspergillus flavus Strains Through Repeated Biological Control Applications. Frontiers in Microbiology, 10(August), 1–7. https://doi.org/10.3389/fmicb.2019.01788

109. Wicklow, D. T., Hesseltine, C.W., Shotwell, O. L., and Adams, G. L. (1980). Interfer- ence competition and aflatoxin levels in corn. Phytopathology, 70, 761–764. https://doi.org/doi: 10.1094/Phyto-70-761

110. Widstrom, N. (1996). the aflatoxins problem with corn grain. Advances in Agronomy. Academic Press.

111. Blount, W.P. (1961). Turkey BX^ disease. J Br Turkey Fed, 9, 55–58.

112. Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V., and Kuca, K. (2009). Biological degradation of aflatoxins. Drug Metabolism Reviews, 41(1), 1–7. https://doi.org/10.1080/03602530802563850

113. Yi, Q., Álvarez-Iglesias, L., Malvar, R. A., Romay, M. C., and Revilla, P. (2021). A worldwide maize panel revealed new genetic variation for cold tolerance. Theoretical and Applied Genetics, 134(4), 1083–1094. https://doi.org/10.1007/s00122-020-03753-3

114. Yu, J., Chang, P., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, G. A., Linz, J. E., Woloshuk, C. P., Bennett, W., Yu, J., Chang, P., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, G. A., Linz, J. E., Woloshuk, C. P., and Bennett, J. W. (2004). Clustered Pathway Genes in Aflatoxin Biosynthesis MINIREVIEW Clustered Pathway Genes in Aflatoxin Biosynthesis. https://doi.org/10.1128/AEM.70.3.1253

Chapter 2:

Multiple year influences of the aflatoxin biocontrol product AF-X1 on A. flavus communities associated with maize production in Italy

Mohamed Ali Ouadhene [1], Alejandro Ortega-Beltran [2], Martina Sanna[3], Peter J. Cotty [4], and Paola Battilani[1,*]

[1] **Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy; mohamedali.ouadhene@unicatt.it**

[2] **International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria; a.beltran@cgiar.org**

[3] **AGROINNOVA- Centre of Competence for the Innovation in the Agro-Environmental Sector. University of Torino, Grugliasco, Italy; martina.sanna@unito.it**

[4] **College of Food Science and Engineering, Ocean University of China, Qingdao, China; cottypj@gmail.com**

* **Correspondence: paola.battilani@unicatt.it**

Note: The present research work was done in collaboration with Corteva Agriscience (Soil Sampling); the International Institute of Tropical Agriculture (VCG) and AGROINNOVA- Centre of Competence for the Innovation in the Agro-Environmental Sector (Real-time PCR)

Abstract

AF-X1 is a commercial aflatoxin biocontrol product containing the atoxigenic strain of *Aspergillus flavus* MUCL54911 (VCG IT006), endemic to Italy, as active ingredient. The present study aimed to evaluate the long-term persistence of VCG IT006 in the treated fields, and the multi-year influence of the biocontrol application on the *A. flavus* population. Soil samples were collected in 2020 and 2021 from 28 fields located in four provinces in North Italy. Vegetative compatibility Analysis was conducted to monitor the occurrence of VCG IT006 on the total of 399 isolates of *A. flavus* collected. IT006 was present in all the fields, mainly in fields treated 1 yr and 2 consecutive yrs (58% and 63%, respectively). The density of toxigenic isolates, detected using *AflR* gene, was low in the treated fields and displaced by the atoxigenic deployment; variability was noticed between the sampled fields and areas. The current findings support long-term durability of application benefits without deleterious effects on the whole fungal populations. Nevertheless, based on the current results, as well as, previous studies, yearly applications of AF-X1 in the Italian commercial maize fields should be continued because of the variability noticed among sampled areas.

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Introduction

Aspergillus flavus Link is one of the most important filamentous fungi worldwide because it can produce aflatoxins in various crops of economic importance. This species is widely distributed in temperate, tropical and subtropical zones (Cotty et al., 1994), including various regions in Europe (Piva et al., 2006; Perrone et al., 2014), and thrives in many agro-ecosystems and diverse natural habitats. Competitive advantages of *A. flavus* increase under several abiotic stresses including high temperature and salinity (Bock et al., 2004; Zuluaga-montero et al., 2010). However, it is as a causal agent of aflatoxin contamination that *A. flavus* is most frequently distinguished. The International Agency for Research on Cancer (IARC, 2002) classifies aflatoxin B1 (AFB1) as a Group 1compound because it is known to be carcinogenic to humans (Marchese et al., 2018). As a result, maximum levels (MLs) for aflatoxins in food and feed have been established in most countries, to prevent commercialization and consumption of unsafe commodities (Ayalew et al., 2017; Singh and Cotty, 2017; Eskola et al., 2020)

The life cycle of *A. flavus* is divided into an opportunistic phase during which plants, animals, and humans are infected and a saprophytic phase where detritus is produced from excrement and through the decay of plant and insect parts and other organic matter (Cotty, et al. 1994). Mycelia, conidia, and sclerotia are produced during both phases providing for reproduction and survival in the absence of nutrients and/or environments conducive to growth (Hedayati et al., 2007; Sepahvand et al., 2011; Horn, 2003; Ojiambo et al., 2018). Many *A. flavus* genotypes produce AFB1 and AFB2, but other genotypes lack abilities to produce aflatoxins (Mehl et al., 2012). Based on the morphology of sclerotia, two morphotypes of *A. flavus* have been distinguished: S morphotype, known for copious production of small sclerotia (<400 µm in diameter), and L morphotype, characterized by few, larger (>400 µm) sclerotia (Cotty, 1989). Virtually all S morphotype produce high aflatoxin concentrations while aflatoxin producing potentials of L morphotype genotypes vary widely with some producing high aflatoxin concentrations more than 100 ppm and others producing no aflatoxins. Genotypes that produce no aflatoxins, termed atoxigenic, are detected in most studies evaluating *A. flavus* diversity (Cotty, 1989; Grubisha and Cotty, 2015; Mauro et al., 2013; Mehl and Cotty, 2010; Ortega-Beltran et al., 2016). Another important aflatoxin producing species is *A. parasiticus* Speare, which produces both B and G aflatoxins (Horn et al., 1996; Klich and Pitt, 1988; Sengun et al., 2008).

There are many *A. flavus* genetic groups, called Vegetative Compatibility Groups (VCGs), which are delimited by a self/non-self-recognition system (Grubisha and Cotty, 2010; Leslie, 1993). VCGs composed entirely of atoxigenic members reflect stable retention of atoxigenicity during clonal evolution (Adhikari et., 2016). Atoxigenicity does not affect the ability of *A. flavus* to infect and decay crops. This attribute led to the suggestion that atoxigenic *A. flavus* might be used as biocontrol agents to competitively exclude aflatoxin producers (Cotty, 1989). This hypothesis led directly to development of an aflatoxin biocontrol technology based on atoxigenic genotypes of *A. flavus*. In 1989, the atoxigenic strain AF36 was applied for the first time to a field, after being tested at laboratory scale, to reduce aflatoxin contamination in cottonseed in Yuma, Arizona; aflatoxin contamination was significantly reduced (Cotty, 1989; Cotty, 1990).

Displacement of aflatoxin producers is an important mechanism by which applied atoxigenic strains reduce aflatoxin contamination (Cotty and Bayman, 1993; Cotty, 1994). From the initial commercial field evaluations of the atoxigenic biocontrol product AF36 starting in 1996, it became evident that atoxigenic biocontrol products shift the *A. flavus* population structure in treated fields and these changes to the population structure may be retained, in part, over multiple years (Cotty, 2006; Cotty et al., 2008). Similar results also occur in small-scale field station studies (Weaver et al, 2019) and in multi-year evaluations of the biocontrol product Aflasafe in commercial maize fields in Kaduna State, Nigeria (Atehnkeng et al., 2022).

There are currently over 50 atoxigenic strains of *A. flavus* registered for use as active ingredients in aflatoxin biocontrol products in various countries (Khan et al., 2021; Moral et al., 2020). However, AF-X1 is the only product currently available in the EU. AF-X1 has been used in Italy since 2015. The active ingredient is *A. flavus* MUCL 54911 which is endemic to Italy and belongs to VCG IT006 (Mauro et al., 2015). All members of this VCG lack the entire aflatoxin biosynthesis gene cluster as the result of a large insertion/deletion event shared in common with a number of other atoxigenic genotypes of *A. flavus* (Mauro et al., 2018; Adhikari, et al. 2016).

In 2003 there was an aflatoxin outbreak in maize grown in North Italy (Piva et al., 2006) which resulted in large quantities of milk being destroyed and significant impact on the regions signature cheese production by the consortia Parmigiano Reggiano and Grana Padano. After 2003, North Italy experienced additional aflatoxin contamination events as did many temperate regions in

South East Europe (Perrone et al., 2014, 2020) causing significant problems for both maize and dairy industries (Anfossi et al., 2012). Further, climate change is predicted to worsen maize contamination in Europe (Battilani et al., 2016). The use of different compounds able to bind AFB1 to reduce its bioavailability was purposed, but this approach is not totally effective and imply side effects (Girolami et al., 2022; Walte et al., 2022). Fortunately, the atoxigenic strain-based product AF-X1 was successfully developed to address the contamination in commercially grown maize in Italy. Initial treatments resulted in substantial relief for the maize industry with aflatoxin contamination reduced more than 90% compared to untreated maize (Mauro et al., 2018; Anonymous 2022ab). AF-X1 is commercialized with a temporary authorization since 2015 while the final registration is still ongoing.

There are several mechanisms through which atoxigenic strains of *A. flavus* may influence contamination. However, the only mechanism demonstrated consistently in agricultural fields is the modification of *Aspergillus* populations to reduce the average aflatoxin-producing potential (Agbetiameh et al., 2020; Bandyopadhyay et al., 2021; Atehnkeng et al., 2022). Atoxigenic strains displace toxigenic strains and thereby reduce aflatoxin content in many crops grown commercially in the United States, Nigeria, Kenya, Senegal, The Gambia, Ghana, and Italy (Cotty and Mellon, 2006; Doster et al., 2014; Bandyopadhyay et al., 2016; Mauro et al., 2018; Ortega-Beltran et al., 2021; Bandyopadhyay et al., 2022).

Aflatoxin concentration is influenced by high temperature, low humidity, and precipitation. Moreover, crop rotation, timing of planting and harvest have strong effects on contamination. These factors make it difficult to determine multiyear efficacy of biocontrol applications based on aflatoxin concentrations alone (Cotty and Jaime-Garcia, 2007; Battilani et al. 2016; Leggieri et al., 2021). In some regions, modifications to fungal populations have been shown to have both multi-year influences and influences beyond the treated fields with increases in incidences of atoxigenic active ingredients in the soil (Cotty et al., 2007; Cotty Jaime-Garcia, 2008; Bandyopadhyay et al., 2016; Abbas et al., 2017; Weaver and Abbas, 2019). However, extents of such influences are dependent on both the atoxigenic genotypes employed and the agroecosystem in which the product is used (Ching'anda et al., 2021). Currently, there are no studies on either long-term efficacy of AF-X1 or influences of the maize-based agroecosystem of Northern and Central Italy on AF-X1 persistence. Residual influences of biocontrol products can be assessed by multi-year monitoring

of atoxigenic active ingredients in agricultural soils. This can be done by examining individual genotypes within the resident fungal population and characterizing those individuals with either culture-based (i.e. vegetative compatibility analysis (VCAs) (Cotty and Bayman, 1993; Atehnkeng et al., 2014)) or molecular tools such as microsatellite analyses or SNP monitoring with pyrosequencing (Das et al., 2008; Grubisha and Cotty, 2009; Islam et al., 2021) or real-time PCR (Ortega et al., 2020). Moreover, several studies have previously identified the role of some aflatoxin biosynthesis pathway genes such as *omt-A* and *AflR* to develop new approaches to estimate the aflatoxin producing capacity in *Aspergillus* spp., like the use of real-time PCR (Shapira et al., 1996; Rodriguez et al., 2012; Ortega et al., 2020). In fact, qPCR was previously used to detect AF36 during pistachio production (Garcia-Lopez et al., 2021). In addition, Cluster Amplification Pattern (CAP) is a multiplex PCR method used to monitor the stability of atoxigenic strains of *A. flavus* (Callicott and Cotty, 2014)

The current study sought to assess long-term effects of commercial applications of the biocontrol product AF-X1 on the structure of *A. flavus* communities' resident in fields frequently cropped to maize in North Italy. The results suggest that AF-X1 applications have beneficial effects on the structure of *A. flavus* communities that extend across multiple years. The residual influences of applications may provide cumulative benefits over multiple seasons and may, in part, explain reduced frequencies of aflatoxin contamination in regions where applications of AF-X1 were previously widely employed.

Materials and Methods

Soil Sampling and field data collection

Soil was sampled in North Italy during April 2020 and 2021 in seven sampling areas distributed across the provinces Rovigo, Modena, Padova, and Venezia. In each area, four fields (28 fields total) were chosen where AF-X1 had been applied either once the previous year (treated n-1), once two years prior (treated n-2), both the previous year and two years prior (treated n-1 and n-2), or not at all (not treated). All applications were made according to label instructions by the farmers. For each treatment year, crops were treated once at 25 kg/ha between BBCH phenological growth stages 33-39 (Meier, 2018). In each region, the approximate percentages of maize farms

where AF-X1 had been applied varied, with 40% in Rovigo, 35% in Padova, 30% in Venezia, and 25% in Modena.

Ten soil samples of ~50 g were collected with a surface disinfected trowel from the top 2 cm at 4 to 10 m intervals across diagonal transects of each of the 28 fields. Distances between sampled fields exceeded 5 km (Cotty, 1997; Senghor et al., 2019). Soil samples were taken to the laboratory, dried in a forced air (40 to 45°C, 48 hr), and stored in plastic bags at 4°C until processed. Information regarding the cropping system (e.g., crop rotation, tillage system, stalk burial and soil texture, provided by the farmers/extension agents) were collected from the sampled fields.

4.2. Aspergillus flavus isolation

Isolation of *A. flavus* from soil samples was performed aseptically following protocols previously reported (Jaime and Cotty, 2006). Briefly, 10 g of soil per sample was mixed with 50 ml double distilled sterile water and stirred for 20 min at 300 RPM. A 100 µl aliquot of the soil suspension was transferred onto MRBA (Cotty, 1994) and incubated at 31°C for 3 d. The colonies of *A. flavus* were identified based on morphology (Klich and Pitt, 1988) and quantified as colony forming units per g soil (CFU/g). From each field, 10 to 15 discrete colonies of *A. flavus* were transferred to the low nutrient agar medium 5/2 (5% V-8 vegetable juice, 2% agar, pH 5.2) (Cotty and Misaghi, 1985) and incubated (5-7 d, in the dark, 31°C). Cultures were saved in sterile water vials at 4°C containing five plugs (3 mm dia) of sporulating agar in 1 ml sterile distilled water (Probst et al., 2011).

In total, 399 isolates (range = 10-15 per field) were used to quantify the persistence of the active ingredient of biocontrol product AF-X1, MUCL 54911, using Vegetative Compatibility Analysis (VCA). In addition, all isolates were subjected to a qPCR assay to evaluate presence or absence of a section of the *AflR* gene. This gene is required for aflatoxin production (see below). The isolates were single spored (i.e., monosporic) through serial dilution on Malt Extract Agar (MEA) (Mauro et al., 2013). After 2 d of incubation at 31°C, one colony per isolate was transferred to 5/2 agar. Single spore transfers were performed in triplicate to ensure culture purity. Five agar plugs from pure mature cultures were saved as above.

4.3. DNA extraction

Monosporic *A. flavus* isolates (399 total) were used to evaluate presence or absence of a section of the *AflR* gene, using a TaqMan qPCR assay developed for *A. flavus* (Ortega et al., 2020). Positive (isolate FS7; aflatoxin producer) and negative controls (isolates FS3, FS5, FS6, and FV9; non-aflatoxin producers) were included. The 399 monosporic isolates were grown on Yeast Extract Sucrose Agar (YES agar) for 7 d at room temperature (Probst and Cotty, 2012). Fresh mycelium from the edges of the colonies were used to extract genomic DNA with the E.Z.N.A. fungal DNA mini kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions. DNA concentrations were measured with NanoDrop 2.0 (ThermoFisher, Wilmington, DE, USA) and adjusted to be less than 100 ng/µl (Ortega et al., 2020).

4.4. qPCR conditions

The two primers, AflF and AflR (Ortega et al., 2020), were used at a concentration of 0.3 µmol, TaqMan probe concentration was 0.1 µmol with 1× of TaqMan universal PCR MasterMix (Applied Biosystems, Loughborough, UK) and 1µl of DNA (100 ng/µl) of the isolate being assayed. StepOne thermal cycler instrument (Applied Biosystems, Loughborough, UK) was used to perform the reaction with the following cycle: initial denaturation at 95°C for 4.5 min, 40 cycles of 15 s at 95°C and 15 s at 60°C. Each reaction was run in triplicate; positive and negative controls were included in each run. The standard curve utilized DNA of *A. flavus* FS7 with serial dilution to test qPCR sensitivity (Ortega et al., 2020). The atoxigenicity of the isolate was assumed based on the CT value generated from the amplification curve of *AflR* and ranged from 20 to 47.61 (CT ≤35= toxigenic; CT > 35 atoxigenic)

4.5. Vegetative Compatibility Analysis (VCA)

To determine the distribution and frequencies of the AF-X1 active ingredient (MUCL 54911), all 399 monosporic isolates were subjected to VCA with the tester pairs of VCG IT006 (Mauro, et al. 2013), the VCG to which MUCL 54911 belongs, following previously published protocols Bayman and Cotty, (1991) and Cotty (1994).

To obtain the nitrate-non-utilizing (nit-) mutants, 10 µl of spore suspension of each isolate was seeded into a well (3 mm diameter) in the center of SEL plates (Cotty, 1994). Sectors auxotrophic for nitrate were visible after 10 to 30 d of incubation at 31°C. Auxotrophs were transferred to MIT,

incubated for 3 d at 31°C (Das et al., 2008), transferred to 5/2 agar, and stored in water vials as described above.

Complementation tests with the tester pair of VCG IT006 were performed on Starch medium (Cotty and Taylor, 2003). Three wells (3 mm in diameter), 1 cm apart, were made in a triangular pattern in the center. Two wells were seeded with 10 µl of spore suspension of each of the testers and the third one was seeded with 10 µl of spore suspension of the nit- mutant of the isolate being analyzed. Compatibility was assessed after 7 d of incubation at 31°C. Wildtype growth at the zone of mycelial interaction indicated that the isolate belonged to VCG IT006 (Bayman and Cotty, 1990).

4.6. Data Analysis

Data on CFU/g of total fungi and *A. flavus* in the soil samples were ln transformed and data on % of atoxigenic isolates and those belonging to IT006, both computed on all *A. flavus* and on *A. flavus* atoxigenic were arcsin transformed before statistical analysis to reduce the heterogeneity in variance. All data obtained were subjected to univariate analysis of variance (ANOVA) using the generalized linear model (GLM) procedure and significant differences between means were determined using Tukey's HSD test ($\alpha = 0.05$). The statistical package IBM SPSS statistics 27 (IBM Corp., Armonk, NY, USA) was used for data analysis.

Results

Cropping systems of the surveyed fields

Most of the fields (70%) included in the current study contained predominantly silt soil (Table 1). Six (21%) fields were predominantly clay and three (11%) were sandy. Several of the sampled fields (25 %) were planted to maize repeatedly without rotation. However, some fields were rotated between maize and either wheat, soybean, rarely with tomato, or pea. Conventional tillage was commonly applied, with conservative approaches (no tillage) reported only for 3 fields in Area 2 in Rovigo. In addition, stalk burial was performed in ~50% of the fields and all four of the fields sampled in Area 6.

Table 1. List of maize fields sampled in this study. Location (district and municipality), sampling year, geographic coordinates (latitude and longitude), AF-X1 treatment regimen (not treated, treated n-2, treated n-2&n-1 and treated n-1; n is the sampling year), soil texture, crop rotation (one or two years before sampling) and stalk burial were reported.

Area N*	Districts **	Municipality ***	Sampling year	Latitude	Longitude	Treatment	Soil texture	One year prior	Two years prior	Tillage	stalk burial
1	Rovigo	Occhiobello	2020	44.9297388	11.62801768	Not treated	Silt	Maize	Tomato	Conventional	No
1	Rovigo	Occhiobello	2020	44.943139	11.567212	n-2	Sandy	Maize	Maize	Conventional	No
1	Rovigo	Occhiobello	2020	44.951586	11.547809	n-1&n-2	Silt	Maize	Maize	Conventional	No
1	Rovigo	Occhiobello	2020	44.955789	11.554532	n-1	Silt	Maize	Tomato	Conventional	No
2	Rovigo	Occhiobello	2020	44.969438	11.70443	Not treated	Silt	Wheat	Pea	Conservation	No
2	Rovigo	Occhiobello	2020	44.93645466	11.62733917	n-2	Clay	Wheat	Maize	Conservation	No
2	Rovigo	Fiesso Umbertino	2020	44.955438	11.589537	n-1&n-2	Silt	Maize	Maize	Conservation	Yes
2	Rovigo	Occhiobello	2020	44.94879764	11.60269669	n-1	Silt	Maize	Wheat	Conventional	Yes
3	Rovigo	Occhiobello	2020	44.94469842	11.49092054	Not treated	Clay	Wheat	Wheat	Conventional	No
3	Rovigo	Occhiobello	2020	44.94834217	11.59027712	n-2	Sandy	Wheat	Maize	Conventional	Yes
3	Rovigo	Fiesso Umbertino	2020	44.973695	11.630536	n-1&n-2	Silt	Maize	Maize	Conventional	Yes
3	Rovigo	Occhiobello	2020	44.96235125	11.6690985	n-1	Silt	Maize	Soybean	Conventional	Yes
4	Modena	Finale Emilia	2021	44.81483	11.217337	Not treated	Clay	Wheat	Maize	Conventional	No
4	Modena	Finale Emilia	2021	44.829477	11.094888	n-2	Silt	Wheat	Maize	Conventional	No
4	Modena	Finale Emilia	2021	44.835933	11.27449	n-1&n-2	Sandy	Maize	Maize	Conventional	No
4	Modena	Finale Emilia	2021	44.86689	11.174496	n-1	Silt	Maize	Wheat	Conventional	No
5	Rovigo	Occhiobello	2021	44.98778	11.698756	Not treated	Clay	Soybean	Wheat	Conventional	No
5	Rovigo	Occhiobello	2021	44.931735	11.629024	n-2	Silt	Soybean	Maize	Conventional	No
5	Rovigo	Occhiobello	2021	44.958125	11.627436	n-1&n-2	Silt	Maize	Maize	Conventional	Yes
5	Rovigo	Occhiobello	2021	44.971765	11.721178	n-1	Silt	Maize	Wheat	Conventional	Yes
6	Padova	Noale	2021	45.54925	12.052739	Not treated	Silt	Soybean	Soybean	Conventional	Yes
6	Padova	Noale	2021	45.549233	12.05297	n-2	Silt	Soybean	Maize	Conventional	Yes

6	Padova	Noale	2021	45.553166	12.045033	n-1&n-2	Clay	Maize	Maize	Conventional	Yes
6	Padova	Noale	2021	45.552759	12.048152	n-1	Clay	Maize	Soybean	Conventional	Yes
7	Venezia	Scorzè	2021	45.563136	12.10695	Not treated	Silt	Soybean	Soybean	Conventional	Yes
7	Venezia	Scorzè	2021	45.569842	12.099649	n-2	Silt	Soybean	Maize	Conventional	Yes
7	Venezia	Scorzè	2021	45.565978	12.09769	n-1&n-2	Silt	Maize	Maize	Conventional	No
7	Venezia	Scorzè	2021	45.56353	12.10323	n-1	Silt	Maize	Soybean	Conventional	No

* Number of Areas, 3 areas in 2020 and 4 Areas in 2021

** 4 Provinces in which sampled fields were located.

*** Different Areas where the soil samples were collected from the surveyed fields

Soil fungal populations

The total fungal community (Table 2) in the soil sampled in 2020 was significantly ($P< 0.01$) influenced by the AF-X1 application schedule, however, no significance was observed in soils sampled in 2021. Further, the interaction between treatment and location was significant ($P<0.01$).

The highest fungal occurrence was noted in Area 4 with 3,443 CFU/g and the lowest in Area 7 with 112 CFU/g (Figure 1 b). Additionally, a CFU/g increase by 47% was noted in all fields treated n-1 compared to the untreated fields. The distribution of the total fungal population varied within the Areas. The highest fungal concentrations occurred in a field treated n-1 in Area 1 (6,027 CFU/g).

The occurrence of *A. flavus* in the soil varied with treatment ($P<0.01$) only in the samples collected in 2020, ranging from 51 to 190 CFU/g (Table 2). Overall results from both sampled years indicated that the lowest average recovery of *A. flavus* occurred in the non-treated fields surveyed in 2020 with 51 CFU/g. Concentrations of *A. flavus* were elevated in both fields treated in a single year and fields treated two years (2020-2021; Table 1). There was a significant interaction between treatments and locations only for data collected in 2021 ($P<0.01$).

Influences of treatment on *A. flavus* density was inconsistent (Figure 1 c, d). On one hand, in the Area 6 the non-treated field had the greatest *A. flavus* concentrations (99 CFU/g), whereas the lowest *A. flavus* concentration was observed in the field treated n-1 (15 CFU/g). On the other hand, in area 5 the highest concentration of *A. flavus* was reported in the field treated n-1 (312 CFU/g).

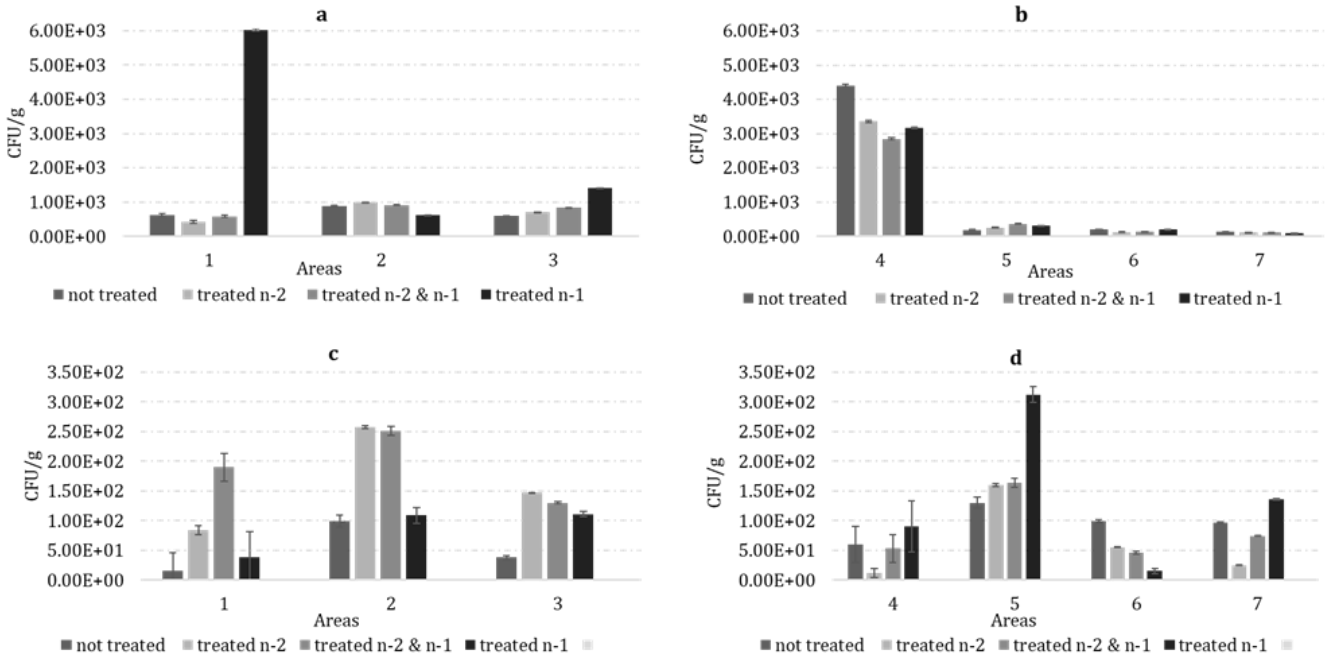

Figure 1. Colony forming unit (CFU /g + confidence interval) of total fungal community resident in the soil samples collected from 3 Areas in 2020 (a); 4 Areas in 2021 (b) and of A. flavus in the same soil sample collected in 2020 (c) and in 2021 (d). The 7 sampling Areas belong to 4 District: 1 (Rovigo), 2 (Rovigo), 3 (Rovigo) in 2020 and 4 (Modena), 5 (Rovigo), 6 (Padova) and 7 (Venezia) in 2021. In each Area 4 different treatment regimens were applied: Not treated, treated n-2, treated n-2&n-1 and treated n-1; n is the sampling year.

Table 2. Results of ANOVA run for colony forming units (CFU /g) of total fungal community and of *A. flavus* resident in the soil samples collected from 3 Areas in 2020 and 4 Areas in 2021. The 7 different Areas belong to 4 District: 1, 2, 3 and 5 Rovigo, 4 Modena, 6 Padova and 7 Venezia. Per each Area 4 different AF-X1 treatment regimen were considered: not treated, treated n-2, treated n-2&n-1 and treated n-1; n is the sampling year. Percentage of atoxigenic strains on all *A. flavus* isolated was also reported, so as the percentage of the VCG IT006 on total *A. flavus* strains and on the atoxigenic strains.

Sample year	**Treatment year**	**N. of isolates**	**Replicates §**	**Total fungi (CFU/g)**	***A. flavus* (CFU/g)**	**% atoxigenic §§**	**IT006 on *A. flavus* %†**	**IT006 on *A. flavus* atox %†**
2020				*	*	*NS*	*NS*	*NS*
	No Treatment	39	3	702 b	51 c	77	37	48
	2018	44	3	704 b	163 ab	75	41	56
	2018&2019	42	3	778 b	190 a	93	72	77
	2019	44	3	2688 a	86 b	86	77	88
2021				*NS*	*NS*	*NS*	**	**
	No Treatment	60	4	1229	96	38	12 b	23 b
	2019	54	4	964	63	75	62 a	86 a
	2019, 2020	60	4	863	84	68	57 a	79 a
	2020	56	4	943	138	72	54 a	74 ab
2020, 2021 Combined				*NS*	*	*	**	**
	No Treatment	99	7	1003	77 b	55 b	23 b	34 b
	Treated 1 yr	198	14	1271	111 a	77 ab	58 a	76 a
	Treated 2 yrs	102	7	826	130 a	79 a	63 a	78 a

§ Each replicate is a separate commercial field

§§ The % of atoxigenic and atoxigenic was calculated based on the total number of isolates of *A. flavus* p (120 and 279 isolates of *A. flavus* recovered in 2020 and 2021, respectively)

** ($p<0.01$), * ($p<0.05$); Different letters indicate significant difference according to Tukey's HSD test.

†Percentages were calculated based on the total number of isolates collected for each treatment

Frequency of atoxigenic and toxigenic *A. flavus*

Overall, 399 *A. flavus* isolates were collected from soil samples. The frequency of isolates of *A. flavus* lacking the *AflR* gene was determined using a qPCR method with TaqMan® probe. Among the 399 *A. flavus* isolates, 28% contained the *AflR* gene and in 72%, the gene was lacking (Table 2). The occurrence of atoxigenic isolates lacking the *AflR* gene was significantly influenced by AF-X1 treatment ($p < 0.05$) only when the two sampling years were combined; fields treated for two years had significantly more atoxigenic isolates compared to untreated fields.

The highest occurrence of isolates lacking *AflR* was noted in all treated fields and ranged from 68% to 93%, among the examined communities of *A. flavus* (Table 2). However, a low occurrence of toxigenic isolates was also noted in some Areas in untreated fields (Areas 1-3; Figure 2 a). The highest occurrence of toxigenic isolates was reported in untreated fields in areas 6 and 7 (Figure 2 b).

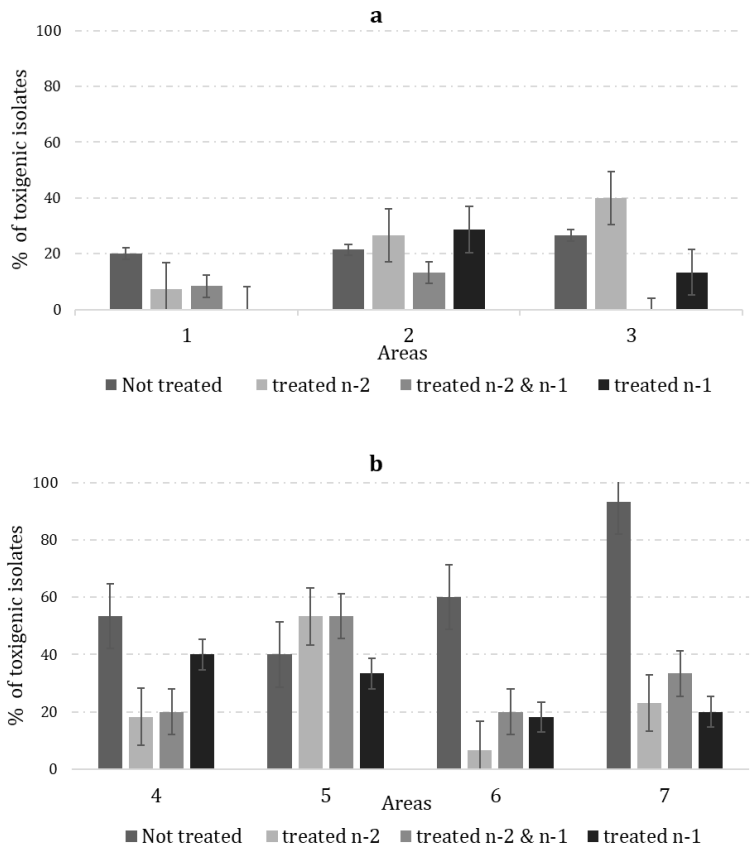

Figure 2. Percentage of toxigenic isolates of *A. flavus* isolates in the soil samples collected from 3 Areas in 2020 (a); 4 Areas in 2021 (b). The 7 sampling Areas belong to 4 District: 1 (Rovigo), 2 (Rovigo), 3 (Rovigo) in 2020 and 4 (Modena), 5 (Rovigo), 6 (Padova) and 7 (Venezia) in 2021. In each Area 4 different treatment regimens were applied: Not treated, treated n-2, treated n-2&n-1 and treated n-1; n is the sampling year.

Frequency of atoxigenic active ingredient strain of the biocontrol AF-X1

All isolates of *A. flavus* belonging to VCG IT006 (200 total) lacked *AflR,* as expected. Whereas, among the 199 isolates not belonging to IT006, only 84 (42%) did not have *AflR*.

The percent of *A. flavus* isolates that belong to the AF-X1 active ingredient VCG was significantly influenced by the treatment regimens ($p<0.01$) only in the fields sampled in 2021; VCG IT006 was significantly lower in untreated fields, both when the incidence was computed on all *A. flavus* or only the atoxigenic isolates (Table 2). When the two years were combined, the significantly lower incidence of VCG IT006 in untreated fields was confirmed.

Similar ranges of IT006 frequency were observed in fields treated two years (20% to 83% IT006) and fields treated only the year before sampling (40% to 93% IT006) (Figure 3 a, b). However, in Areas 1 and 3 the frequencies of IT006 in untreated fields were higher than in fields treated two years prior (+29% versus +17%) (Figure 3 a).

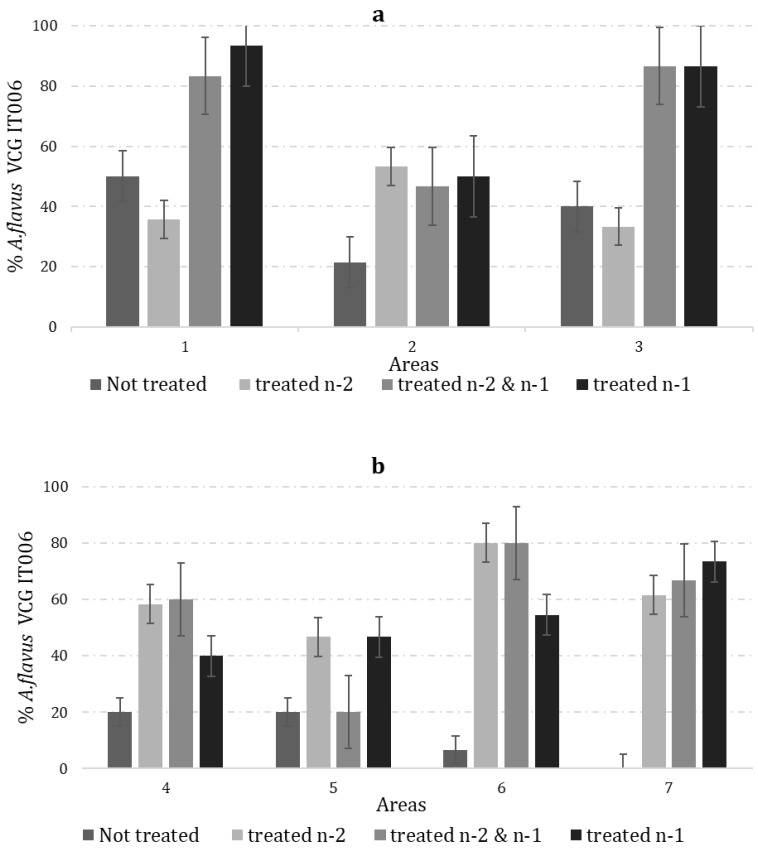

Figure 3. Incidence (%) of *A. flavus* strains belonging to the atoxigenic VCG IT006 on total number of *A. flavus* isolates, the active ingredient of AF-X1 in soil sample collected in 2020 (a) and 2021 (b); soil samples were collected from 3 Areas in 2020 (a) and 4 Areas in 2021 (b) belonging to 4 District: 1 (Rovigo), 2 (Rovigo), 3 (Rovigo) in 2020 and 4 (Modena), 5 (Rovigo), 6 (Padova) and 7 (Venezia) in 2021. In each Area 4 different treatment regimens were applied: Not treated, treated n-2, treated n-2&n-1 and treated n-1; n is the sampling year

Impact of cropping system on the soil fungal population

Crop rotation was the only factor among the cropping system that influenced significantly the fungal population isolated from soil, with a significantly higher CFU/g with wheat grown before

maize compared to soybean. The incidence of atoxigenic isolates, so as the incidence of IT006, was the highest with maize as preceding crop (data not shown).

Discussion

Farmers, industries, and regulatory authorities have questioned if applications of the aflatoxins biocontrol product might have long-term benefits (Anonymous, 2022a; Anonymous, 2022b). The current study provides observations that suggest applications of AF-X1 have influences that extend to the next season and the season after, and even to nearby fields never treated. Soils collected in 2020 and 2021 from fields located in Northern Italian maize production areas where AF-X1 was previously applied contained significant frequencies of the VCG to which MUCL 54911, the active ingredient of AF-X1, belongs. The results (Table 2) indicate that: I) use of AF-X1 has a residual effect that improves the structure of *A. flavus* resident in both the treated fields and in neighboring not-treated fields, so that the atoxigenic active ingredient is more common and the frequency of aflatoxin-producers is reduced; and II) the application of AF-X1 promotes the creation of these safer *Aspergillus* populations with no significant effects on the total fungal communities. These results suggest follow-up studies should be used to determine frequencies and distributions of AF-X1 applications required for the levels of cost-effective aflatoxin management required by North Italy's maize industry to provide grain that is consistently safe for the region's vital dairy industry. To assess residual effects of AF-X1 applications, VCA was chosen to monitor the active ingredient MUCL- 54911 in the current study despite being a labor-intensive, time-consuming technique; this has been judged the most reliable and accurate method available and the only method which has been successfully applied to identifying MUCL-54911 in field samples (Das et al., 2008; Mauro et al., 2013). Significant occurrence of the active ingredient MUCL-54911 in all treated areas was reported. Similarly, application of single atoxigenic *A. flavus* isolates of the aflatoxin biocontrol products Afla-Guard® and AF36 resulted in persistence overtime. In addition the most extensive carry over studies involving thousands of isolates were carried out in the US with AF36 (Cotty, et al,. 2007; Weaver and Abbas, 2019; Molo et al., 2022). Similar carry-over was also observed on African small holder farms with Aflasafe, a biocontrol product containing four atoxigenic strains as active ingredients (Atehnkeng et al. 2022).

Several studies have shown not only survival, but also an increased frequency of atoxigenic biocontrol product VCGs beyond the treatment season (Dorner, 2004; Cotty et al., 2007; Bandyopadhyay et al., 2016). On the other hand, the study of Weaver and Abbas, (2019) and Atehnkeng et al. (2022) showed a decline in frequencies of biocontrol VCGs when follow-up treatments are delayed by one or two years. This suggests that biocontrol carry-over effects may change from area to area and carry-over effects must continue to be investigated.

The current study revealed some unexpected results. In two Areas (Figure 2) prevalence of VCG IT006 in untreated fields was comparable with fields treated two years prior. Field to field variation in microenvironment, agronomic practice, or predation by insects may have contributed to these observations (Dowd, 2003).

The isolate of *A. flavus* MUCL 54911, belonging to VCG IT006, was identified and validated as the most efficient atoxigenic strain among those included in the Italian fungal collection by Mauro and coworkers in 2013 and 2018. The study of Mauro et al. (2018) highlighted the benefits of MUCL 54911, active ingredient of AF-X1, in reducing aflatoxin in maize. Mauro and colleagues showed that IT006 is the largest VCG in the Italian population from which the active ingredient was chosen, and it was found in 4 out of 5 Northern Italian regions where our current study was conducted. Areas 1 and 3 belong to district of Rovigo where 40% of fields had been treated with AF-X1. Examined samples from Rovigo had larger proportions of IT006, suggesting that aerially dispersed and insect transmitted conidia may be factors facilitating the active ingredient movement (Horn, 2003; Bock et al.,2004). Recovery of VCG IT006 in relatively high proportions in untreated fields supports the approach of selecting VCGs native in and well adapted to target regions for use as active ingredients of biocontrol formulations for improved persistence. Adaptation to target areas plus dispersal from treated to untreated fields are useful characteristics for biocontrol active ingredients.

Data on cropping systems, such as rotation, soil texture, and other agricultural practices, might be relevant in explaining the observed variability among fields. Several studies have examined the link between previous crop and *A. flavus* population (Abbas et al., 2004; Jaime and Cotty, 2006; Jaime and Cotty, 2010). In the prior studies, the highest densities of *A. flavus* were found in soil after maize, followed by wheat, cotton, and sorghum. Results from the current study agree with

these prior studies. One field treated two years prior to sampling with a prior crop of wheat had the lowest *A. flavus* density observed. Furthermore, soil texture is associated with variability in *A. flavus* communities. Clay soil and *A. flavus* are positively correlated while sandy soil is negatively correlated (Jaime and Cotty, 2006). Even if not statistically significant, the lowest incidence of atoxigenic isolates and those belonging to IT006 were detected in sandy soil. In Area 1, in the current study, a field with sandy soil treated two years prior had low incidence (35%) of IT006 with 84 CFU/g of total *A. flavus* population (Figure 2 a).

Conservation tillage combined with stalk burial, which increases the organic matter in the soil, were highly correlated with *A. flavus* density and contributes to maintaining a reservoir of *A. flavus* (Abbas et al., 2008; Donner et al., 2015). In this study significant differences were not detected, probably because of few fields with conservation tillage, but results from Area 2 are in agreement with this statement; the density of *A. flavus* (251 CFU/g) was greater under conservation tillage with stalk burial than under conventional tillage (109 CFU/g) (Figure 1 a).

In the present study, the fields or Areas were chosen randomly to obtain diverse conditions. Therefore, a large variation in the cropping system may be a barrier to establishing a link between the cropping system and *A. flavus* density, as well as the occurrence of IT006. As expected, the carry-over experiment had no significant effect on the global fungal communities other than proportions of toxigenic and atoxigenic *A. flavus* residing in the soil. Bhandari et al, (2020) found that the application of the commercial biocontrol product FourSure™ had no overall impact on microbiome composition of treated and untreated crops. Aflatoxin biocontrol application has been reported to have no increase in *Aspergillus* density (Agbetiameh et al., 2020; Atehnkeng et al., 2022) and no influence on composition of other fungal species and contamination with fumonisins (Mauro et al., 2018; Reis et al., 2020)

Tracking of biocontrol active ingredients has been carried out by first classifying *A. flavus* isolates by morphotype (L strain and S strain), and then conducting VCA in L morphotype isolates with tester pairs specific to the VCGs of the active ingredients (Cotty, 1989; Moral et al., 2020). A qPCR technique has resulted in useful information on hazelnuts and pistachio because of its specificity, sensitivity and accurate detection properties in accordance with the international EPPO standard (PM7/98) (Ortega et al., 2020; Garcia-Lopez et al., 2021). Usefulness of qPCR for

detecting isolates lacking the gene, *AflR*, from the aflatoxin biosynthesis cluster was confirmed in the current study, and this is the first time that tracking of a biocontrol isolate using qPCR directed at the mechanism for atoxigenicity has been reported for maize. The current work found predominance of atoxigenic fungi in all the surveyed areas and a higher incidence of atoxigenic fungi in most fields in which a prior year biocontrol application was made. Similar results have been reported by Atehnkeng and coworkers (2022) with other biocontrol fungi on small-holder farms in Africa. Our results show shifts in the *A. flavus* population following application of the atoxigenic *A. flavus* based biocontrol product, AF-X1. This has previously been demonstrated under various conditions in both small-scale and large commercial-scale agriculture (Cotty, 1994; Weaver and Abbas, 2019; Moral et al., 2020; Atehnkeng et al., 2022). Nevertheless, a wide variability was observed among studied fields.

Examining the proportion of the biocontrol active ingredients post-application, over multiple years, is an important criterion to evaluate success of atoxigenic strain-based biocontrol. This study provides valuable data regarding the performance, as well as the stability of the active ingredient of AF-X1 in Italian agroecosystems for sustainable aflatoxin management. Also, this study confirms that once the biocontrol is distributed in a field over the years the movement of the active ingredient may occur beyond the treated field which can provide a protection from aflatoxin contamination in untreated fields. The Area size and the percentage of biocontrol application are highly correlated with the dispersal to not-treated fields, as well as, the persistence of the active ingredient over the years (Jaime et al., 2017; Atehnkeng et al., 2022).

In conclusion aflatoxin biological control utilizing products with atoxigenic *A. flavus* active ingredients is the most successful technique for aflatoxin management, demonstrating considerable adaptability in the field by strains native to target regions as active ingredients. The current findings support long-term durability of application benefits without deleterious effects on fungal populations. The primary detected influence of AF-X1 applications is a switch in *A. flavus* community structure towards increased incidences of atoxigenic *A. flavus*. Based on the current results, as well as, previous studies, yearly applications of AF-X1 in the Italian commercial maize fields should be anyway continued until additional data is available to determine what timing and distribution of applications will provide the most cost-effective treatment regimen.

References

- Abbas, H.K.; Accinelli, C.; Thomas Shier, W. Biological control of aflatoxin contamination in U.S. crops and the use of bioplastic formulations of *Aspergillus flavus* biocontrol strains to optimize application strategies. *J. Agric. Food Chem.* **2017**, *65*, 7081–7087, doi:10.1021/acs.jafc.7b01452.
- Abbas, H.K.; Accinelli, C.; Zablotowicz, R.M.; Abel, C.A.; Bruns, H.A.; Dong, Y.; Shier, W.T. Dynamics of mycotoxin and *Aspergillus flavus* levels in aging Bt and non-Bt corn residues under mississippi no-till conditions. *J. Agric. Food Chem.* **2008**, *56*, 7578–7585, doi:10.1021/jf801771a.
- Adhikari, B.N.; Bandyopadhyay, R.; Cotty, P.J. Degeneration of aflatoxin gene clusters in *Aspergillus flavus* from Africa and North America. *AMB Express* **2016**, *6*, doi:10.1186/s13568-016-0228-6.
- Agbetiameh, D.; Ortega-Beltran, A.; Awuah, R.; Atehnkeng, J.; Elzein, A.; Cotty, P.; Bandyopadhyay, R. Field efficacy of two atoxigenic biocontrol products for mitigation of aflatoxin contamination in maize and groundnut in ghana. *Biol. Control* **2020**, *150*, 1-13, doi:10.1016/j.biocontrol.2020.104351.
- Anfossi, L.; Baggiani, C.; Giovannoli, C.; D'Arco, G.; Passini, C.; Giraudi, G. Occurrence of aflatoxin M 1 in Italian cheese: Results of a survey conducted in 2010 and correlation with manufacturing, production season, milking animals, and maturation of cheese. *Food Control* **2012**, *25*, 125–130, doi:10.1016/j.foodcont.2011.10.027.
- Anonymous Biocontrollo Aflatossine, La Soluzione Contro Le Aflatossine. https://www.corteva.it/content/dam/dpagco/corteva/eu/it/it/files/cp/brochures/DF-AF-X1_2022-volantino.pdf.Accessed December 17, **2022**.
- Anonymous Agente Di Biocontrollo per La Riduzione Delle Aflatossine Nel Mais. https://www.granapadano.it/it-it/af-x1-2022-agente-di-biocontrollo-per-la-riduzione-delle-aflatossine-nel-mais.Accessed December 17, **2022**.
- Atehnkeng, J.; Ojiambo, P.; Ortega-Beltran, A.; Augusto, J.; Cotty, P.; Bandyopadhyay, R. Impact of frequency of application on the long-term efficacy of the biocontrol product aflasafe in reducing aflatoxin contamination in maize. *Front. Microbiol.* **2022**, 1–14, doi:10.3389/fmicb.2022.1049013.
- Atehnkeng, J.; Ojiambo, P.S.; Cotty, P.J.; Bandyopadhyay, R. Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link : Fr Vegetative Compatibility Groups in preventing aflatoxin contamination in maize (*Zea mays* L .). *Biol. Control* **2014**, *72*, 62–70, doi:10.1016/j.biocontrol.2014.02.009.
- Ayalew, A.; Kimanya, M.; Matumba, L.; Bandyopadhayay, R.; Menkir, A.; Cotty, P. Controlling aflatoxins in maize in africa: strategies, challenges and opportunities for improvement. In *Achieving*

sustainable cultivation of maize cultivation techniques, pest, and disease control. Volume 2; Watson, D., Ed.; Burleigh Dodds Science: Cambridge, UK, 2017; pp. 1–24.

- Bandyopadhyay, R.; Ortega-Beltran, a.; Akande, a.; Mutegi, C.; Atehnkeng, J.; Kaptoge, L.; Senghor, a. L.; Adhikari, B.N.; Cotty, P.J. Biological control of aflatoxins in africa: current status and potential challenges in the face of climate change. *World Mycotoxin J.* **2016**, *9*, 771–789, doi:10.3920/WMJ2016.2130.
- Bandyopadhyay, R.; Ortega-Beltran, A.; Konlambigue, M.; Kaptoge, L.; Falade, T.D.O.; Cotty, P.J. Development and scale-up of bioprotectants to keep staple foods safe from aflatoxin contamination in africa. In *Microbial bioprotectants for plant disease management*; Kohl, J. and Ravensverg, W, J., Ed.; 2022; pp. 587–628.
- Battilani, P.; Toscano, P.; Van Der Fels-Klerx, H.J.; Moretti, A.; Camardo Leggieri, M.; Brera, C.; Rortais, A.; Goumperis, T.; Robinson, T. Aflatoxin B1 contamination in maize in europe increases due to climate change. *Sci. Rep.* **2016**, *6*, 1–7, doi:10.1038/srep24328.
- Bayman, P.; Cotty, P.J. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. *Can. J. Bot.* **1991**, *69*, 1707–1711, doi:10.1139/b91-216.
- Bhandari, K.B.; Longing, S.D.; West, C.P. Soil microbial communities in corn fields treated with atoxigenic aspergillus flavus. *Soil Syst* **2020**, 4–35.
- Bock, C.H.; Mackey, B.; Cotty, P.J. Population Dynamics of *Aspergillus flavus* in the Air of an Intensively Cultivated Region of South-West Arizona. *Plant Pathol.* **2004**,*5*, 422–433, doi:10.1111/j.1365-3059.2004.01015.x.
- Callicott, K.A.; Cotty, P.J. Method for monitoring deletions in the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* with multiplex PCR. **2014**,*10*, 60–65, doi:10.1111/lam.12337.
- Ching'anda, C.; Atehnkeng, J.; Bandyopadhyay, R.; Callicott, K.A.; Orbach, M.J.; Mehl, H.L.; Cotty, P.J. Temperature influences on interactions among aflatoxigenic species of *Aspergillus* section *Flavi* during maize colonization. *Front. Fungal Biol.* **2021**, *2*, 1–12, doi:10.3389/ffunb.2021.720276.
- Cotty, P. J.; Bayman, D. S.; Egel, D. S.; Elias, K.S. Agriculture , Aflatoxins and Aspergillus. In *The Genus Aspergillus*; K. Powell, Eds.; Plenum Press: New York, 1994; pp. 1–2.
- Cotty, P. Virulence and cultural characteristics of two A*spergillus flavus* strains pathogenic on cotton. *Phytopathology* **1989**, *79*, 808–814.
- Cotty, P.J.; Bayman, P. Competitive Exculusion of a Toxigenic Strain of Aspergillus Flavus by and Atoxigenic Strain. *Postharvest Pathol. Mycotoxins* **1993**,*83*,1283-1287.
- Cotty, P.J.; Mellon, J.E. Ecology of Aflatoxin producing fungi and biocontrol of aflatoxin contamination. *Mycotoxin Res.* **2006**, *22*, 110–117, doi:10.1007/BF02956774.

- Cotty, P.J. Biocompetitive exclusion of toxigenic fungi. In *The mycotoxin factbook: food and feed topics*; Barug D, Bhatnagar D, van Egmond HP, van der Kamp JW, van Osen- bruggen WA, V.A., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2006; pp. 179–197.
- Cotty, P.J.; Probst, C.; Jaime-Garcia, R. Etiology and management of aflatoxin contamination. In *Mycotoxins: detection methods, management, public health and agri- cultural trade*; Leslie JF, Bandyopadhyay R, V.A., Ed.; CABI Publ: Wallingford, 2008; pp. 287–299.
- Cotty, P.J.; Jaime-Garcia, R. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Int. J. Food Microbiol.* **2007**, *119*, 109–115, doi:10.1016/j.ijfoodmicro.2007.07.060.
- Cotty, P.J.; Antilla, L.; Wakelyn, P.J. Competitive exclusion of aflatoxin producers: farmer-driven research and development. *Biol. Control a Glob. Perspect.* **2007**, 241–253.
- Cotty, P. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the population of *A.flvaus* infecting cotton bolls and on the aflatoxin content of cottonseed. *Biol. Control* **1994**, *84*, 1270–1277.
- Cotty, P.J. Aflatoxin-producing potential of communities of *Aspergillus* Section *flavi* from cotton producing areas in the United States. *Mycol. Res.* **1997**, *101*, 698–704, doi:10.1017/S0953756296003139.
- Cotty, P.J. Comparison of Four media for the isolation of *Aspergillus flavus* group fungi. *Mycopathologia* **1994**, *125*, 157–162, doi:10.1201/b17871.
- Cotty,P.J.; Misaghi I.J. Effect of Light on the Behavior of Alternaria Tagetica in Vitro and in Vivo. *Am. Phytopathol. Soc. Plant Dis.* **1985**, *75*, 366–370.
- Cotty, P.J.; Taylor, D.R. Influence of complementation medium composition on vegetative compatibility analyses of *Aspergillus flavus*. In Proceedings of the Proceedings of the American Phytopathological Society Annual Meeting; 2003; p. 18.
- Das, M.K.; Ehrlich, K.C.; Cotty, P.J. Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. *Phytopathology* **2008**, *98*, 282–288, doi:10.1094/PHYTO-98-3-0282.
- Dorner, J.W. Biological control of aflatoxin contamination of crops. *J. Toxicol. - Toxin Rev.* **2004**, *23*, 425–450, doi:10.1081/TXR-200027877.
- Donner, M.; Lichtemberg, P.S.F.; Doster, M.; Picot, A.; Cotty, P.J.; Puckett, R.D.; Michailides, T.J. Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of california, United States. *Plant Dis.* **2015**, *99*, 1161–1169, doi:10.1094/PDIS-05-14-0450-RE.
- Doster, M.A.; Cotty, P.J.; Michailides, T.J. Evaluation of the atoxigenic *Aspergillus flavus* Strain AF36 in pistachio orchards. *Plant Dis.* **2014**, *98*, 948–956, doi:10.1094/PDIS-10-13-1053-RE.

- Dowd, P.F. Insect management to facilitate preharvest mycotoxin management. *Toxin Rev.* **2003**,*22*, 327–350.
- Eskola, M.; Kos, G.; Elliott, C.T.; Hajšlová, J.; Mayar, S.; Krska, R. Worldwide contamination of food-crops with mycotoxins: validity of the widely cited 'fao estimate' of 25%. *Crit. Rev. Food Sci. Nutr.* **2020**,*60*, 2773–2789, doi:10.1080/10408398.2019.1658570.
- Garcia-Lopez, M.T.; Luo, Y.; Ortega-Beltran, A.; Jaime, R.; Moral, J.; Michailides, T.J. Quantification of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in soil and in nuts and leaves of pistachio by Real-Time PCR. *Plant Dis.* **2021**, *105*, 1–9, doi:10.1094/PDIS-05-20-1097-RE.
- Girolami, F.; Barbarossa, A.; Badino, P.; Ghadiri, S.; Cavallini, D.; Zaghini, A.; Nebbia, C. Effects of Turmeric Powder on Aflatoxin M1 and Aflatoxicol Excretion in Milk from Dairy Cows Exposed to Aflatoxin B1 at the EU Maximum Tolerable Levels. Toxins (Basel). 2022, 14, 1–11, doi:10.3390/toxins14070430.
- Grubisha, L.C.; Cotty, P.J. Genetic analysis of the *Aspergillus flavus* Vegetative Compatibility Group to which a biological control agent that limits aflatoxin contamination in U.S. crops belongs. *Appl. Environ. Microbiol* **2015**, *81*, 5889–5899, doi:10.1128/AEM.00738-15
- Grubisha, L.C.; Cotty, P.J. Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus *Aspergillus flavus*. *Mol. Ecol.* **2010**, *19*, 269–280, doi:10.1111/j.1365-294X.2009.04467.x.
- Hedayati, M.T.; Pasqualotto, A.C.; Warn, P.A.; Bowyer, P.; Denning, D.W. *Aspergillus flavus* : Human pathogen , allergen and mycotoxin producer. *Microbiology.* **2007**, 1677–1692, doi:10.1099/mic.0.2007/007641-0.
- Horn, B.W. Ecology and population biology of aflatoxigenic fungi in soil. *J. Toxicol. - Toxin Rev.* **2003**, *22*, 351–379, doi:10.1081/TXR-120024098.
- Grubisha, L.C.; Cotty, P.J. Twenty-Four microsatellite markers for the aflatoxin-producing fungus *Aspergillus flavus*. *Mol. Ecol. Resour.* **2009**, *9*, 264–267, doi:10.1111/j.1755-0998.2008.02378.x.
- Horn, B.W.; Greene, R.L.; Sobolev, V.S.; Dorner, J.W.; Powell, J.H.; Layton, R.C. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. Parasiticus* , and *A. Tamarii* . *Mycologia* **1996**, *88*, 574–587, doi:10.1080/00275514.1996.12026688.
- Islam, M.S.; Callicott, K.A.; Mutegi, C.; Bandyopadhyay, R.; Cotty, P.J. Distribution of active ingredients of a commercial aflatoxin biocontrol product in naturally occurring fungal communities across kenya. *Microb. Biotechnol.* **2021**, *14*, 1331–1342, doi:10.1111/1751-7915.13708.

- Jaime-Garcia, R.; Cotty, P.J. Spatial distribution of *Aspergillus flavus* and its toxigenic strains on commercial cottonseed from south texas and its relationship to aflatoxin contamination. *Plant Pathol.* **2006**, *55*, 358–366, doi:10.1111/j.1365-3059.2006.01358.x.
- Jaime-Garcia, R.; Cotty, P.J. Crop rotation and soil temperature influence the community structure of *Aspergillus flavus* in soil. *Soil Biol. Biochem.* **2010**, *42*, 1842–1847, doi:10.1016/j.soilbio.2010.06.025.
- Jaime, R.; Leisner, L.; Antilla, L.; Mehl, H.L.; Cotty, P.J. Area-Wide programs for aflatoxin mitigation: treatments to cotton can be cost effective. In Proceedings of the Proceedings of the Beltwide Cotton Prod. Res. Conference; **2017**; pp. 327–350.
- Khan, R.; Ghazali, F.M.; Mahyudin, N.A.; Samsudin, N.I.P. Aflatoxin biosynthesis, genetic regulation, toxicity, and control strategies: A Review. *J. Fungi* **2021**, *7*,1-13, doi:10.3390/jof7080606.
- Klich, M. A.; Pitt, J. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Trans. Br. Mycol. Soc.* **1988**, *91*, 99–108, doi:10.1016/S0007-1536(88)80010-X.
- Leggieri, M.C.; Toscano, P.; Battilani, P. Predicted aflatoxin B1 increase in Europe due to climate change: Actions and reactions at global level. *Toxins* **2021**, *13*, 1–21, doi:10.3390/TOXINS13040292.
- Leslie, J.F. Fungal Vegetative Compatibility. *Annu. Rev. Phytopathol.* **1993**, *31*, 127–150, doi:10.1146/annurev.py.31.090193.001015.
- Mauro, A.; Battilani, P.; Cotty, P.J. Atoxigenic *Aspergillus flavus* endemic to Italy for biocontrol of aflatoxins in maize. *BioControl* **2015**, *60*, 125–134, doi:10.1007/s10526-014-9624-5.
- Mauro, A.; Garcia-cela, E.; Pietri, A.; Cotty, P.J.; Battilani, P. Biological control products for aflatoxin prevention in italy : commercial field evaluation of atoxigenic *Aspergillus flavus* active ingredients. *Toxins* **2018**,*10*,1–14, doi:10.3390/toxins10010030.
- Mahuku, G.; Mauro, A.; Pallangyo, B.; Nsami, E.; Boni, S.B.; Koyano, E.; Mponda, O.; Ortega-Beltran, A.; Atehnkeng, J.; Aquiline, F. Atoxigenic-based technology for biocontrol of aflatoxin in maize and groundnuts for tanzania. *World Mycotoxin J.* **2022**, 1–16, doi:10.3920/wmj2021.2758.
- Mauro, A.; Battilani, P.; Callicott, K.A.; Giorni, P.; Pietri, A.; Cotty, P.J. Structure of an *Aspergillus flavus* population from maize kernels in northern italy. *Int. J. Food Microbiol* **2013**, *162*, 1–7, doi:10.1016/j.ijfoodmicro.2012.12.021.
- Marchese, S.; Polo, A.; Ariano, A.; Velotto, S.; Costantini, S.; Severino, L. Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins (Basel).* **2018**, *10*, 1–19, doi:10.3390/toxins10060214.

- Mehl, H.L.; Jaime, R.; Callicott, K.A.; Probst, C.; Garber, N.P.; Ortega-Beltran, A.; Grubisha, L.C.; Cotty, P.J. *Aspergillus flavus* diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. *Ann. N. Y. Acad. Sci.* **2012**, *1273*, 7–17, doi:10.1111/j.1749-6632.2012.06800.x.
- Mehl, H.L.; Cotty, P.J. Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection. *Phytopathology* **2010**, *100*, 150–159, doi:10.1094/PHYTO-100-2-0150.
- Meier, U. Growth Stage of mono- and dicoyle-donous plants. BBCH monograph. In *Growth stage of mono- and dicoyle-donous plants. BBCH Monograph*; Meier, U., Ed.; Julius Kuhn-Institute (JKI): Quedlinburg, Germany, **2018** ISBN 9783955470715.
- Molo, M.S.; White, J.B.; Cornish, V.; Gell, R.M.; Baars, O.; Singh, R.; Carbone, M.A.; Isakeit, T.; Wise, K.A.; Woloshuk, C.P.; Bluhm, B.H.; Horn. B.W.; Heiniger, R.W.; Carbone, I. Asymmetrical Lineage introgression and recombination in populations of *Aspergillus flavus*: Implications for biological control. *PLoS One* **2022**, *10*, 1–41, doi:10.1371/journal.pone.0276556.
- Moral, J.; Garcia-Lopez, M.T.; Camiletti, B.X.; Jaime, R.; Michailides, T.J.; Bandyopadhyay, R.; Ortega-Beltran, A. Present status and perspective on the future use of aflatoxin biocontrol products. *Agronomy* **2020**, *10*, doi:10.3390/agronomy10040491.
- Ojiambo, P.S.; Battilani, P.; Cary, J.W.; Blum, B.H.; Carbone, I. Cultural and genetic approaches to manage aflatoxin contamination: recent insights provide opportunities for improved control. *Phytopathology* **2018**, *108*, 1024–1037, doi:10.1094/PHYTO-04-18-0134-RVW.
- Ortega-Beltran, A.; Grubisha, L.C.; Callicott, K. a.; Cotty, P.J. The Vegetative Compatibility Group to which the us biocontrol agent *Aspergillus flavus* AF36 belongs is also endemic to Mexico. *J. Appl. Microbiol.* **2016**, *120*, 986–998, doi:10.1111/jam.13047.
- Ortega-Beltran, A.; Kaptoge, L.; Senghor, A.L.; Aikore, M.O.S.; Jarju, P.; Momanyi, H.; Konlambigue, M.; Falade, T.D.O.; Bandyopadhyay, R. Can it be all more simple? manufacturing aflatoxin biocontrol products using dry spores of atoxigenic isolates of *Aspergillus flavus* as active ingredients. *Microb. Biotechnol.* **2021**, doi:10.1111/1751-7915.13802.
- Ortega, S.F.; Siciliano, I.; Prencipe, S.; Gullino, M.L.; Spadaro, D. Development of PCR, LAMP and QPCR assays for the detection of aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* in hazelnut. *Toxins.* **2020**, *12*, 1–27, doi:10.3390/toxins12120757.
- Perrone, G.; Gallo, A.; Logrieco, A.F. Biodiversity of *Aspergillus* Section *Flavi* in Europe in relation to the management of aflatoxin risk. *Front. Microbiol.* **2014**, *5*, 1–5, doi:10.3389/fmicb.2014.00377.

- Perrone, G.; Ferrara, M.; Medina, A.; Pascale, M.; Magan, N. Toxigenic fungi and mycotoxins in a climate change scenario: ecology, genomics, distribution, prediction and prevention of the risk. *Microorganisms* **2020**, *8*, 1–20, doi:10.3390/microorganisms8101496.
- Piva, G.; Battilani, P.; P. Emerging issues in southern Europe: Aflatoxins in Italy. In *Emerging issues in southern Europe: aflatoxins in Italy*; Barug, D.; Bhatnagar, D.; Van Egmond, H.P.; Van der Kamp, J.W.; Van Ossenbruggen, W.A.; Visconti, A., Eds.; Wageningen Academic Publishers: Wgeningen, the Netherland, 2006; pp. 139–135.
- Probst, C.; Bandyopadhyay, R.; Price L.E.; Cotty, P.J. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in kenya. *Plant Dis.* **2011**, *95*, 212–218.
- Probst, C.; Cotty, P.J. Relationships between in *vivo* and in *vitro* aflatoxin production: Reliable prediction of fungal ability to contaminate maize with aflatoxins. *Fungal Biol.* **2012**, *116*, 503–510, doi:10.1016/j.funbio.2012.02.001.
- Reis, T.A.; Oliveira, T.D.; Zorzete, P.; Faria, P.; Corrêa, B. A non-toxigenic *Aspergillus flavus* strain prevents the spreading of fusarium verticillioides and fumonisins in maize. *Toxicon* **2020**, *181*, 6–8, doi:10.1016/j.toxicon.2020.04.091.
- Rodríguez, A.; Rodríguez, M.; Luque, M.I.; Martín, A.; Córdoba, J.J. Real-Time PCR assays for detection and quantification of aflatoxin-producing molds in foods. *Food Microbiol.* **2012**, *31*, 89–99, doi:10.1016/J.FM.2012.02.009.
- Sengun, I.; Yaman, D.; Gonul, S. Mycotoxins and mould contamination in cheese: A Review. *World Mycotoxin J.* **2008**, *1*, 291–298, doi:10.3920/wmj2008.x041.
- Senghor, L.A.; Ortega-Beltran, A.; Atehnkeng, J.; Callicott, K.A.; Cotty, P.J.; Bandyopadhyay, R. The Atoxigenic biocontrol product aflasafe SN01 is a valuable tool to mitigate aflatoxin contamination of both maize and groundnut cultivated in senegal. *Plant Dis.* **2019**, PDIS-03-19-0575, doi:10.1094/pdis-03-19-0575-re.
- Sepahvand, A.; Shams-ghahfarokhi, M. A survey on distribution and toxigenicity of *Aspergillus flavus* from indoor and outdoor hospital environments. *Folia Microbiol.* **2011**, *56*, 527–534, doi:10.1007/s12223-011-0078-1.
- Shapira, R.; Paster, N.; Eyal, O.; Menasherov, M.; Mett, A.; Salomon, R. Detection of aflatoxigenic molds in grains by PCR. *Appl. Environ. Microbiol.* **1996**, *62*, 3270–3273, doi:10.1128/aem.62.9.3270-3273.1996.
- Singh, P.; Cotty, P.J. Aflatoxin contamination of dried red chilies: contrasts between the united states and nigeria, two markets differing in regulation enforcement. *Food Control* **2017**, *80*, 374–379, doi:10.1016/j.foodcont.2017.05.014.
- Walte, H.-G.; Knappstein, K.; Maul, R.; Steinberg, P. Re-Evaluation of Aflatoxin M1 Transfer

into Milk of High-Yielding Cows Considering Ration Composition. J. Anim. Feed Sci. 2022, 343–351, doi:10.22358/jafs/151045/2022.

- Weaver, M.A.; Abbas, H.K. Field displacement of aflatoxigenic *Aspergillus flavus* strains through repeated biological control applications. *Front. Microbiol.* **2019**, *10*, 1–7, doi:10.3389/fmicb.2019.01788.
- World Health Organistaion; International Agency for Research on Cancer. Aflatoxins. In *IARC Monographs on the Evaluation of* 443 *carcinogenc Risks to Humans;* IARC Press: Lyon, France, **2002**; Volumes 82, pp. 171-300.
- Zuluaga-montero, A.; Rami, L.; Rauscher, J.; Bayman, P. Marine Isolates of *Aspergillus flavus* : Denizens of the Deep or Lost at Sea ? *Fungal Ecol.* **2010**, *3*, 5–10, doi:10.1016/j.funeco.2010.05.003.

Chapter 3:

Structure of Aspergillus flavus populations associated with maize in Greece, Spain, and Serbia; implications for aflatoxin biocontrol on a regional scale

Mohamed Ali Ouadhene[1], Kenneth A. Callicott[2], Alejandro Ortega-Beltran[3], Hillary L. Mehl[2], Peter J. Cotty[4] and Paola Battilani[1*]

[1] **Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy**

[2] **United States Department of Agriculture, Agricultural Research Service, Tucson, AZ, United States**

[3] **International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria**

[4] **College of Food Science and Engineering, Ocean University of China, Qingdao, China**

Note: The present research work was done in collaboration with Corteva Agriscience (maize grain sampling); the International Institute of Tropical Agriculture (*A.flavus* isolation and DNA extraction) and USDA's Tucson Aflatoxin Biocontrol Lab in Arizona (SSR genotyping analysis)

Abstract

The ubiquitous fungus *Aspergillus flavus* is the most frequently identified producer of aflatoxins. This species is divided into two morphotypes, the L and S strains, with the L strain being the most widely distributed. Members of the L strain without ability to produce aflatoxins, atoxigenic strains, are used on several continents as active ingredients of biopesticides directed at preventing aflatoxin contamination. However, the diversity of the *A. flavus* L strain in Europe has not received detailed attention. The current research examined genetic diversity of *A. flavus* across southern Europe in order both to gain insight on this species population structure and evolution in Europe and to evaluate the prevalence of MUCL54911, the active ingredient of the commercial biocontrol product AF-X1. *A.flavus* isolates (2,173) recovered from maize collected across Greece, Spain, and Serbia in 2020 and 2021 were subjected to SSR genotyping which revealed high diversity within and among countries and dozens of haplotypes shared among countries. Linkage disequilibrium analysis showed the asexual reproduction and clonal evolution of *A. flavus* resident in southern Europe. Moreover, haplotypes closely related to MUCL54911 were found to belong to the same vegetative compatibility group (VCG) and that group, IT006, was common in all three countries. The results indicate that IT006 is endemic and well-adapted to the environment of southern Europe and should be utilized as an aflatoxin mitigation tool for maize across the region.

Key words: Aflatoxins, *Aspergillus flavus*, Genetic diversity, Biocontrol, atoxigenic strain

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Introduction

Maize (*Zea mays* L.) is one of the world's most important crops in terms of production, international trade, and provision of calories for livestock and humans. Maize is an important component of international trade and contributes to the socio-economic balance in many countries (Wu, 2015). However, maize is susceptible to aflatoxin contamination from crop development through food until ultimately consumed (Palumbo et al., 2020; Strosnider et al., 2006). Aflatoxins frequently occur in tropical and subtropical regions, while in Europe aflatoxin outbreaks were first identified in 2003 (Piva et al., 2006) and ultimately considered as an emerging problem due to climate change, particularly in maize-growing regions of southern Europe including Italy, Spain, Greece, and Serbia (Battilani et al., 2016; Curtui et al., 2004; Moretti et al., 2019). Aflatoxin contamination of maize is now recurrent, with aflatoxin concentration in some years sufficiently high to interfere with commercial use of the crop (Dobolyi et al., 2013; Levic et al., 2013; Udovicki, 2019).

Aflatoxins (AFB1, AFB2, AFG1, AFG2, and AFM1) are particularly potent mutagenic and carcinogenic mycotoxins that are naturally produced by several species in *Aspergillus* section *Flavi* (Janić Hajnal et al., 2017; JECFA, 2017). AFB1 is a highly potent liver carcinogen in humans and several domestic animal species. AFB1 is also immunotoxic and hepatotoxic and contributes to impaired productivity and reproductive efficiency in livestock (IARC, 2007; Kollia et al., 2017; Valencia-Quintana et al., 2020).

Aspergillus flavus, the most commonly reported aflatoxin-producer, is divided into two morphologically distinct strains, the L and S strains, with the L strain most readily identified and widely distributed (Cotty, 1989; Cotty et al., 2008). This haploid species is well known as an abundant saprophyte and opportunistic pathogen and is widely distributed in warm environments (Klich, 2002; Horn, 2003; Grubisha and Cotty, 2009). The life cycle of this asexual species is characterized by profuse production of haploid conidia (Papa, 1986; Islam et al., 2018). However, several studies have reported that sexual recombination occurs under experimental conditions between *A. flavus* genotypes with different mating type loci. The two mating types, MAT1-1 and MAT1-2, co-occur in natural populations (Geiser et al., 1998; Pál et al., 2007; Ramirez-prado et al., 2008; Horn et al., 2009; Moore et al., 2009; Olarte et al., 2012), but the frequency of sexual reproduction in natural populations is less clear.

Genetic diversity of *A. flavus* is high, with many different genotypes that can produce aflatoxins at varying levels (Chang and Ehrlich, 2010). Natural populations of *A. flavus* are complex and still not fully described (Grubisha and Cotty, 2009). Isolates can be segregated into many vegetative compatibility groups (VCGs) on the basis of complementation between nitrate non-utilizing auxotrophs (Bayman and Cotty, 1991,1993). Genetic information can be exchanged within a VCG through an asexual recombination process referred to as the parasexual cycle (Papa, 1986; Leslie, 1993; Ehrlich et al., 2007; Grubisha and Cotty, 2015), that includes hyphal fusions between fungi with identical heterokaryon incompatibility alleles. Characterizing genetic diversity within *A. flavus* has allowed identification of VCGs containing exclusively non-aflatoxigenic ("atoxigenic") strains. Naturally occurring, native atoxigenic strains have been used for decades to displace aflatoxin producers as a form of biocontrol that is highly effective at reducing aflatoxin contamination in the United States, Africa, and Europe (Italy) (Cotty, 1994; Dorner and Lamb, 2006; Mauro et al., 2018; Ojiambo et al., 2018; Moral et al., 2020;). Assessment of genetic diversity in *A. flavus* populations is often carried out in order to ensure that exotic strains with potentially negative ecological effects are not introduced into target agroecosystems (Probst et al., 2011; Mehl et al., 2012). Such characterization also allows for identification of *A. flavus* VCGs particularly well adapted to crop practices in target agroecosystems (Islam et al., 2020a).

In Europe, investigations into the composition of *Aspergillus* section *Flavi* communities have provided useful information on the relative role of key species in the contamination process as well as the ability of specific fungi to produce sclerotia and aflatoxins (Giorni et al., 2007). Work on distributions of *A. flavus* L strain fungi in maize growing regions in Italy (Mauro et al., 2013, 2018) identified an atoxigenic isolate of *A. flavus*, MUCL54911, belonging to VCG IT006, which is an effective biocontrol active ingredient that has been selected and then validated in laboratory and field trials, where a product utilizing this genotype reduced aflatoxin contamination in the field by over 90%. The product utilizing MUCL54911 is currently commercialized under the name AF-X1.

Despite numerous studies of diversity in *A. flavus* in several countries, little information is available on *A. flavus* L strain population structure in Europe (Gallo et al., 2012; Perrone et al., 2014). Prior studies have developed numerous methods to study *A. flavus* diversity, and these have been used to determine the predominant causal agent of aflatoxin contamination of various crops

and to optimize selection of atoxigenic genotypes in target agroecosystems (Mehl et al., 2012; Alejandro Ortega-Beltran et al., 2020; Vlajkov et al., 2021). Vegetative compatibility analysis (VCA) using pairs of complementary nitrate non-utilizing auxotrophs to test membership of *A. flavus* isolates in a specific VCG have been used both to characterize populations of *A. flavus* (Bayman and Cotty, 1991) and define atoxigenic *A. flavus* active ingredient of biocontrol products (Cotty, 1994; Ehrlich and Cotty, 2004). Despite its accuracy, VCA is time-consuming and laborious (Das et al., 2008; Sweany et al., 2011). Consequently, several molecular methods have been developed to characterize *A. flavus* isolates more rapidly. To identify atoxigenic isolates, cluster amplification pattern markers are used to monitor large deletions in the aflatoxin biosynthesis gene cluster of *A. flavus* through multiplex PCR (Callicott and Cotty, 2015; Vlajkov et al., 2021). In addition, to characterize *A. flavus* populations on a finer scale, detect diversity within VCGs and contrast competitiveness and adaptability among specific genotypes of interest, many typing schemes using simple sequence repeats (SSR) or inter-simple sequence repeats (ISSRs) have been developed (Tran-Dinh and Carter, 2000; Grubisha and Cotty, 2010; Hadrich et al., 2010; Hatti et al., 2010; Sweany et al., 2011; Wang et al., 2012; Molo et al., 2022).

The current study examined the population structure and genetic diversity of *A. flavus* recovered from maize growing areas in three European countries considered hotspots for aflatoxin contamination: Greece, Spain, and Serbia. Seventeen SSR markers developed by Grubisha and Cotty, (2009) and used to characterize *A. flavus* populations in Africa and North America (Grubisha and Cotty, 2010; Ortega-Beltran et al., 2016; Islam et al., 2018, 2020) were applied to European populations for the first time in order to I) study genetic diversity among and within countries, II) obtain insight into divergence of *A. flavus* populations and distribution of common haplotypes among the three countries, and III) investigate the frequency and distribution of MUCL54911, the active ingredient of AF-X1 to support efforts to extend regulatory approval for use of AF-X1 beyond Italy, the country from which the active ingredient was initially isolated.

Material and methods

Maize sample collections

Grain samples were collected in areas known to have periodic *A. flavus* contamination across Greece (n=128), Spain (n=153), and Serbia (n=165) (Figure 1). The grain was sampled either from the combine at harvest or upon receipt at an elevator before the drying process. Grain

collection was initiated in late August 2020 and early September 2021. Each sample consisted of 30 sub-samples (about 100 g of kernels, 3 kg total). After drying, samples were stored at 0-5°C and shipped to Italy within 3 days. Grain samples were ground, mixed to homogenize, partitioned into 2 aliquots of ~100g, and stored at 5°C until processing for mycotoxin analysis and fungal isolation.

Figure 1. Geographical distribution of the samples collected from Greece (128), Spain (153) and Serbia (165) during maize harvest in either 2020 or 2021

Mycotoxins analysis

For each sample, 5 g flour was mixed with 20 ml acetonitrile/water/formic acid solution (59:20:1), vortexed for 30 min at 2500 rpm, and passed through a FPTE 0.20 µm filter. Mycotoxins were separated by loading 7 µl of the extract into an Ultimate HPLC machine (Thermo Scientific, Milford, MA, USA). Mycotoxins were identified with a calibrated Liquid Chromatography-Mass Spectrometer (LC-MS) coupled with a Q Exactive Focus Orbitrap (Thermo Scientific). Aflatoxin concentrations were reported as the sum of AFB1, AFB2, AFG1, and AFG2. The limits of detection were 0.9 µg/kg and 2.71 µg/kg for LOD and LOQ, respectively.

Aspergillus flavus isolation

Maize flour was serially diluted and plated on modified Rose Bengal agar (3 g sucrose, 3 g $NaNO_3$, 0.75 g KH_2PO_4, 0.25 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 10 g NaCl, 1 mL of micronutrients, 0.025 g Rose Bengal, 0.05 g chloramphenicol, 0.05 g streptomycin, 0.01 g dichloran , 20 g Bacto agar, 1 l water) (Cotty, 1994). After 3-5 days of incubation at 35°C in the dark (Cotty, 1994), colony forming units (CFU/g) were counted, both for all fungi in section *Flavi* and for *A. flavus.* Plates with less than 10 colonies were selected to recover *A. flavus* isolates, with no more than three isolates taken from any plate and up to 15 isolates per sample/field. Serial dilution was repeated to get a suitable number of isolates per plate. Selected isolates were transferred to 5/2 Agar (5% V8 juice and 2% agar, pH 6.0) and incubated for 5-7 days at 31°C (Jaime-Garcia and Cotty, 2004). Single spore isolation was done on the total set of 2,173 isolates of *A. flavus* recovered from Greece, Spain, and Serbia; all monosporic isolates were saved in water vials for further use (Mauro et al., 2013; Ortega-Beltran and Cotty, 2018).

DNA extraction

All single spore isolates were grown on 5/2 agar and incubated for 7 days at 31°C. Spores were harvested from colonies by swab, after which DNA was extracted following the protocol of Callicott and Cotty, 2015. In brief, spores were lysed in a buffer containing detergent and EDTA using a combination of heat and agitation. DNA was then extracted using a standard ethanol-ammonium acetate precipitation. DNA concentration was determined with a NanoDrop 2.0 spectrophotometer (ThermoFisher, Wilmington, DE, USA) and diluted to adjust the concentration to 5 ng/µl.

SSR genotyping

Seventeen SSR loci were examined in this study (AF28, AF13, AF43, AF22, AF31,AF53, AF34, AF42, AF8, AF16, AF54, AF17, AF11, AF66, AF64, AF63 and AF55) previously characterized by Grubisha and Cotty, (2009). SSR analyses followed the protocol described by Islam et al. (2018). SSRs amplicons were free of PCR artifacts and had single peaks in the expected size range per locus based on Grubisha and Cotty, (2009). Amplification and data analysis were performed by the USDA's Tucson Aflatoxin Biocontrol Lab in Arizona and amplified markers were separated on an ABI 3730 at the University of Arizona's Arizona Genetics Core in Tucson, AZ.

Population genetic analyses

Two Italian isolates (MUCL54911 and MPVP A2321) were included in the SSR analyses as references. The atoxigenic strain MUCL54911 belongs to VCG IT006 (Mauro et al., 2013) and possesses the MAT1-1 mating idiomorph. MPVP A2321 is atoxigenic and has the MAT1-2 idiomorph (Mauo et al., 2018).

For population analyses, incomplete SSR genotypes were excluded. The remaining SSR data from 2,011 isolates out of 2,173 and the two reference isolates (MUCL54911 and MPVP A2321) was processed with GENODIVE 3.06 (Meirmans, 2020). SplitsTree 4.8 (Huson and Bryant, 2006) was used to generate neighbor net trees using the Cavalli-Sforza chord distance matrix generated by GENODIVE, following the protocol described by Ortega-Beltran et al. (2020). GENODIVE 3.06 was also used to quickly identify both shared and closely related multilocus genotypes among the three countries using a selected threshold among genetic distances. Haplotypes closely related to the reference isolates were identified.

All isolates of *A. flavus* were sorted by province and country. Where sample size was less than 10 individuals, two or more adjacent provinces were combined to produce more reliable estimates of genetic diversity. GenAlEx version 6.5 (Peakall and Smouse, 2012) was used to evaluate number of alleles, number of private alleles and haploid genetic diversity (H) and to produce a principal coordinate analysis (PCoA) based on pairwise genetic distance matrix (Alejandro Ortega-Beltran et al., 2020; Weaver et al., 2022)

HAPLOTYPE-ANALYSIS version 1.05 (Eliades and Eliades, 2009) was used to identify multilocus SSR haplotypes (genotypes), their frequency within and among the populations, the number of private haplotypes (P_h), the number of different haplotypes observed (Nh, individual population contribution to genetic diversity within populations ($H_S(j)$), and individual population contribution to the total diversity among populations ($D_{ST}(j)$) (Finkeldey and Murillo, 1999).

To determine the genetic relationships among countries, discriminant analysis of principal components (DAPC) was generated by the *adegenet* package (Jombart, 2008; Jombart and Ahmed, 2011) in R software. This clustering analysis method was used for performing the first PCA, followed by a discriminant analysis on the PCA scores. The function *find.cluster*s in R was used to determine the right number of retained principle components for the DAPC analysis (Jombart et al., 2010). G'_{ST} (Hedrick, 2005) was used to determine the standardized genetic differentiation

among alleles and was obtained from mmod package (Winter, 2012) in R. The *poppr* package (Kamvar et al., 2014) in R was used to evaluate the evenness of genotype frequency within groups based on the E_5 calculation (Grünwald et al., 2003).

Linkage disequilibrium (LD) analysis was used to estimate the degree of clonality within populations. Multilocus genotypic LD within each country was calculated after clone correction by the *poppr* package using the unbiased estimator $\bar{r}_d$ (Agapow and Burt, 2001). Analysis of Molecular Variance (AMOVA; Excoffier et al., 2005) using *poppr* was performed to estimate population differentiation.

Vegetative compatibility analysis (VCA)

The neighbor net trees revealed that 19 haplotypes from Greece, Spain, and Serbia were closely related to MUCL54911 and were evaluated for membership in VCG IT006. These haplotypes differed from the haplotype of MUCL 54911 at only 1 to 3 loci (Table 4). To determine whether a haplotype belongs to VCG IT006, isolates from the selected haplotype were subjected to VCA using previously generated IT006 tester pair mutants (*cnx* and *NiaD*), following the protocol described by (Das et al., 2008)

Results

Aflatoxin contamination, fungal densities, and number of isolates recovered.

The percentage of grain samples positive for aflatoxin was 5% and 6% in Greece, 0% and 2% in Spain, and 0% and 35% in Serbia, for 2020 and 2021, respectively. The maximum contamination was 52.6 µg/kg (mean 15.4 µg/kg in samples with detectable aflatoxins) in Greece. In Serbia, one sample from 2021 contained 1,148 µg/kg total aflatoxins. However, the mean aflatoxin concentration in samples with detectable aflatoxins in Serbia was 109.5 µg/kg. Only one Spanish sample was positive for aflatoxins with 2.64 µg/kg. The mean *A. flavus* CFU/g was similar in the three countries: 4.3×10^3 in Greece, 1.6×10^3 in Spain, and 7.8×10^3 in Serbia. A total of 800 (Greece), 627 (Spain), and 758 (Serbia) *A. flavus* isolates were recovered and used for analyses.

Allelic and haplotypic diversity

SSR loci were found to be highly variable in amplicon size, with individual loci having between eight and 47 unique alleles (Table 1). High genetic diversity was detected among the 2,011 isolates from the three countries. Haploid diversity (H) per locus ranged from 0.190 to 0.723. Evenness, which describes how evenly alleles for each SSR marker were divided, ranged from 0.40 to 0.79. Additionally, G'_{ST} of each marker varied between 0.134 and 0.666 (Table 1).

Table 1. Characteristics of 17 SSR makers on 2,011 isolates of *A. flavus* recovered from maize sampled from Greece, Spain, and Serbia

Locus Name	Repeat Motif and Scaffold (Grubisha and Cotty, 2009)	Alleles *	Size range (bp)**	Diversity (H)†	Evenness ††	G'_{ST} †††
AF8	$(AAG)_{16}$/2911	35	147-267	0.722	0.64	0.664
AF11	$(AAG)_{12}$/2504	38	103-281	0.683	0.54	0.486
AF13	$(CTT)_{9}$/1866	23	115-200	0.669	0.67	0.627
AF16	$(TTG)_{10}$/2541	22	161-393	0.437	0.54	0.450
AF17	(AGA)4 $(AGG)_{10}$/1918	18	330-405	0.690	0.79	0.561
AF22	$(TTTA)_{8}$/2911	12	144-208	0.537	0.63	0.487
AF28	$(TTG)_{11}$/2504	15	110-161	0.455	0.63	0.460
AF31	$(TTC)_{31}$/2634	32	290-415	0.588	0.41	0.478
AF34	(GTC)4 $(GTT)_{8}$/2911	22	290-425	0.561	0.67	0.476
AF42	$(TTC)_{16}$/2634	34	139-336	0.666	0.61	0.583
AF43	$(GAG)_{13}$/2634	30	365-451	0.723	0.65	0.666
AF53	$(TCT)_{8}$/1918	17	126-182	0.523	0.54	0.468
AF54	$(ACAT)_{8}$/1918	9	145-192	0.190	0.40	0.267
AF55	$(GT)_{10}$/1739	23	159-212	0.702	0.76	0.589
AF63	$(AT)_{7}$/2856	8	121-137	0.217	0.40	0.134
AF64	$(AC)_{16}$/2856	47	153-271	0.682	0.46	0.602
AF66	$(AT)_{12}$/1569	14	198-279	0.589	0.78	0.543

* Number of Alleles at the SSR locus
** Range of SSR size based on the variation at SSR repeat numbers across the isolates included in this study
†Per locus haploid genetic diversity (H) generated from the program GenAlEx6.5 (Peakall and Smouse, 2012)
†† Evenness obtained from the poppr package in R
††† Standardized genetic differentiation (G'_{ST}; Hedrick, 2005) obtained from mmod package in R

High haplotypic diversity was seen in Greece, Spain, and Serbia, with 363, 134, and 209 haplotypes observed from 766, 574, and 671 isolates, respectively (Table 2). When the three countries were analyzed together, only 645 haplotypes overall were detected, illustrating the large number of haplotypes present in more than one country (Figure 2). Evenness, computed on haplotypes within the countries (Table 2; Grünwald et al., 2003) were 0.440, 0.412, and 0.483 for Greece, Spain, and Serbia, respectively.

Table 2. Overview of the genetic diversity of *A. flavus* recovered from three countries Greece, Spain and Serbia during the 2020 and 2021 growing seasons.

Country	N	*A priori* populations	Ncc	Nh	P_h	H_S	H′	E_5
Greece	766	23	511	363	304	0.906	1.29	0.440
Spain	574	15	261	134	98	0.872	1.33	0.412
Serbia	671	23	355	209	154	0.857	0.98	0.483
Three countries	2,011	61	1127	645	504	0.880	1.18	0.364

N, the total number of isolates
Ncc, number of isolates after clone correction by using poppr package in R (Kamvar et al., 2014)
Nh, number of haplotypes
P_h, number of private haplotypes
H_S , within population genetic diversity from HAPLOTYPE-ANALYSIS V1.04
H(Shannon, 1948), the Shannon information index calculated by GenAlex 6.503
E_5 (Grűnwald et al., 2003), evenness calculated using the poppr package in R

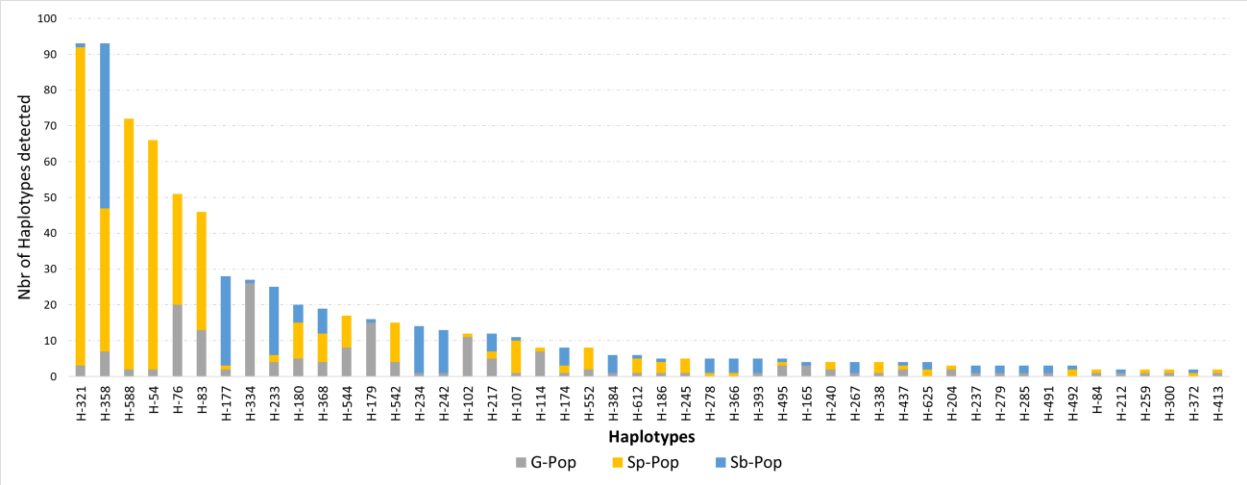


Figure 2. Distribution of the most frequent haplotypes detect in multiple countries (G-Pop: Greece; Sp-Pop: Spain and Sb-Pop: Serbia). Not shown are the many singleton haplotypes present in the dataset.

Clone correction by population illustrates the extent of haplotype diversity, with the number of haplotypes equating to 71%, 51%, and 59% of the clone corrected samples for Greece, Spain, and Serbia, respectively. The Spanish population had the most haplotypes shared among multiple populations, and this resulted in 55% of the original isolates eliminated through population-based clone correction. Similar corrections resulted in elimination of 33% for Greece and 47% for Serbia. After clone correction, gene diversity (H_S) among countries ranged from 0.857 to 0.906 with Greece being the most diverse (Table 2). The contribution of samples to within-population the $H_S(j)$ ranged from 0.001 to 0.043 while the $D_{ST}(j)$ was ranged from 0 to 0.005 (Figure 3). Higher $H_S(j)$ indicates those samples are more diverse, while higher $D_{ST}(j)$ indicates that those samples are more diverged from the other samples. Samples G_pop4, Sp_Pop7, and Sb_pop15 represent samples with higher internal diversity, while samples such as G_pop19 Sp_Pop7, and Sb_pop2 are less like other samples in the dataset (Figure 3). At the country level, the estimation of Shannon-Wiener diversity index H (Table 2) among the populations per country reflects high haplotypic diversity for all three countries (Greece = 1.29, Spain = 1.33 and Serbia = 0.98 respectively), with no dominance by any haplotype.

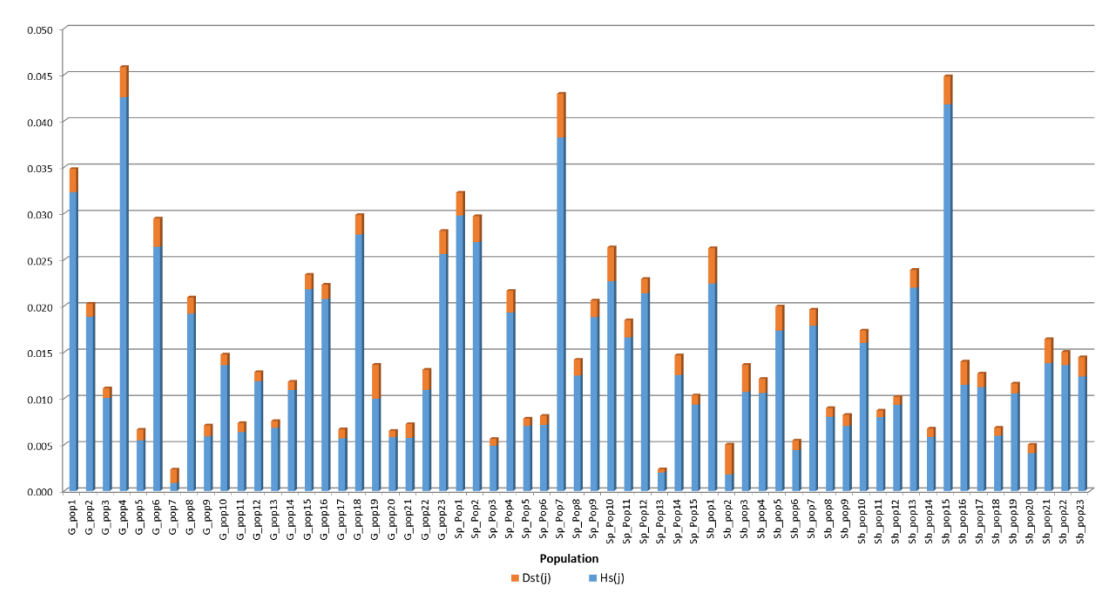


Figure 3. Contribution of each population to the genetic differentiation within ($H_S(j)$) and between ($D_{ST}(j)$) populations from each country (Greece= 23 populations; Spain= 15 population and Serbia= 23 population)

Population structure, reproduction and evolution

PCoA of the isolates showed a largely similar makeup among the three countries, with the most common group present in all populations (Figure 4). The two axes explain 82.56% of the variation in the dataset. Likewise, DAPC (Figure 5) showed extensively overlapping genetic variation, but it also revealed different central tendencies for each country, indicating significant genetic differentiation (Figure 6) even as these populations remain closely related. This genetic divergence can also be seen in an AMOVA performed on the entire dataset using countries and populations within countries as additional strata (Table 3).

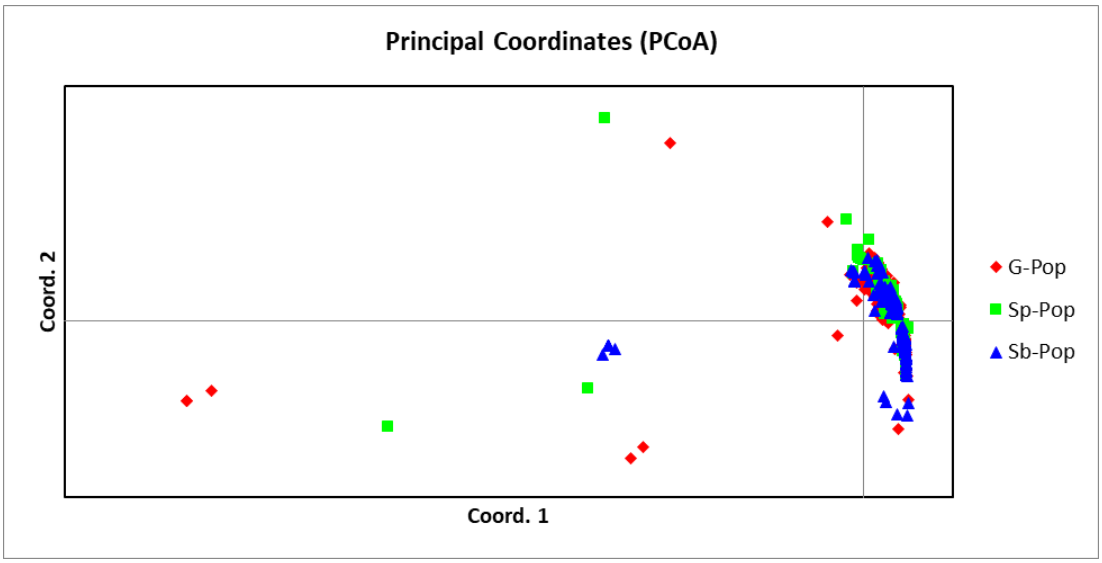


Figure 4. Principal coordinate analysis of *A. flavus* SSRs. Individual points represent individual haplotypes *A. flavus* obtained from three different countries Greece (red, G-Pop), Spain (green, Sp-Pop) and Serbia (blue, Sb-Pop)f. Principle coordinates 1 and 2 explain 68.6% and 13.95% of the genetic variation, respectively

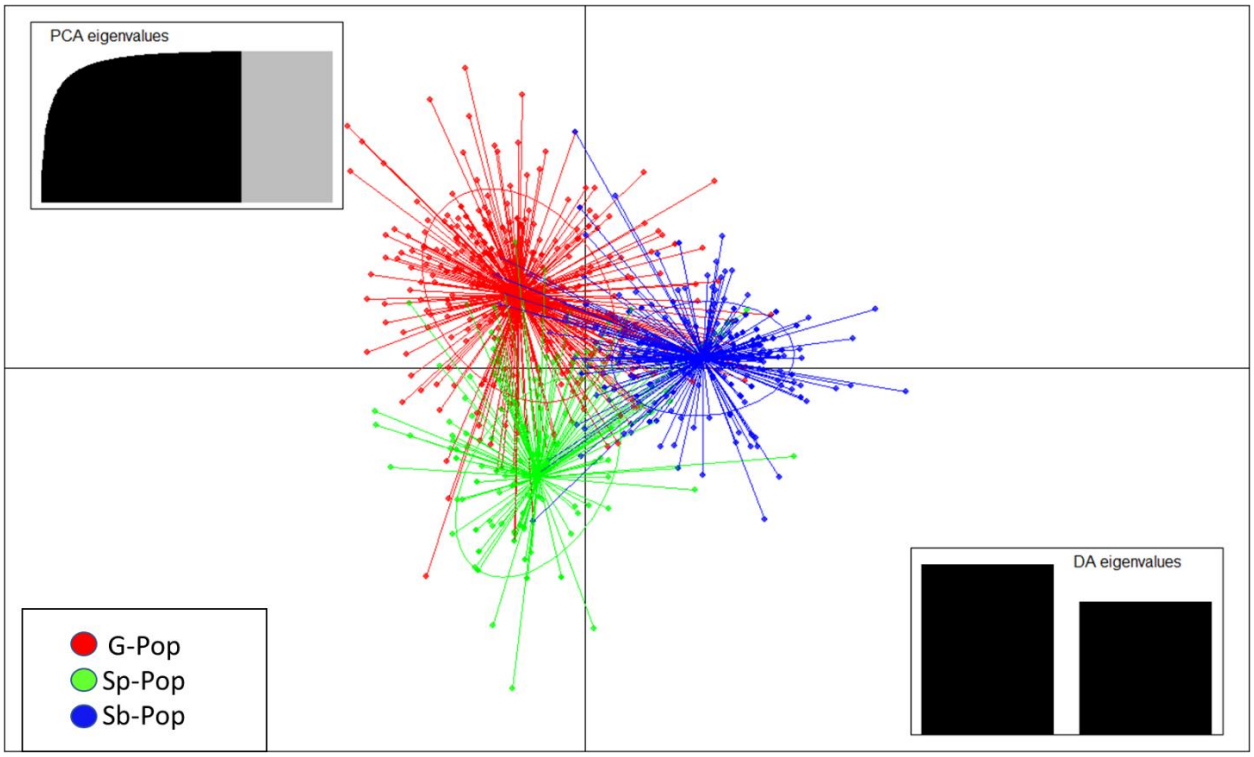


Figure 5. Scatter plot of three countries based on discriminant analysis of principle components (DAPC) on *Aspergillus flavus* isolates recovered from maize flour samples collected across three countries: Greece (red, G-Pop= 766 isolates), Spain (Green, Sp-Pop= 574 isolates) and Serbia (Blue, Sb-Pop= 671 isolates)

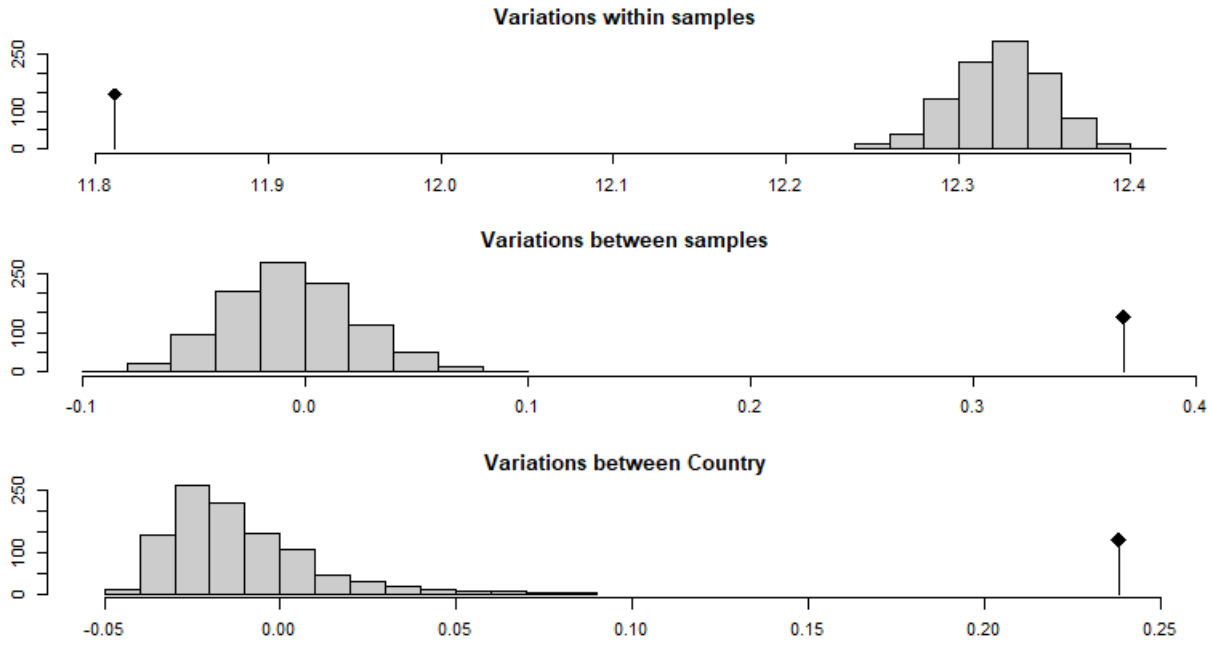
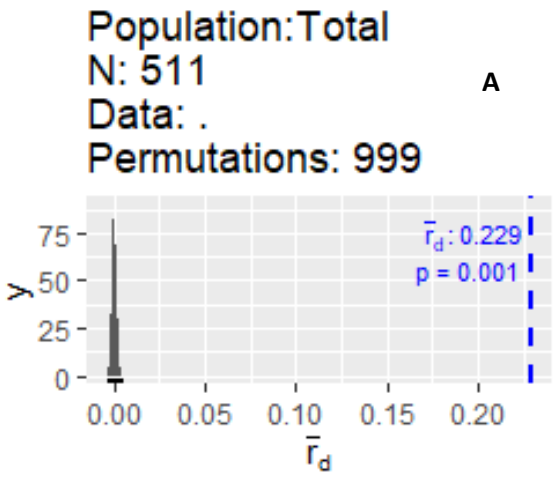


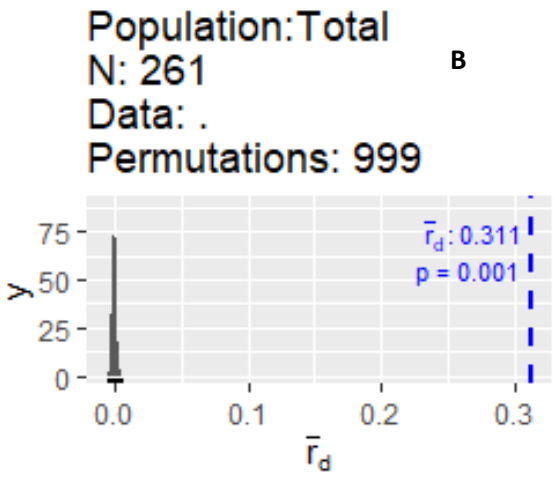
Figure 6 Measured Φ (black lines) versus simulated panmictic populations (histograms) across the three levels of AMOVA analysis.

Table 3. Analysis of molecular variance (AMOVA) of *A. flavus* isolates recovered across the three countries (Greece, Spain and Serbia)

Source of Variation	Df	Sum Sq	Variance components	Percentage of Variation	Φ	P-value
Among countries	2	785.1572	0.234	1.92	0.04	0.001
Among a priori populations within countries	58	3015.116	0.367	2.96	0.03	0.001
Within a priori populations	1950	20601.32	11.81	95.11	0.01	0.001

The results of LD obtained after clone correction (Figure 7), showed the unbiased indices of association, $\bar{r}_d$, are significantly ($p < 0.01$) greater than what is expected under sexual recombination in Greece, Spain, and Serbia (0.229, 0.311, and 0.270 respectively) and supportive of mutation driven divergence among lineages utilizing predominantly asexual reproduction.





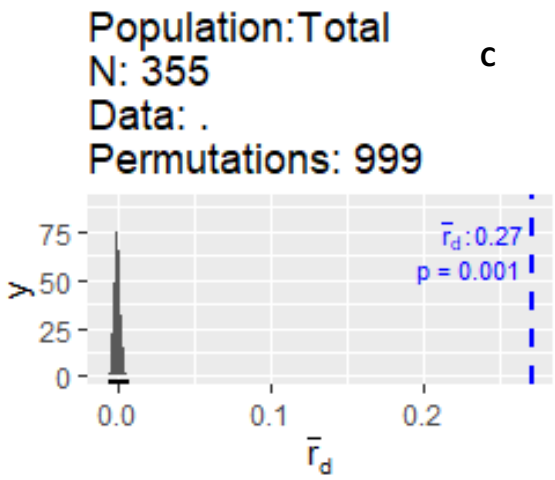


Figure 7. Standardized index of association $\bar{r}_d$ as the measure of multilocus genotypic linkage disequilibrium (LD) in the clone-corrected samples from Greece (A), Spain (B) and Serbia (S). The dotted blue line indicates the calculated value for the actual data, while the histogram represents data from simulated recombining populations with the same allele frequencies.

Genotype distribution and genetic differentiation

Although DAPC shows some differences among countries, many haplotypes were detected in more than one country (Figure 5). H-588, H-321, H-358, H-54, H-76, H-83, H-177, H-233, and H-180 are the most frequent haplotypes found in more than one country and collectively represent more than 70% of all isolates before clone correction. Among the 645 haplotypes detected 13 (2%) were detected in the three countries, and 35 (5%) haplotypes were detected in 2 countries (Spain and Greece, Spain and Serbia, or Greece and Serbia). The remaining 597 (93%) haplotypes were detected in one country (Figure 2, 8). Number of haplotypes detected per country was higher in Greece (A = 363) and Serbia (A = 209) but markedly lower in Spain (A = 134) reflecting the extensive clone correction for that country (Table 2). The two haplotypes detected in the most samples were H76 (18 samples with 7 in Greece and 11 in Spain) and H588 (17 samples with 1 in Greece and 16 in Spain). Haplotypes detected in only a single population (private haplotypes) composed 78% of the 645 haplotypes detected among the 2,011 isolates. This ranged from 73% in Spain and 74% in Serbia to 84% in Greece (Table 2). The AMOVA results (Table 3) show both separation among the three countries and the overwhelming diversity found within populations, representing over 95% of the total variation in the dataset. The variation among countries (1.92%) and among populations within countries (2.96%) are minute by comparison. These small levels of

variation in higher levels of organization are still significant: Φ (calculated by AMOVA) increased at higher strata, ranging from 0.01 within populations to 0.03 among populations within countries to 0.04 among the countries themselves (Table 3). Increasing Φ indicates increasing coalescent times among or within populations with increasing genetic distance.

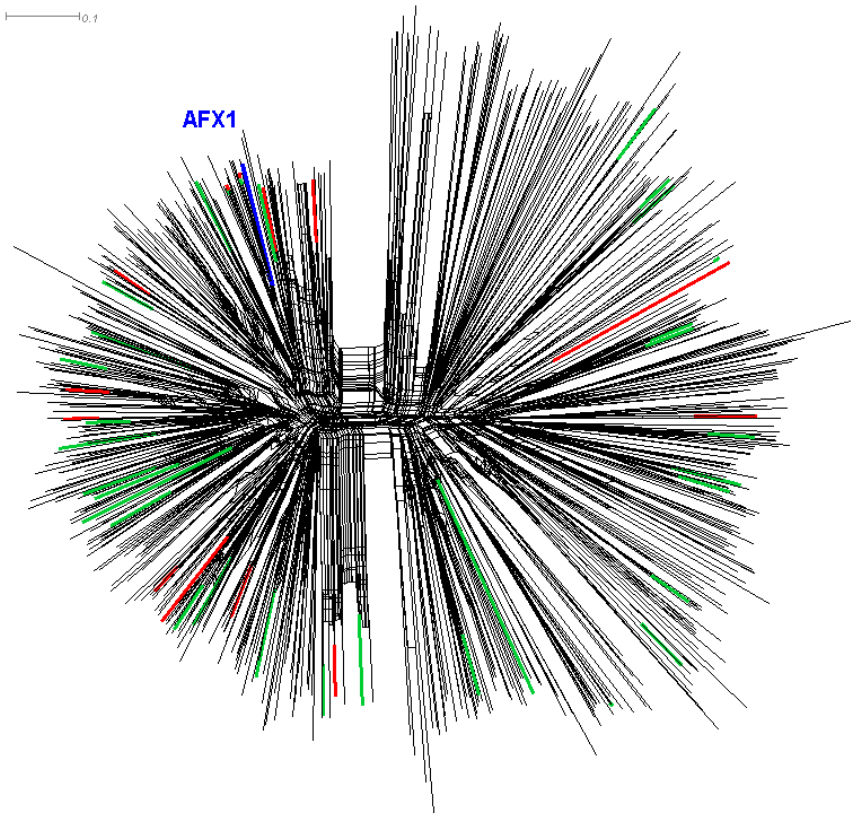


Figure 8: Neighbor Net tree (from SplitsTree 4) of Greek, Spanish and Serbian haplotypes: Black: haplotypes unique to individual countries; Red: haplotypes shared among all three countries; Green: shared haplotypes between 2 countries (Greece & Spain, Greece & Serbia, or Spain & Serbia); Blue: Italian biocontrol haplotype of AF-X1.

The prevalence of MUCL54911

Networks generated in SplitsTree were used to display variation among haplotypes (Figure 8, 9) and to identify haplotypes closely related to the haplotype of isolate MUCL54911 (active ingredient of biocontrol product AF-X1). VCA revealed that isolates with the 13 haplotypes most closely related to that `of MUCL54911 belong to VCG IT006 and that IT006 is present in all three countries. The frequencies of IT006 were highest in Spain (8.9%) but still reasonably frequent in

Greece (2%) and Serbia (1.6%) (Table 4). VCA also revealed that isolates with two haplotypes (H-373 and H-644) differing from MUCL 54911 at 2 or 8 loci respectively do not belong to VCG IT006. As with MUCL54911, several haplotypes closely related to the haplotype of MPVP A2321 were found in multiple countries (data not shown).

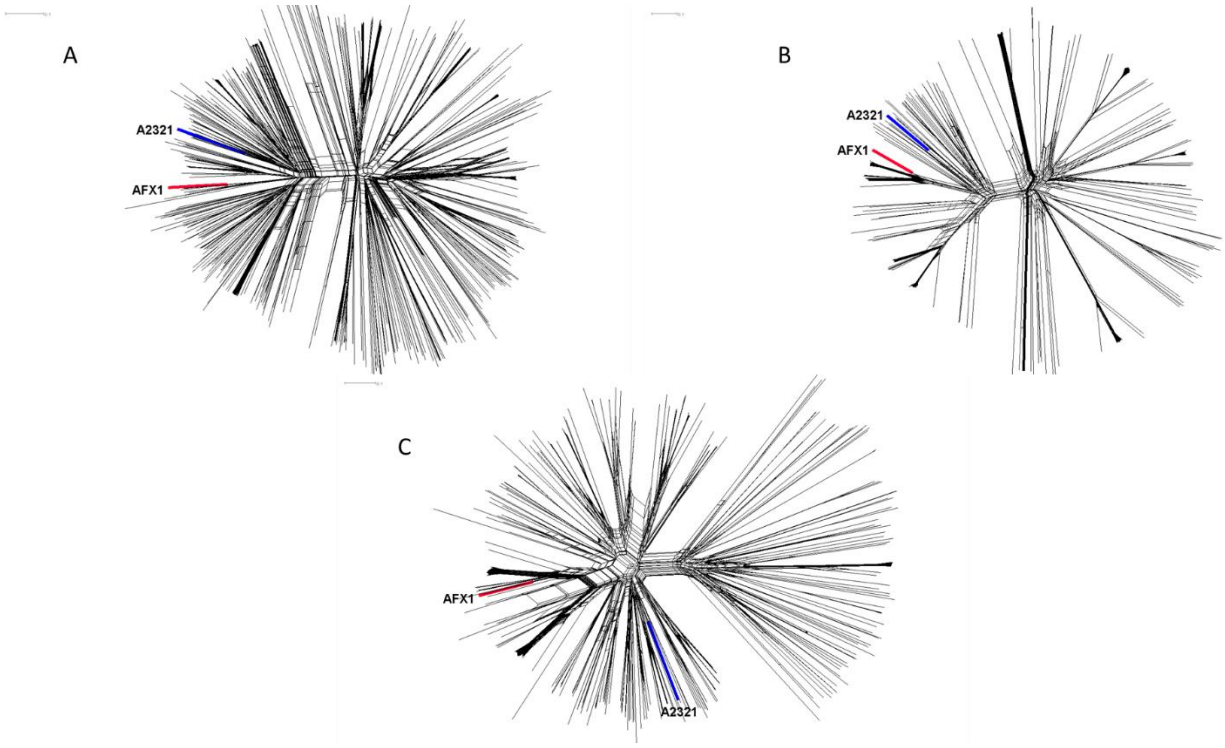


Figure 9. Neighbor networks for each country, generated by SplitsTree 4. A. Greece. B. Spain. C. Serbia. AF-X1 should be the sample color in Figure 8 and Figure 9.

Table 4. Haplotypes belonging to VCG IT006, the VCG to which the active ingredient of AFX1, MUCL54911, belongs.

Haplotype	Country	Isolates* (#)	AF28**	AF13	AF43	AF22	AF31	AF53	AF34	AF42	AF8	AF16	AF54	AF17	AF11	AF66	AF64	AF63	AF55
MUCL54911	Italy	1	119	141	399	144	312	131	296	150	166	169	161	368	135	271	161	127	172
H-358	Greece Spain	7 40	119	141	399	144	312	131	296	150	166	169	161	368	135	271	161	127	180
H-368	Greece Serbia Spain	4 7 8	119	141	399	144	312	131	301	150	166	169	161	368	135	271	161	127	180
H-360	Greece	1	119	141	399	144	312	131	296	150	166	169	161	368	135	271	163	127	180
H-376	Greece	1	119	141	399	144	312	131	323	150	166	169	161	368	135	271	161	127	180
H-367	Greece	1	119	141	399	144	312	131	301	150	166	169	161	368	132	271	161	127	180
H-346	Greece	1	119	141	399	144	309	131	301	150	166	169	161	368	135	271	161	127	180
H-375	Serbia	1	119	141	399	144	312	131	317	150	166	169	161	368	135	271	161	127	182
H-359	Serbia	1	119	141	399	144	312	131	296	150	166	169	161	368	135	271	161	127	182
H-378	Serbia	1	119	141	399	144	312	131	425	150	166	169	161	368	135	271	161	127	180
H-278	Serbia	1	119	141	399	144	312	131	310	150	166	169	161	368	135	271	161	127	180
H-370	Spain	1	119	141	399	144	312	131	307	153	166	169	161	368	135	271	161	127	180
H-372	Spain	1	119	141	399	144	312	131	310	150	166	169	161	368	135	271	161	127	180
H-374	Spain	1	119	141	399	144	312	131	317	150	166	169	161	368	135	271	161	127	180

*Number isolates belonging to this haplotype. All members of this haplotype were tested by VCA and found to belong to VCG IT006.

**SSR loci and allele sizes. Alleles differing from MUCL-54911 are shaded.

Discussion

Careful deployment of an atoxigenic strain of *A. flavus* as a biocontrol agent for aflatoxin mitigation requires knowledge of the distribution of the active ingredient in the target country. Use of native genotypes which are well adapted to the target agroecosystem should allow for more effective competition with aflatoxin producers and thus greater reductions in aflatoxin concentrations in the target crop (Cotty and Mellon, 2006; Abbas et al., 2011; Moral et al., 2020; Peles et al., 2021). The present work provides new insights on populations of *A. flavus* resident in a large area spanning the European regions most susceptible to aflatoxin contamination (Spain, Greece, and Serbia; Figure 1). At the same time, this study describes the distribution of VCG IT006, the active ingredient of the biocontrol product AF-X1, in southern Europe.

The current study had three major findings: I) the genetic diversity of *A. flavus* across southern Europe is very high with local evolution of clonal lineages in each of the three sampled countries and dispersal of both common and rare (detected in only two populations) haplotypes between countries; II) complete LD was observed in all countries, supporting clonal evolution of *A. flavus* populations in southern Europe, as seen in other studies of natural populations of *A. flavus* (Hadrich et al., 2013; Grubisha and Cotty, 2015; Islam et al., 2018, 2020; Picot et al., 2018; Ortega-Beltran et al., 2020); and III) members of the VCG to which MUCL54911 belongs occur in all the sampled regions revealing natural distribution of this biocontrol agent across southern Europe and opening the potential for use of MUCL54911 in the mitigation of aflatoxin contamination throughout this region.

Atoxigenic *A. flavus* active ingredients of biocontrol products are typically defined by VCG, and VCA is used to track the active ingredients on crops, in the environment, and over seasons and to verify identity during manufacture (Cotty, 1994; Cotty et al., 2007; Atehnkeng et al., 2016). *A. flavus* L strain populations are complex, with individual agricultural fields typically containing hundreds of VCGs (Bayman and Cotty, 1993; Barros et al., 2005). These VCGs diverged over thousands of years and during those periods mutation caused variability that can be detected at SSR loci both within and among VCGs (Grubisha and Cotty, 2010, 2015; A. Ortega-Beltran and Cotty, 2018). Such mutations were detected in IT006 resulting in several closely related haplotypes belonging to that VCG (Table 4). VCGs used for biocontrol are

selected so that all members of the VCG are atoxigenic. During evolution of these atoxigenic VCGs mutations accumulate in the 70 kb aflatoxin biosynthesis gene cluster causing multiple lesions that independently may result in loss of the ability to produce aflatoxins (Adhikari et al., 2016). The result of these aflatoxin gene cluster mutations is a highly stable atoxigenic phenotype within the biocontrol VCGs (Adhikari et al., 2016). Parasexual recombination within VCGs increases the diversity of SSR haplotypes and contributes to the great diversity detected within *A. flavus* populations (Papa, 1986; Leslie, 1993; ; Grubisha and Cotty, 2010; Mehl et al., 2012;). The diverse population structure of Southern European *A. flavus* populations is reflected in the high allelic diversity (Table 1) and high haplotypic diversity within the three studied countries (Table 2) similar to the diversity reported in other studies utilizing these SSR markers to study *A. flavus* populations in the U.S. (Grubisha and Cotty, 2010) and Kenya (Islam et al., 2018). The high frequency of haplotypes belonging to VCG IT006 suggests that the biocontrol product AF-X1 can be safely applied in Southern Europe without introducing *A. flavus* lineages that are not naturally occurring. It also suggests that AF-X1 is a readily available, ecologically safe tool for providing highly effective aflatoxin mitigation (Mauro et al., 2013, 2015, 2018).

Richness, haplotypic diversity, evenness, and Shannon's index (Table 2) all reflect a dataset comprised of a large number of haplotypes, most of which occur at very low frequency (Figure 2). The number of haplotypes equals 57% of the total number of clone-corrected isolates within the entire dataset, and private haplotypes (those seen in only one population) were 78% of all haplotypes. This variation in the dataset is reflected in genetic diversity measures, and the large number of singleton haplotypes is reflected in Shannon's index and the evenness (Table 2). While most haplotypes were somewhat closely related (Figure 4, 8), there is a small number of much more divergent haplotypes (Figure 4). The importance of these divergent lineages to aflatoxin contamination in Europe is unknown. Considering the level of variation found among individuals, it is no surprise to find a great deal of variation among populations and countries. Gene diversity for each country, like the measure for the dataset as a whole, is quite high, and the skewed frequency distribution of haplotypes is reflected in evenness and Shannon's index (Table 2). This pattern of diversity mirrors that seen in earlier population studies using VCA (e.g. Bayman and Cotty, 1993; Mauro et al., 2013) and also later studies using SSRs (e.g. Islam et al., 2018; Ortega-Beltran and Cotty, 2018).

In the examined European *A. flavus* populations, nearly all genetic variation is found within populations, as shown by AMOVA (Table 3). Nonetheless, the 3% of variation found among populations within countries and the 2% of variation found among countries suggests some divergence among these groups. Φ, an estimator of fixation indices, shows significant population structure at all levels of the AMOVA (Table 3; Figure 6). These results suggest local, mutation-driven, clonal evolution as seen with the DAPC scatterplot (Jombart et al., 2010), with dispersal of both common and rare haplotypes across the sampled region of southern Europe (Figure 5). The very high haplotype diversity detected in each sampled country reduced the ability of this study to fully describe the distributions of many haplotypes. As a result, even with the large sample size of over 1,100 clone-corrected isolates, only a small minority (9%) of the 645 haplotypes were detected in more than one of the 61 *a priori* populations. However, the geographic range over which haplotypes detected in multiple populations were dispersed included the entire area sampled from Spain in southwest Europe to Greece and Serbia in southeastern Europe. Although diversity was so great that most (78%) haplotypes were detected only in one sample, dispersal of the haplotypes detected in multiple populations supports the three sampled countries having in common a single highly diverse community of *A. flavus* clonal lineages. Both rare (detected in only 2 of the 61 *a priori* populations) and relatively common *A. flavus* haplotypes were found dispersed across the southern portion of the European continent at various frequencies. This wide distribution suggests that atoxigenic haplotypes found anywhere in Europe might be used as active ingredients in biocontrol products for use across the continent without concern about introducing a novel haplotype into a vulnerable habitat with detrimental impact (Probst et al., 2011; Islam et al., 2020).

Aspergillus flavus is a ubiquitous anamorphic fungus species that produces abundant asexual conidia on many organic substrates including material associated with several crops that are also susceptible to aflatoxin contamination (Klich, 2002; Ojiambo et al., 2018). However, several experimental studies suggest frequent sexual reproduction and its concomitant recombination (Horn et al., 2009, 2016; Moore et al., 2013), Recently, Molo et al., (2019, 2022) reported a genetic exchange and sexual recombination when biocontrol strains of opposite mating types are used in the same formulation. They also reported possible sexual recombination in microplot trials with the two biocontrol products registered for use in the US, AF36 and Afla-Guard. In contrast, the data presented here show complete LD across the three

countries (Figure 7). This indicates natural population structures in Greece, Spain and Serbia result predominantly from asexual reproduction. This clonal population structure has previously been reported for *A. flavus* populations in Kenya and Mexico where similar LD was measured (Islam et al., 2018; Ortega-Beltran et al., 2020). Likewise, LD analyses showed no evidence of genetic exchange with other VCGs or sexual recombination for the VCG containing AF36, the first atoxigenic *A. flavus* active ingredient used in the US (Grubisha and Cotty, 2015). The VCG of AF36 is naturally distributed across North America (Ortega-Beltran et al., 2016) in a manner similar to what is reported in the current study for IT006. AF36 has been widely utilized as a biocontrol agent in commercial agriculture in the United States since 1996 (Cotty et al., 2007) with no health or environmental ill effects, suggesting that widespread adoption of AF-X1 to control aflatoxins across Southern Europe should also be safe and appropriate.

Two specific haplotypes, H76 and H588, had the greatest distribution across southern Europe but were only detected in Spain and Greece. It could be that adaptive characteristics caused these haplotypes to be more successful in certain environments. Such adaptive traits should be shared across all haplotypes within their respective VCGs through parasexual recombination. Since VCGs often have significant haplotypic diversity (Islam et al., 2021; Grubisha and Cotty, 2010, 2015; Ortega-Beltran et al., 2020), adaptive success would be reflected in all haplotypes making up the VCG being present at high frequency, rather than a single haplotype. A second, and more likely explanation, may be rapid transient shifts in the composition of *A. flavus* communities across a broad portion of southern Europe initiated by founder events (Ortega-Beltran and Cotty, 2018; Ortega-Beltran, et al. 2020). Two similar independent events have been described in North America in association with maize production in Sonora, Mexico and Louisiana, USA (Ortega-Beltran and Cotty 2018; Sweany, et al., 2011). Although the founder events in Mexico were initially described using VCA, the population shift in Mexico was later shown to be caused by a single haplotype similar to what was observed in the current study with H76 and H588 (Ortega-Beltran et al., 2020).

While the exact haplotype of MUCL54911, the active ingredient in the biocontrol product AF-X1 was not observed in any country studied here, there are close relatives in each of the three countries (Figure 9). Testing of these related haplotypes using VCA showed that like MUCL54911, isolates with these closely related haplotypes belong to VCG IT006 (Table 4; Mauro et al., 2018). Since members of VCGs are clonally related (Leslie, 1993; Atehnkeng

et al., 2016; Grubisha and Cotty, 2010, 2015), the presence of VCG IT006 in all three countries suggests it is endemic and well adapted to environments across this region. A similar situation was reported by Ortega-Beltran et al., (2016), where several haplotypes belonging to YV36, the VCG to which the U.S. biocontrol active ingredient AF36 belongs, are endemic in maize growing regions in Mexico and across the southern US. This all suggests that AF-X1 is an environmentally safe product that will likely be effective in all three countries.

References

Abbas, H. K., Weaver, M. A., Horn, B. W., Carbone, I., Monacell, J. T., and Shier, W. T. (2011). Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. *Toxin Reviews*, *30*(2–3), 59–70. https://doi.org/10.3109/15569543.2011.591539

Adhikari, B. N., Bandyopadhyay, R., and Cotty, P. J. (2016). Degeneration of aflatoxin gene clusters in Aspergillus flavus from Africa and North America. *AMB Express*, *6*(1). https://doi.org/10.1186/s13568-016-0228-6

Agapow, P. M., and Burt, A. (2001). Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, *1*(1–2), 101–102. https://doi.org/10.1046/j.1471-8278.2000.00014.x

Atehnkeng, J., Donner, M., Ojiambo, P. S., Ikotun, B., Augusto, J., Cotty, P. J., and Bandyopadhyay, R. (2016). Environmental distribution and genetic diversity of vegetative compatibility groups determine biocontrol strategies to mitigate aflatoxin contamination of maize by *Aspergillus flavus*. *Microbial Biotechnology*, *9*(1), 75–88. https://doi.org/10.1111/1751-7915.12324

Barros, G., Torres, A., and Chulze, S. (2005). *Aspergillus flavus* population isolated from soil of Argentina's peanut-growing region. Sclerotia production and toxigenic profile. *Journal of the Science of Food and Agriculture*, *85*(14), 2349–2353. https://doi.org/10.1002/jsfa.2257

Battilani, P., Toscano, P., Van Der Fels-Klerx, H. J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T., and Robinson, T. (2016). Aflatoxin B 1 contamination in maize in Europe increases due to climate change. *Scientific Reports*, *6*(April), 1–7. https://doi.org/10.1038/srep24328

Bayman, P., and Cotty, P. J. (1991). Vegetative compatibility and genetic diversity in the Aspergillus flavus population of a single field. *Canadian Journal of Botany*, *69*(8), 1707–1711. https://doi.org/10.1139/b91-216

Bayman, Paul, and Cotty, P. J. (1993). Genetic diversity in Aspergillus flavus : association with aflatoxin production and morphology . *Canadian Journal of Botany*, *71*(1), 23–31. https://doi.org/10.1139/b93-003

Callicott, K. A., and Cotty, P. J. (2015). Method for monitoring deletions in the aflatoxin

biosynthesis gene cluster of Aspergillus flavus with multiplex PCR. *Letters in Applied Microbiology*, *60*(1), 60–65. https://doi.org/10.1111/lam.12337

Chang, P. K., and Ehrlich, K. C. (2010). What does genetic diversity of Aspergillus flavus tell us about Aspergillus oryzae? *International Journal of Food Microbiology*, *138*(3), 189–199. https://doi.org/10.1016/j.ijfoodmicro.2010.01.033

Cotty, P. J., Probst, C., and Jaime-Garcia, R. (2008). Etiology and management of aflatoxin contamination. In and A. V. J. F. Leslie, R. Bandyopadhyay (Ed.), *Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade* (pp. 287–299). CAB International.

Cotty, P.J., Antilla, L. and Wakelyn, P.J. (2007). Competitive exclusion of aflatoxin producers: Farmer driven research and development. In: Vincent, C., Goettel, N. and Lazarovits, G. (eds.) Biological control: a global perspective. CAB International, Wallingford, UK, pp. 242-253.

Cotty, P.J. (1989). Virulence and cultural characteristics of two Aspergillus flavus strains pathogenic on cotton. *Phytopathology*, *79*, 808–814.

Cotty, P. J., and Mellon, J. E. (2006). Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. *Mycotoxin Research*, *22*(2), 110–117. https://doi.org/10.1007/BF02956774

Cotty, P. J. (1994). Comparison of four media for the isolation of Aspergillus flavus group fungi. *Mycopathologia*, *125*, 157–162. https://doi.org/10.1201/b17871

Cotty, P.J. (1994). Influence of Field Application of an Atoxigenic Strain of Aspergillus flavus on the population of A.flvaus Infecting Cotton Bolls and on the Aflatoxin Content of Cottonseed. *Biological Control*, *84*(11), 1270–1277.

Curtui, V., Seidler, C., Schneider, E., and Usleber, E. (2004). Mycotoxins in Food in Germany. *An Overview on Toxigenic Fungi and Mycotoxins in Europe*, 109–121. https://doi.org/10.1007/978-1-4020-2646-1_8

Das, M. K., Ehrlich, K. C., and Cotty, P. J. (2008). Use of pyrosequencing to quantify incidence of a specific Aspergillus flavus strain within complex fungal communities associated with commercial cotton crops. *Phytopathology*, *98*(3), 282–288.

https://doi.org/10.1094/PHYTO-98-3-0282

Dobolyi, C., Sebok, F., Varga, J., Kocsube, S., Szigeti, G., Baranyi, N., Szecsi, A., Toth, B., Varga, M., Kriszt, B., Szoboszlay, S., Krifaton, C. and Kukolya, J. (2013). Occurrence of aflatoxin producing Aspergillus flavus isolates in maize kernel in Hungary. *Acta Alimentaria*, *42*, 451–459.

Dorner, J. W., and Lamb, M. C. (2006). Development and commercial use of afla-guard®, an aflatoxin biocontrol agent. *Mycotoxin Research*, *22*(1), 33–38. https://doi.org/10.1007/BF02954555

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of Aspergillus flavus used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. *Applied Microbiology and Biotechnology*, *65*(4), 473–478. https://doi.org/10.1007/s00253-004-1670-y

Ehrlich, K. C., Montalbano, B. G., and Cotty, P. J. (2007). Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain Aspergillus flavus vegetative compatibility groups. *FEMS Microbiology Letters*, *268*(2), 231–236. https://doi.org/10.1111/j.1574-6968.2006.00588.x

Eliades, N-G., Eliades, D. G. (2009). HAPLOTYPE ANALYSIS: software for analysis of haplotypes data. Distributed by the authors. In *Forest Genetics and Forest Tree Breeding*. Georg-Augst University.

Excoffier, L., Laval, G., Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Bioinformatics*, *1*, 47–50.

Finkeldey, R., and Murillo, O. (1999). Contributions of subpopulations to total gene diversity. *Theoretical and Applied Genetics*, *98*(3–4), 664–668. https://doi.org/10.1007/s001220051118

Gallo, A., Stea, G., Battilani, P., Logrieco, A. F., and Perrone, G. (2012). Molecular characterization of an Aspergillus flavus population isolated from maize during the first outbreak of aflatoxin contamination in Italy. *Phytopathologia Mediterranea*, *51*(1), 198–206.

Geiser, D. M., Pitt, J. I., and Taylor, J. W. (1998). Cryptic speciation and recombination in

the aflatoxin-producing fungus Aspergillus flavus. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(1), 388–393. https://doi.org/10.1073/pnas.95.1.388

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., and Battilani, P. (2007). Studies on Aspergillus section Flavi isolated from maize in northern Italy. *International Journal of Food Microbiology*, *113*(3), 330–338. https://doi.org/10.1016/j.ijfoodmicro.2006.09.007

Grubisha, L. C., and Cotty, P. J. (2009). Twenty-four microsatellite markers for the aflatoxin-producing fungus Aspergillus flavus. *Molecular Ecology Resources*, *9*(1), 264–267. https://doi.org/10.1111/j.1755-0998.2008.02378.x

Grubisha, L. C., and Cotty, P. J. (2010). Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus Aspergillus flavus. *Molecular Ecology*, *19*(2), 269–280. https://doi.org/10.1111/j.1365-294X.2009.04467.x

Grubisha, L. C., and Cotty, P. J. (2015). Genetic analysis of the Aspergillus flavus vegetative compatibility group to which a biological control agent that limits aflatoxin contamination in U.S. crops belongs. *Applied and Environmental Microbiology*, *81*(17), 5889–5899. https://doi.org/10.1128/AEM.00738-15

Grünwald, N. J., Goodwin, S. B., Milgroom, M. G., and Fry, W. E. (2003). Analysis of genotypic diversity data for populations of microorganisms. *Phytopahthology*, *93*, 738–746.

Hadrich, I., Amouri, I., Neji, S., Mahfoud, N., Ranque, S., Makni, F., and Ayadi, A. (2013). Genetic structure of Aspergillus flavus populations in human and avian isolates. *European Journal of Clinical Microbiology and Infectious Diseases*, *32*(2), 277–282. https://doi.org/10.1007/s10096-012-1740-5

Hadrich, Inès, Makni, F., Ayadi, A., and Ranque, S. (2010). Microsatellite typing to trace Aspergillus flavus infections in a hematology unit. *Journal of Clinical Microbiology*, *48*(7), 2396–2401. https://doi.org/10.1128/JCM.01269-09

Hatti , A.D., Taware, S.D., Taware, A. S., Pangrikar, P.P., Chavan, A.M. and Mukadam, D. S. (2010). Genetic Diversity of Toxigenic and Non-Toxigenic Aspergillus Flavus Strains Using Issr Markers. *International Journal of Current Research*, *5*, 61–66.

Hedrick, P. W. (2005). A standardized genetic differentiation measure. *Evolution*, *59*(8), 1633–1638. https://doi.org/10.1111/j.0014-3820.2005.tb01814.x

Horn, B. W. (2003). Ecology and population biology of aflatoxigenic fungi in soil. *Journal of Toxicology - Toxin Reviews*, *22*(2–3), 351–379. https://doi.org/10.1081/TXR-120024098

Horn, B. W., Gell, R. M., Singh, R., Sorensen, R. B., and Carbone, I. (2016). Sexual reproduction in aspergillus flavus sclerotia: Acquisition of novel alleles from soil populations and uniparental mitochondrial inheritance. *PLoS ONE*, *11*(1), 1–22. https://doi.org/10.1371/journal.pone.0146169

Horn, B. W., Moore, G. G., and Carbone, I. (2009). Sexual reproduction in Aspergillus flavus. *Mycologia*, *101*(3), 423–429. https://doi.org/10.3852/09-011

Huson, D. H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, *23*(2), 254–267. https://doi.org/10.1093/molbev/msj030

IARC. (2007). INTERNATIONAL AGENCY FOR RESEARCH ON CANCER IARC Monographs on the Evaluation of Carcinogenic Risks to Humans VOLUME 90 Human Papillomaviruses. In *Iarc Monographs On The Evaluation Of Carcinogenic Risks To Humans* (Vol. 90).

Islam, M. S., Callicott, K. A., Mutegi, C., Bandyopadhyay, R., and Cotty, P. J. (2018). Aspergillus flavus resident in Kenya: High genetic diversity in an ancient population primarily shaped by clonal reproduction and mutation-driven evolution. *Fungal Ecology*, *35*, 20–33. https://doi.org/10.1016/j.funeco.2018.05.012

Islam, M. S., Callicott, K. A., Mutegi, C., Bandyopadhyay, R., and Cotty, P. J. (2020). Distribution of active ingredients of a commercial aflatoxin biocontrol product in naturally occurring fungal communities across Kenya. *Microbial Biotechnology*. https://doi.org/10.1111/1751-7915.13708

Jaime-garcia, R., and Cotty, P. J. (2004). *Aspergillus flavus in Soils and Corncobs in South Texas : Implications for Management of Aflatoxins in Corn – Cotton Rotations*. *88*(12), 1366–1371.

Janić Hajnal, E., Kos, J., Krulj, J., Krstović, S., Jajić, I., Pezo, L., Šarić, B., and Nedeljković,

N. (2017). Aflatoxins contamination of maize in Serbia: the impact of weather conditions in 2015. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *34*(11), 1999–2010. https://doi.org/10.1080/19440049.2017.1331047

JECFA. (2017). Evaluation of certain contaminants in food, Prepared by the Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In *WHO Technical Report Series* (Issue 1002). http://www.ncbi.nlm.nih.gov/pubmed/29144071

Jombart, T. (2008). Adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Jombart, T., and Ahmed, I. (2011). adegenet 1.3-1: New tools for the analysis of genome-wide SNP data. *Bioinformatics*, *27*(21), 3070–3071. https://doi.org/10.1093/bioinformatics/btr521

Kamvar, Z.N., Tabima, J.F., Grunwald, N. (2014). The poppr R package for genetic analysis of populations with mixed (clonal/sexual) reproduction. *Peer J*.

Klich, M. A. (2002). *Biogeography of Aspergillus species in soil and litter*. *5514*, 20–27. https://doi.org/10.1080/15572536.2003.11833245

Kollia, E., Proestos, C., Zoumpoulakis, P., and Markaki, P. (2017). Inhibitory effect of Cynara cardunculus L. extract on aflatoxin B1 production by Aspergillus parasiticus in sesame (Sesamum indicum L.). *International Journal of Food Properties*, *20*(6), 1270–1279. https://doi.org/10.1080/10942912.2016.1206928

Leslie, J. F. (1993). Fungal Vegetative Compatibility. *Annual Review of Phytopathology*, *31*(1), 127–150. https://doi.org/10.1146/annurev.py.31.090193.001015

Levic, J., Gosic-Dondo, S., Ivanovic, D., Stankovic, S., Krnjaja, V., Bocarov-Stancic, A., and Stepanic, A. (2013). An outbreak of Aspergillus species in response to environmental conditions in Serbia. *Pesticidi i Fitomedicina*, *28*(3), 167–179. https://doi.org/10.2298/pif1303167l

Mauro, A., Battilani, P., Callicott, K. A., Giorni, P., Pietri, A., and Cotty, P. J. (2013). International Journal of Food Microbiology Structure of an Aspergillus fl avus population from maize kernels in northern Italy. *International Journal of Food*

Microbiology, *162*(1), 1–7. https://doi.org/10.1016/j.ijfoodmicro.2012.12.021

Mauro, A., Battilani, P., and Cotty, P. J. (2015). Atoxigenic Aspergillus flavus endemic to Italy for biocontrol of aflatoxins in maize. *BioControl*, *60*(1), 125–134. https://doi.org/10.1007/s10526-014-9624-5

Mauro, A., Garcia-cela, E., Pietri, A., Cotty, P. J., and Battilani, P. (2018). Biological Control Products for Aflatoxin Prevention in Italy : Commercial Field Evaluation of Atoxigenic Aspergillus flavus Active Ingredients. *Toxins*, 1–14. https://doi.org/10.3390/toxins10010030

Mehl, H. L., Jaime, R., Callicott, K. a., Probst, C., Garber, N. P., Ortega-Beltran, A., Grubisha, L. C., and Cotty, P. J. (2012). Aspergillus flavus diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. *Annals of the New York Academy of Sciences*, *1273*(1), 7–17. https://doi.org/10.1111/j.1749-6632.2012.06800.x

Meirmans, P. G. (2020). genodive version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. *Molecular Ecology Resources*, *20*(4), 1126–1131. https://doi.org/10.1111/1755-0998.13145

Molo, M. S., Heiniger, R. W., Boerema, L., and Carbone, I. (2019). Trial summary on the comparison of various non-aflatoxigenic strains of aspergillus flavus on mycotoxin levels and yield in maize. *Agronomy Journal*, *111*(2), 942–946. https://doi.org/10.2134/agronj2018.07.0473

Molo, M. S., White, J. B., Cornish, V., Gell, R. M., Baars, O., Singh, R., Carbone, M. A., Isakeit, T., Wise, K. A., Woloshuk, C. P., Bluhm, B. H., Horn, B. W., Heiniger, R. W., and Carbone, I. (2022). Asymmetrical lineage introgression and recombination in populations of Aspergillus flavus: implications for biological control. *PLoS ONE*, *10*(17), 1–41. https://doi.org/10.1371/journal.pone.0276556

Moore, G. G., Elliott, J. L., Singh, R., Horn, B. W., Dorner, J. W., Stone, E. A., Chulze, S. N., Barros, G. G., Naik, M. K., Wright, G. C., Hell, K., and Carbone, I. (2013). Sexuality Generates Diversity in the Aflatoxin Gene Cluster: Evidence on a Global Scale. *PLoS Pathogens*, *9*(8). https://doi.org/10.1371/journal.ppat.1003574

Moore, G. G., Singh, R., Horn, B. W., and Carbone, I. (2009). Recombination and lineage-specific gene loss in the aflatoxin gene cluster of Aspergillus flavus. *Molecular Ecology*, *18*(23), 4870–4887. https://doi.org/10.1111/j.1365-294X.2009.04414.x

Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., and Ortega-Beltran, A. (2020). Present status and perspective on the future use of aflatoxin biocontrol products. *Agronomy*, *10*(4). https://doi.org/10.3390/agronomy10040491

Moretti, A., Pascale, M., and Logrieco, A. F. (2019). Mycotoxin risks under a climate change scenario in Europe. *Trends in Food Science and Technology*, *84*(June 2017), 38–40. https://doi.org/10.1016/j.tifs.2018.03.008

Ojiambo, P. S., Battilani, P., Cary, J. W., Blum, B. H., and Carbone, I. (2018). Cultural and genetic approaches to manage aflatoxin contamination: Recent insights provide opportunities for improved control. *Phytopathology*, *108*(9), 1024–1037. https://doi.org/10.1094/PHYTO-04-18-0134-RVW

Olarte, R. A., Horn, B. W., Dorner, J. W., Monacell, J. T., Singh, R., Stone, E. A., and Carbone, I. (2012). Effect of sexual recombination on population diversity in aflatoxin production by Aspergillus flavus and evidence for cryptic heterokaryosis. *Molecular Ecology*, *21*(6), 1453–1476. https://doi.org/10.1111/j.1365-294X.2011.05398.x

Ortega-Beltran, A., and Cotty, P. J. (2018). Frequent shifts in aspergillus flavus populations associated with maize production in Sonora, Mexico. *Phytopathology*, *108*(3), 412–420. https://doi.org/10.1094/PHYTO-08-17-0281-R

Ortega-Beltran, A., Grubisha, L. C., Callicott, K. a., and Cotty, P. J. (2016). The vegetative compatibility group to which the US biocontrol agent Aspergillus flavus AF36 belongs is also endemic to Mexico. *Journal of Applied Microbiology*, *120*(4), 986–998. https://doi.org/10.1111/jam.13047

Ortega-Beltran, Alejandro, Callicott, K. A., and Cotty, P. J. (2020). Founder events influence structures of Aspergillus flavus populations. *Environmental Microbiology*, *22*(8), 3522–3534. https://doi.org/10.1111/1462-2920.15122

Ortega-Beltran, A., Callicott, K. A., and Cotty, P. J. (2020). Founder events influence

structures of Aspergillus flavus populations . *Environmental Microbiology*, *June*. https://doi.org/10.1111/1462-2920.15122

Pál, K., Van Diepeningen, A. D., Varga, J., Hoekstra, R. F., Dyer, P. S., and Debets, A. J. M. (2007). Sexual and vegetative compatibility genes in the aspergilli. *Studies in Mycology*, *59*, 19–30. https://doi.org/10.3114/sim.2007.59.03

Palumbo, R., Gonçalves, A., Gkrillas, A., Logrieco, A., Dorne, J. Lou, Dall'asta, C., Venâncio, A., and Battilani, P. (2020). Mycotoxins in maize: mitigation actions, with a chain management approach. *Phytopathologia Mediterranea*, *59*(1), 5–27. https://doi.org/10.14601/Phyto-11142

Papa, K. E. (1986). Heterokaryon Incompatibility in Aspergillus flavus. *Mycologia*, *78*(1), 98. https://doi.org/10.2307/3793383

Peakall, R., and Smouse, P. E. (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, *28*(19), 2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Peles, F., Sipos, P., and Kov, S. (2021). Biological Control and Mitigation of Aflatoxin Contamination in Commodities. *Toxin Reviews*, *13*, 1–20.

Perrone, G., Gallo, A., and Logrieco, A. F. (2014). Biodiversity of Aspergillus section Flavi in Europe in relation to the management of aflatoxin risk. *Frontiers in Microbiology*, *5*(JULY), 1–5. https://doi.org/10.3389/fmicb.2014.00377

Picot, A., Doster, M., Islam, M. S., Callicott, K., Ortega-Beltran, A., Cotty, P., and Michailides, T. (2018). Distribution and incidence of atoxigenic Aspergillus flavus VCG in tree crop orchards in California: A strategy for identifying potential antagonists, the example of almonds. *International Journal of Food Microbiology*, *265*(October 2017), 55–64. https://doi.org/10.1016/j.ijfoodmicro.2017.10.023

Piva, G., Battilani, P. and P. (2006). Emerging issues in southern Europe: aflatoxins in Italy. In A. Barug, D., Bhatnagar, D., Van Egmond, H.P., Van der Kamp, J.W., Van Ossenbruggen, W.A. and Visconti (Ed.), *Emerging issues in southern Europe: aflatoxins in Italy* (pp. 139–135). Wageningen Academic Publishers.

Probst, C., and Bandyopadhyay, R. Price L.E. Cotty, P. J. (2011). Identification of

Atoxigenic Aspergillus flavus Isolates to Reduce Aflatoxin Contamination of Maize in Kenya. *Plant Disease*, *95*(2), 212–218.

Ramirez-prado, J. H., Moore, G. G., Horn, B. W., and Carbone, I. (2008). *Characterization and population analysis of the mating-type genes in Aspergillus flavus and Aspergillus parasiticus*. *45*, 1292–1299. https://doi.org/10.1016/j.fgb.2008.06.007

Shannon, C. . (1948). mathematical theory of communication. *The Bell System Technical*, *27*(3), 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R. V., Breiman, R., Brune, M. N., DeCock, K., Dilley, A., Groopman, J., Hell, K., Henry, S. H., Jeffers, D., Jolly, C., Jolly, P., Kibata, G. N., Lewis, L., Liu, X., Luber, G., McCoy, L., … Wilson, D. (2006). Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. *Environmental Health Perspectives*, *114*(12), 1898–1903. https://doi.org/10.1289/ehp.9302

Sweany, R. R., Damann, K. E., and Kaller, M. D. (2011). Comparison of soil and corn kernel Aspergillus flavus populations: Evidence for niche specialization. *Phytopathology*, *101*(8), 952–959. https://doi.org/10.1094/PHYTO-09-10-0243

Thibaut Jombart, Sébastien Devillard, F. B. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, *11*(94), 1–15. https://doi.org/10.1371/journal.pcbi.1000455

Tran-Dinh, N., Carter, D. (2000). Characterization of microsatellite loci in the aflatoxigenic fungi Aspergillus flavus and Aspergillus parasiticus. *Molecular Ecology*, *9*(12), 2170–2172. https://doi.org/http://dx.doi.org/10.1046/j.1365-294X.2000.10539.x

Udovicki, B. I. D. ;Jasenka G. K. M. P. A. S. J. D. A. R. (2019). Exposure assessment and risk characterization of aflatoxins intake through consumption of maize products in the adult populations of Serbia, Croatia and Greece. *FOOD ADDITIVES and CONTAMINANTS:*, *1.13*.

Valencia-Quintana, R., Mili´cmili´c, M., Jakši´c, D. J., Šegvi´c, M., Klari´c, Š. K., Tenorio-Arvide, G., Alejandro Pérez-Flores, G., Bonassi, S., and Sánchez-Alarcón, J. (2020).

Environment Changes, Aflatoxins, and Health Issues, a Review. *International Journal of Environemnt Research and Public Health*, *17*(7850), 1–10. https://doi.org/10.3390/ijerph17217850

Vlajkov, V., Grahovac, M., Budakov, D., Loc, M., Pajčin, I., Milić, D., Novaković, T., and Grahovac, J. (2021). Distribution, genetic diversity and biocontrol of aflatoxigenic Aspergillus flavus in Serbian maize fields. *Toxins, 13*, 687-711

Wang, D. Y., Hadj-Henni, L., Thierry, S., Arné, P., Chermette, R., Botterel, F., Hadrich, I., Makni, F., Ayadi, A., Ranque, S., Huang, W. Y., and Guillot, J. (2012). Simple and Highly Discriminatory VNTR-Based Multiplex PCR for Tracing Sources of Aspergillus flavus Isolates. *PLoS ONE*, *7*(9), 18–21. https://doi.org/10.1371/journal.pone.0044204

Weaver, M. A., Callicott, K. A., Mehl, H. L., Opoku, J., Park, L. C., Fields, K. S., and Mandel, J. R. (2022). Characterization of the Aspergillus flavus Population from Highly Aflatoxin-Contaminated Corn in the United States. *Toxins*, *14*(11), 755. https://doi.org/10.3390/toxins14110755

Winter, D. J. (2012). MMOD: An R library for the calculation of population differentiation statistics. *Molecular Ecology Resources*, *12*(6), 1158–1160. https://doi.org/10.1111/j.1755-0998.2012.03174.x

Wu, F. (2015). Global impacts of aflatoxin in maize: Trade and human health. *World Mycotoxin Journal*, *8*(2), 137–142. https://doi.org/10.3920/WMJ2014.1737

Chapter 4:

Post-harvest and long-term efficacy of biocontrol application of AF-X1 in Italian maize

Background

Aflatoxins are secondary metabolites produced by *Aspergillus* fungi and one the most frequent mycotoxins contaminating grains. Maize, considered as the main staple food for many nations worldwide, is one the crops most often affected by aflatoxin contamination during pre and post-harvest stages(Palumbo et al., 2020; Kortei et al., 2021; Dey et al., 2022). Aflatoxins are difuranocoumarin derivatives synthetised through the polyketide pathway and characterized by a polycyclic structure derived from a coumarin nucleus attached to a bifuran system. Furthermore, the solubility of aflatoxins is high in moderately polar solvent like chloroform, methanol and dimethylsulfoxide and low in water (Nakai et al., 2008; Turner et al., 2009). Four major aflatoxins have been identified: AFB1, AFB2, AFG1 and AFG2, in addition AFM1 is another type of aflatoxin derived from AFB1 commonly found in dairy products (Wu et al., 2009; Eskola et al., 2020).

Aspergillus flavus and *A. parasiticus* are the main producer of aflatoxins, in particular AFB1 is mainly produced by *A. flavus* (Tran-Dinh et al., 1999; Amaike and Keller, 2011). High temperature provides favourable conditions for fungal growth and a delay in the drying process and poor agricultural practices together lead to an increase in aflatoxin contamination (Hell et al., 2000).

The use of atoxigenic strains of *A. flavus* is the most effective strategy to reduce aflatoxin contamination in maize and other crops (Cotty and Bayman, 1993; Mehl et al., 2012). Currently, in Italy the use of the commercial product AF-X1 has reduced more than 90% the aflatoxin content in treated maize. The active ingredient of AF-X1 is the atoxigenic strain MUCL54911endemic to Italy and belonging to the VCG IT006 (Mauro et al., 2013, 2018). The present experiment aimed to evaluate the long term and postharvest efficacy of the AF-X1 preharvest treatment in commercial maize grown in Italy.

Material and Methods

Sample collection

The samples were collected from commercial maize harvested between end of August and beginning of September. Maize treated with AF-X1 was stored in silobag, while untreated maize was stored in big silos (Figure 1 a, b).

Every month, starting from October 2020 up to 8 months, samples were collected from both the big silo and the silo one bag. Regarding the big silo, a sample of about 10 kg was discharged and 3 subsamples of 500 were picked, after accurately mixing the grains. Regarding the silo bag, 3 subsamples (~500g/sample) were collected using a drill (Figure 1b). All samples were ground and stored at 4°C before analysis.

Figure 1. Overview on where the samples have been collected: storehouse (a) and Silo bag (b)

Fungal enumeration in maize samples

All the grounded samples were managed to determine colony forming units (CFU/g). One g of maize flour was mixed with 10 ml of sterile water added with 10µl of 10% TWEEN®80. The bottles were placed on the shaker at 175 RPM for 20 min. Serial dilution was conducted on the Dichloran Rose-Bengal Chloramphenicol (DRBC) Agar (Thermo Fisher), with three replicates per each dilution; all plates were incubated for 5 days in the dark at 31°C. Fungal colonies were identified based on morphological characteristics (Klich, 2002) and the result was reported as CFU/g of *A. flavus* and other fungal species.

Aflatoxin analysis

All maize samples were analysed for aflatoxin content. Five grams of flour were randomly taken from each sample and mixed with 20 mL acetonitrile/water/formic acid solution (59:20:1) and filtered with FPTE 0.20 µm filter after being vortexed for 30 min at 2500 rpm. Liquid

Chromatography- mass spectrometry (LC-MS) (Thermo Scientific, Milford, MA, USA) was used after calibration to identify all types of mycotoxins. Simultaneously, 7 µL of the extraction mix was injected into Ultimate HPLC machine (Thermo Scientific) to separate all mycotoxins. The limits of detection for aflatoxin B1, B2, G1 and G2 were 0.9 µg/kg and 2.71 µg/kg for LOD and LOQ respectively.

Result

Maize Fungal Population

The results of the total fungal population density (CFU/g) (Figure 2a) showed the high occurrence of the fungal community in the samples collected from the big silo, not treated with AF-X1. The highest density was noted after 7 and 8 months of the storage ($3.84*10^6$ CFU/g and $5.3*10^5$ CFU/g, respectively). However, the lowest density was reported during the 6^{th} month of the storage with $9.01*10^2$ CFU/g.

The samples treated with AF-X1 showed a low density of fungal community during all the storage period and ranged from $1.11 *10^1$ CFU/g to $9.83*10^2$ CFU/g, the lowest density was observed during the 4^{th} month of storage whilst the higher density was in 7^{th} month (Figure 2a).

The occurrence of *A. flavus* (Figure 2 b) in the maize flour was higher in the untreated than the treated samples. The highest incidence of *A. flavus* was noted in 7^{th} month of storage with $9.96*10^3$ CFU/, while in the treated samples the density was ranged from 0 to $1.94*10^2$ CFU/g. The absence of *A. flavus* colonies was reported in 4^{th} and 6^{th} months whether in treated or untreated samples.

Colony forming units followed a comparable trend in grain treated and untreated with AF-X1, but the population was always higher in untreated grain.

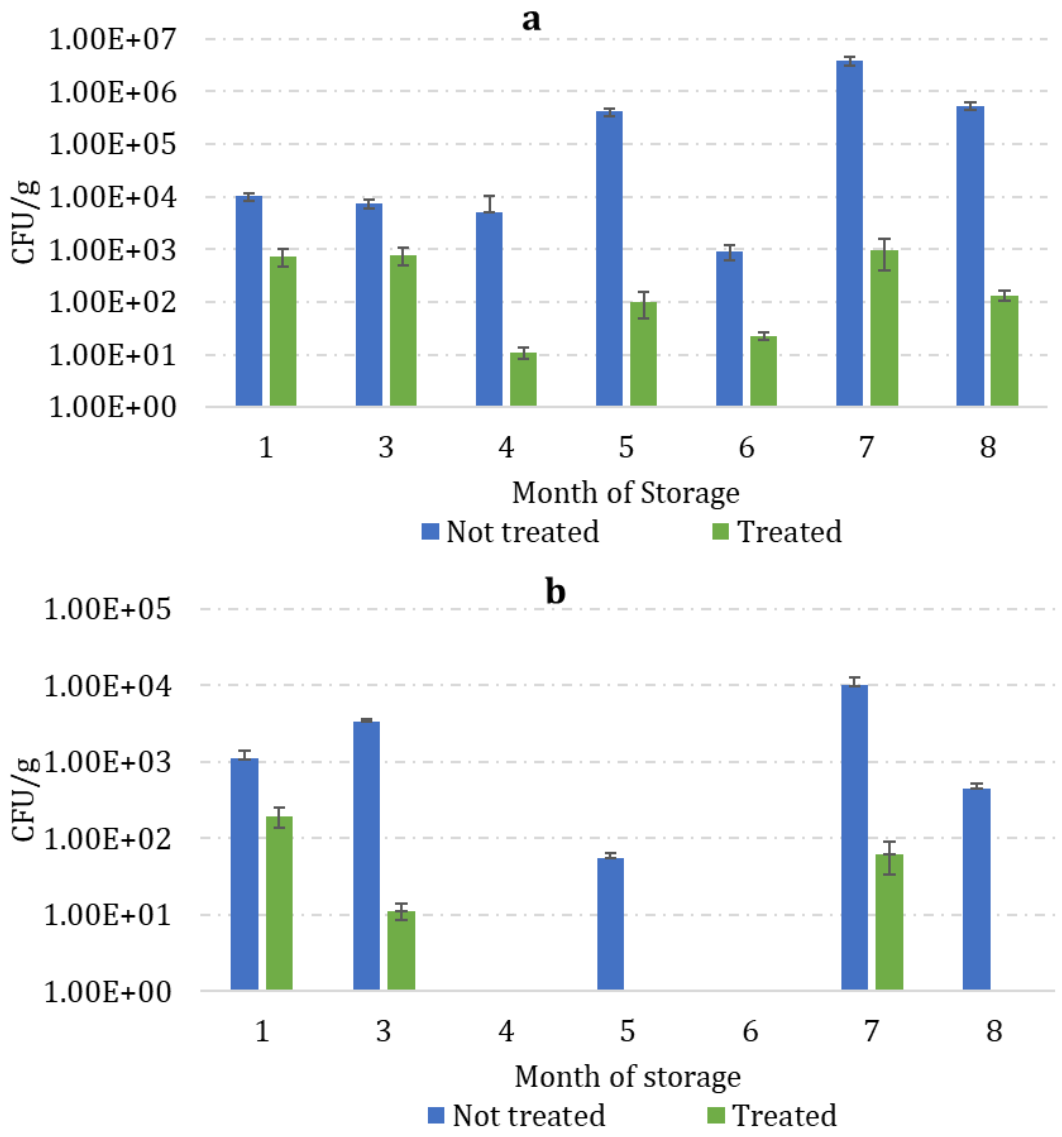


Figure 2. Colony forming unit (CFU /g + confidence interval) of total fungal community (a) and *A. flavus* (b) isolated from the maize flour during 8 months of storage (silo bag= Treated by AF-X1; Storehouse =not treated)

Aflatoxin quantification

During the whole storage period, aflatoxins were lower than 0.04 µg/kg, whether in treated or untreated samples. However, aflatoxins were detected in the 8th month of the storage in the untreated grain with 0.1 µg/kg.

Discussion

The main objective of this experimental trial was to evaluate the long term efficacy of the pre-harvest treatment by AF-X1 on the fungal community, in particular on *A.flavus* population as

well as the aflatoxin contamination along the post-harvest stage. The results of this trial do not contribute to conclude if the AF-X1 treatment pre-harvest had a positive effect on the fungal community and aflatoxins contamination. One of the most challenging issue we faced during the trial was to convince technicians to organize the post-harvest trial and repeatedly sampling the grain in order to have large scale experiment and collect more useful data.

The infection starts from the field and continue during post-harvest stage with aflatoxin production in favorable conditions (Temperature, humidity and storage hygiene condition…) (Palumbo et al., 2020; Shabeer et al., 2022). Applying good agricultural practices, such drying and maintaing storehouse in good condition with prior disinfection and aeration, contribute to prevent mycotoxin production, in particular aflatoxin, avoiding production lost (Magan and Aldred, 2007;Moore, 2021).

Due to the application by the deployment of atoxigenic strain the active ingredient of AF-X1, one would expect the untreated samples could have a high occurrence of *A.flavus* with low level of aflatoxin contamination. Many reasons could be involved in the low concentration of aflatoxins in the untreated samples such us the closeness of the fields (treated and untreated), which can facilitate the dispersal of the active ingredient of AF-X1. Or the environmental and the storage conditions are not favorable for the aflatoxin's contamination in that year. The study of Kinyungu et al., (2019), revealed that the preharvest application of atoxigenic biocontrol products had a positive impact to reduce aflatoxin content in maize during the post-harvest stage.

In conclusion, the application of AF-X1 in the field has a positive effect on aflatoxins concentration during the storage which our study support somehow this fact. Further investigation regarding the dynamic of applied atoxigenic strain during the storage should be done with a large experimental scale period in order to gain more data to validate the long-term efficacy of the biocontrol application by AF-X in the pre-harvest period.

The biocontrol approach by using the atoxigenic strain during the pre-harvest period is highly effective to reduce aflatoxins contamination in food and feed worldwide (Agbetiameh et al., 2020; Doster et al., 2014; Mauro et al., 2018; Moral et al., 2020)

Reference

Agbetiameh, D., Ortega-Beltran, A., Awuah, R., Atehnkeng, J., Elzein, A., Cotty, P., & Bandyopadhyay, R. (2020). Field efficacy of two atoxigenic biocontrol products for mitigation of aflatoxin contamination in maize and groundnut in Ghana. *Biological Control*, *150*(June), 104351. https://doi.org/10.1016/j.biocontrol.2020.104351

Amaike, S., & Keller, N. P. (2011). Aspergillus flavus . In *Annual Review of Phytopathology* (Vol. 49, Issue 1). https://doi.org/10.1146/annurev-phyto-072910-095221

Cotty and BAyman. (1993). Competitive Exculusion of a Toxigenic Strain of Aspergillus flavus by and Atoxigenic Strain. *Postharvest Pathology and Mycotoxins*.

Dey, D. K., Kang, J. I., Bajpai, V. K., Kim, K., Lee, H., Sonwal, S., Simal-Gandara, J., Xiao, J., Ali, S., Huh, Y. S., Han, Y. K., & Shukla, S. (2022). Mycotoxins in food and feed: toxicity, preventive challenges, and advanced detection techniques for associated diseases. *Critical Reviews in Food Science and Nutrition*, *0*(0), 1–22. https://doi.org/10.1080/10408398.2022.2059650

Doster, M. A., Cotty, P. J., & Michailides, T. J. (2014). Evaluation of the atoxigenic Aspergillus flavus strain AF36 in pistachio orchards. *Plant Disease*, *98*(7), 948–956. https://doi.org/10.1094/PDIS-10-13-1053-RE

Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2020). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%. *Critical Reviews in Food Science and Nutrition*, *60*(16), 2773–2789. https://doi.org/10.1080/10408398.2019.1658570

Hell, K., Cardwell, K. F., Setamou, M., & Poehling, H. M. (2000). The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of Benin, West Africa. *Journal of Stored Products Research*, *36*(4), 365–382. https://doi.org/10.1016/S0022-474X(99)00056-9

Kinyungu, S., Isakeit, T., Ojiambo, P. S., & Woloshuk, C. P. (2019). Spread of Aspergillus flavus and aflatoxin accumulation in postharvested maize treated with biocontrol products. *Journal of Stored Products Research*, *84*, 101519.

https://doi.org/10.1016/j.jspr.2019.101519

Klich, M. A. (2002). *Biogeography of Aspergillus species in soil and litter*. *5514*, 20–27. https://doi.org/10.1080/15572536.2003.11833245

Kortei, N. K., Annan, T., Akonor, P. T., Richard, S. A., Annan, H. A., Kyei-Baffour, V., Akuamoa, F., Akpaloo, P. G., & Esua-Amoafo, P. (2021). The occurrence of aflatoxins and human health risk estimations in randomly obtained maize from some markets in Ghana. *Scientific Reports*, *11*(1), 1–13. https://doi.org/10.1038/s41598-021-83751-7

Magan, N., & Aldred, D. (2007). Post-harvest control strategies: Minimizing mycotoxins in the food chain. *International Journal of Food Microbiology*, *119*(1–2), 131–139. https://doi.org/10.1016/j.ijfoodmicro.2007.07.034

Mauro, A., Battilani, P., Callicott, K. A., Giorni, P., Pietri, A., & Cotty, P. J. (2013). International Journal of Food Microbiology Structure of an Aspergillus fl avus population from maize kernels in northern Italy. *International Journal of Food Microbiology*, *162*(1), 1–7. https://doi.org/10.1016/j.ijfoodmicro.2012.12.021

Mauro, A., Garcia-cela, E., Pietri, A., Cotty, P. J., & Battilani, P. (2018). Biological Control Products for Aflatoxin Prevention in Italy : Commercial Field Evaluation of Atoxigenic Aspergillus flavus Active Ingredients. *Toxins*, 1–14. https://doi.org/10.3390/toxins10010030

Mehl, H. L., Jaime, R., Callicott, K. A., Probst, C., Garber, N. P., Ortega-Beltran, A., Grubisha, L. C., & Cotty, P. J. (2012). Aspergillus flavus diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. *Annals of the New York Academy of Sciences*, *1273*(1), 7–17. https://doi.org/10.1111/j.1749-6632.2012.06800.x

Moore, G. G. (2021). Practical considerations will ensure the continued success of pre-harvest biocontrol using non-aflatoxigenic Aspergillus flavus strains. *Critical Reviews in Food Science and Nutrition*, *0*(0), 1–18. https://doi.org/10.1080/10408398.2021.1873731

Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., & Ortega-Beltran, A. (2020). Present status and perspective on the future use of aflatoxin biocontrol products. *Agronomy*, *10*(4).

https://doi.org/10.3390/agronomy10040491

Nakai, V. K., de Oliveira Rocha, L., Gonçalez, E., Fonseca, H., Ortega, E. M. M., & Corrêa, B. (2008). Distribution of fungi and aflatoxins in a stored peanut variety. *Food Chemistry*, *106*(1), 285–290. https://doi.org/10.1016/j.foodchem.2007.05.087

Palumbo, R., Gonçalves, A., Gkrillas, A., Logrieco, A., Dorne, J. Lou, Dall'asta, C., Venâncio, A., & Battilani, P. (2020). Mycotoxins in maize: mitigation actions, with a chain management approach. *Phytopathologia Mediterranea*, *59*(1), 5–27. https://doi.org/10.14601/Phyto-11142

Shabeer, S., Asad, S., Jamal, A., & Ali, A. (2022). *Aflatoxin Contamination, Its Impact and Management Strategies: An Updated Review*. https://doi.org/10.3390/toxins14050307

Tran-Dinh, N., Pitt, J. I., & Carter, D. A. (1999). Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of Aspergillus flavus and A. parasiticus. *Mycological Research*, *103*(11), 1485–1490. https://doi.org/10.1017/S0953756299008710

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta*, *632*(2), 168–180. https://doi.org/10.1016/j.aca.2008.11.010

Weaver, M. A., & Abbas, H. K. (2019). Field Displacement of Aflatoxigenic Aspergillus flavus Strains Through Repeated Biological Control Applications. *Frontiers in Microbiology*, *10*(August), 1–7. https://doi.org/10.3389/fmicb.2019.01788

Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V., & Kuca, K. (2009). Biological degradation of aflatoxins. *Drug Metabolism Reviews*, *41*(1), 1–7. https://doi.org/10.1080/03602530802563850

Chapter 5

Conclusion and Perspective

Final Remarks and perspective

Aflatoxin is becoming the main food safety concern for many European countries in particular in Italy and South Europe, for several commodities, mainly in maize. The use of atoxigenic strains of *A.flavus* has been demonstrated as an effective tool to consistenly reduce aflatoxin content in different crops. Since 2003, significant effort has been devoted by researchers in Italy to investigate the occurrence of *A. flavus* in different regions across the country to understand the structure and their potential to produce aflatoxin. Additionally, previous studies have led to select an atoxigenic strain to be an active ingredient of the commercial product available for Italian maize producers under the name AF-X1. AF-X1 received temporary authorization with 120 day validity, annually renewed, till when it will be finally accepted by the European authorities. This PhD research is seeking to providing new insights to support the further process of AF-X1 registration documents as a biopesticide to control aflatoxin contamination in Italy and southern Europe.

C**hapter 2** focused on the carry-over and evaluation of the multi-year influence of AF-X1, the commercial product with MUCL54911, an atoxigenic strain of *A. flavus* as active ingredient, on the fungal communities, mainly on *A. flavus*. A three year study was conducted by collecting soils from maize fields with different biocontrol treatment history located in different provinces in North Italy. The Vegetative Compatibility Analysis (VCA) demonstrated that the VCG IT006 where the active ingredient MUCL54911 of AF-X1 is included persists in the soil over the years after being applied in maize fields. Moreover, the analysis of the fungal community (CFU/g) and qPCR confirmed that there is no ecological side effect in the soil on the microbiome communities where AF-X1 was applied. The influence of the AF-X1 applications is a switch in the *A. flavus* community structure towards increased incidences of *A. flavus* non-aflatoxin producer. Therefore, only positive effects were noticed in the soil with the application of AF-X1. However, the persistence of IT006 is variable between years and areas. Therefore, in agreement with reports from other studies, the annual application of AF-X1 to commercial maize fields should be maintained, not yet possible to suggest a different treatment regime. More data should be collected to define solid bases for the optimal timing and distribution of AF-X1 that provide the most cost-effective treatments.

The ongoing approval of AF-X1 will cover the Southern Europe area. Therefore, a study was planned to scientifically support the extension of the application of AF-X1 in Southern Europe "Greece, Spain and Serbia". Therefore, the analysis of the genetic diversity and population structure of *A. flavus* was conducted in the **Chapter 3.** More than two thousand *Aspergillus flavus* isolates, 800 from Greece, 627 from Spain, and 758 from Serbia, were recovered from maize collected from different maize growing areas across the three countries. The analysis of natural populations of *A. flavus* showed a high diversity in Greece, Spain and Serbia, with predominance of clonal reproduction. Moreover, there were some haplotypes shared between the countries which confirm their adaptability in the Southern European climate. The analysis of VCA highlighted the presence of the active ingredient MUCL54911 of AF-X1 in all studied countries with the close relative haplotypes belonging to VCG IT006. The confirmed mode of action of *A. flavus* strains unable to produce aflatoxins as biocontrol agents is a competitive exclusion of toxigenic strains. Therefore, adaptability to the geographic area where they are planned to be distributed is crucial. Based on the results of this part of the thesis, it is confirmed that after approval AF-X1 can be distributed in the cited countries because VCG IT006 is naturally occurring, and it is expected to effectively play its biocontrol role.

To study the long-term efficacy of the biocontrol application of AF-X1 in field during the post-harvest period, an experimental trial was conducted and reported in **Chapter 4.** *Aspergillus flavus* density as well as aflatoxin concentration were checked monthly on maize grains collected from a silobag filled with grain collected in a field treated with AF-X1 and a big silo containing grain coming from untreated fields. The results obtained showed low concentration of aflatoxin in both treated and untreated grain; the variability of fungal communities was high in grain coming from untreated fields. The study was not run in optimal experimental conditions, and it was run for only 1 year due to the problem faced in finding technicians available to support the study following the suggested guidelines. Therefore, the results from this part of the thesis do not add the expected information.

The result obtained in these three years of PhD have led to investigate and provide new insights regarding the use of atoxigenic based product to reduce aflatoxin concentration in maize in southern Europe. The carry-over study of AF-X1 in the Italian maize growing areas provides valuable information that support the registration process to the European authorities for plant protection product and its placement in the market. All the effects reported of AF-X1 distribution are positive and very helpful in preventing aflatoxin contamination in maize so as

in understanding how the product behave in the field after its deployment. This should support the final decision of European Commission regarding the registration of the product.

The outcome of the study of *A. flavus* population in Greece, Spain and Serbia gave deeper knowledge about the community of *A. flavus* L strain, which agrees with the previous research conducted in the U.S. and Africa. Hence, these results will support researcher for further investigation on *A. flavus* community resident in other European nations. Furthermore, the results will open new perspective to use the biocontrol product AF-X1 in Greece, Spain and Serbia for aflatoxin mitigation.

Furthermore, the post-harvest efficacy trial did not give sound conclusion to understand the long-term effect of AF-X1 during the storage stage. Further studies are needed to gain more knowledge in the large-scale experiment by involving more farmers from different Italian maize growing areas. Finally, other research activities have been conducted but still ongoing and not reported in this thesis due to the time limit and some delay caused by the COVID pandemic.

Spore viability of the active ingredients of biocontrol product "Aflasafe (Africa) and AF-X1 (Italy)" by testing different solvent as well as dry spore preparation to improve the inoculum shelf life during the long-distance shipment were managed. Results are promising, even if not complete, and will help to optimize the biocontrol preparation and delivery reducing related costs.

Another part of the research was focused on the co-occurrence of *Fusarium verticillioides* (producer of fumonisins) and *A. flavus* in maize grain collected in Greece, Spain and Serbia, so as their related mycotoxins. This is a hot topic because the co-occurrence is stressed due to climate change and could significantly increase the related health risks due to additive effects of the co-occurring mycotoxins. Data available, even if not complete confirm the need to investigate co-occurrence of fungi and mycotoxins and account for this aspect in toxicological studies.

Additionally, the original idea was to study the population structure and genetic diversity of *A.flavus* isolates from Turkish maize. The delay in grain delivery did not make possible to analyse the population and include these data in the thesis, but this important tassel of information will be completed in the next future.

In perspective, following this study, genetic characterization of the global population of *A. flavus* using the SSR data obtained from Europe, USA and Africa will be managed to have a global overview.

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Thanks