UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXIV

S.S.D. AGR12/AGR13

ATOXIGENIC *ASPERGILLUS FLAVUS* ISOLATES as CANDIDATE BIOCONTROL AGENTS in MAIZE

Candidate: Mauro Antonio
Matr. n.: 3710430

Academic Year 2010/2011

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alla mia famiglia

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CHAPTER 1

Maize and *Aspergillus flavus*

1. Maize

Maize (*Zea mays* L.) was unknown in Europe until Cristoforo Colombo brought it from America. In particular, the oldest maize plant was found in Mexico and the name means literally “that which sustains life”. Besides for humans and animals nowadays maize is also used to produce starch, oil, alcoholic beverages and food sweeteners.

1.1 Botanical description

Maize (commonly called corn) is an annual plant that belongs to the family of Poaceae (formerly known as Gramineae) (Table 1).

Table 1. Systematics of maize

Kingdom	Plantae
Order	Poales
Family	Poaceae (Gramineae)
Subfamily	Panicoideae
Tribe	Andropogoneae
Genus	Zea
Species	*Z. mays*

Corn is a tall-growing plant with a single stalk, usually two-three meters high, but can vary from one to six meters. It can have anywhere between eight to forty-eight leaves. Each corn plant contains both female (ear) and male (tassel) reproductive organs located in separate places (Fig. 1a and 1b). After cross-pollination, seeds develop in the ears/cobs, often one on each stalk (Fig. 1c). Colour (red, black, white or yellow), number of rows (12 to 16) and weight of kernels can also differ (FAO, 1992). The root system consists of a single primary root, a variable number of seminal roots and shoot borne roots that are formed at consecutive shoot nodes (Fig. 1d).

Fig. 1. Structure of maize: a) ear; b) tassel; c) stalk; d) roots.

1.2 Life cycle

The development of maize can be divided into two physiological stages, vegetative and reproductive. The Leaf Collar Method determines specific stages in corn by counting the number of leaves on a plant with visible leaf collars. This method subdivides the vegetative stage (V) into V1, V2, V3, through V (n) where (n) represent the final number of leaves (usually 16-23) before vegetative tasseling. The first and the last V stages are abbreviated as VE (vegetative emergence) and VT (vegetative tasseling). The (n) will fluctuate with corn variety and environmental conditions. The reproductive stage concerns the development of seeds and is designated with R1 through R6 (Ritchie et al., 1993).

The general description of vegetative and reproductive stages is shown in Table 2.

Table 2. Vegetative and reproductive stages in maize

Stage	Features
VE	Emergence. The coleoptile emerges from the soil surface
V1	First leaf. The collar of the first leaf is visible
V2	Second leaf. The collar of the second leaf is visible
V3	Third leaf. The collar of the third leaf is visible
V (n)	Nth leaf. The collar of the leaf number 'n' is visible.
VT	Tasseling. The last branch of the tassel is completely visible
R0	Anthesis or male flowering. Pollen shed begins
R1	Silking. Silks are visible
R2	Blister. Kernels are filled with clear fluid and the embryo can be seen
R3	Milk. Kernels are filled with a white, milky fluid
R4	Dough. Kernels are filled with a white paste. The embryo is about half as wide as the kernel
R5	Den. If the genotype is a dent type, the grains are dented. The 'milk line' is close to the base
R6	Physiological maturity. Grain moisture is usually about 35%

1.3 The seed

Maize produces one seeded fruit, which is a caryopsis, but commonly called kernel. A mature kernel has three major parts: pericarp/ hull, endosperm (nutriment storage organ), and embryo or germ. The pericarp is a thin layer of maternal tissue that encloses the entire seed. The endosperm or food storage organ consists primarily of starch, which is digested into sugar upon germination and growth. The embryo or germ contains most of the fats, vitamins, and minerals. A thin layer of tissue (aleurone) covers the endosperm (Fig. 2).

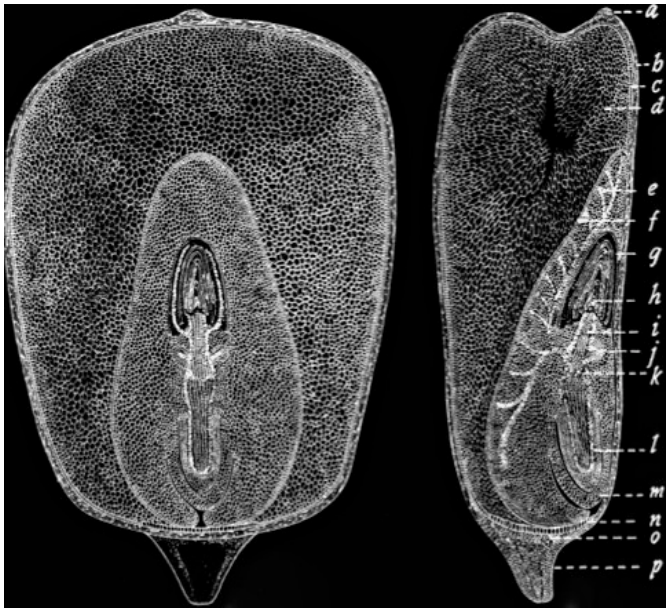


Fig. 2. Seed structure in maize: a) silk scar; b) pericarp; c) aleurone; d) endosperm; e) scutellum; f) glandular layer of scutellum; g) coleoptile; h) plumule with stem and leaves; i) first internode; j) lateral seminal root; k) scutellar node; l) primary root; m) coleorhiza; n) basal conducting cells of endosperm; o) brown abscission layer; and p) pedicel or flower stalk. Adapted from Coe (2001).

Two major structures of the seed, i.e. endosperm and embryo, constitute approximately 80% and 10% of the mature seed dry weight, respectively (Table 3). The mature seed is composed of 71% to 73% starch, which is principally stored in the endosperm, 12% to 13% protein and 4% to 5% oil. Proteins and oil are 18% and 30% in the embryo, respectively (Hasjim et al., 2009).

Table 3. Main parts of a seed

Structure	Weight distribution (%)
Endosperm	80-85
Embryo	10-12
Pericarp	5-6
Aleurone	2-3

1.4 Maize in the World

Maize can grown in temperate, subtropical and tropical regions, from 58° N in Canada to 35° S in South America. It is produced from sea level up to terraced plots 3960 m high in the Andean mountain of South America. It cannot however be grown above the freezing line (Bradburn et al., 1993). High adaptation to growth in diverse climates, numerous uses and genetic improvement has contributed to the dispersal of maize worldwide. Effectively, corn represent the most produced crop in the world, with about 817 millions of tons in 2009 (Table 4) (FAO, 2009).

Table 4. First 10 countries producer of maize in the world (millions of tons) (FAO, 2009)

Country	2007	2008	2009	World production (%)*
Argentina	21,8	22,0	13,1	1,6
Brazil	52,1	58,9	51,2	6,3
Canada	11,6	10,6	9,6	1,2
China	152,4	166,0	163,1	20,0
EU	48,9	62,9	57,8	7,1
India	19,0	19,7	17,3	2,1
Indonesia	13,3	16,3	17,6	2,2
Mexico	23,5	24,3	20,2	2,5
South Africa	7,1	12,7	12,1	1,5
USA	331,2	307,1	333,0	40,8
World	789,5	826,2	817,1	

Symbol (*) represents % of year 2009.

Moreover, maize is a very important crop in Italy where about one million ha is dedicated to this cereal with a production of about 8.5 million of tons. The growing area represents almost the 15% of the available agricultural area in the nation. Around 90% of the area dedicated to maize cultivation is located principally in northern Italy. Most of the yearly production (86%) is destined to animal feed and the rest to human food (5%) and starch production (9%) (Istat, 2011).

Several mycotoxigenic fungi can infect maize and their presence is strictly related to meteorological conditions in the growing area. In particular, this important cereal is susceptible to a range of different Fusaria, including *Fusarium graminearum, F. verticillioides* and *F. moniliforme* as well as by *Aspergillus ochraceus* and *A. flavus*. These pathogens are able to produce different types of toxins dangerous for both humans and animals. Especially *A. flavus* can produce the most toxic naturally occurring carcinogens known (CAST, 2003).

1.5 *Aspergillus* section *Flavi*

The *Aspergillus* conidiophore consists of an elongated stalk or stipe culminating in an expanded bulbous region variously called the columella or vesicle on which are borne one or two layers of cells that generate the asexual spores or conidia. The base of the conidiophore is 'T' or 'L' shaped and is called the foot cell, even though it is not a separate cell. The foot cell is a diagnostic feature of the *Aspergillus* conidiophore (Scheidegger and Payne, 2003).

Antonio Micheli (1679-1736) described for the first time the genus *Aspergillus* in his publication, *Nova plantarum*, published in 1729. The name originated from the similarity of fungus conidiosphore to the aspergillum, a liturgical brush used to sprinkle holy water (Bennett, 2009). More than 200 species, which reproduces by making asexual spores, have been assigned to this genus (Amaike and Keller, 2011; Bennett, 2009). These species are principally common in tropical, subtropical and warm climates (Scheidegger and Payne, 2003), but were also isolates in Antarctic soil (Mercantini et al., 2004) and in the Mir spacecraft (Novikova, 2004). Ubiquitous is the key to describe the widely distribution of these fungi (Bennett, 2009).

Among the numerous known species, *A. parasiticus* and *A. flavus* are the most relevant in safety issue because are pathogenic for humans and produce the most strong natural carcinogenic known toxins: the aflatoxins (AFs) (Bennett, 2009). Different oil-

containing crops are infected by these potent toxins producers. Although these two species are closely related with 96% of identical genetic sequences (Cary and Ehrlich, 2006), *A. parasiticus* is common particularly on peanuts while *A. flavus* represent the principally source of contaminations on maize (Horn and Dorner, 1998).

Researchers have frequently failed to distinguish between the two species but macro and micro differences can be observed between them. *A. parasiticus* grown on Czapex agar medium appear darken green contrary *A. flavus* is yellow to dark yellowish-green. Micro observation showed conidial head in *A. parasiticus* is usually uniseriate (Fig. 1A-B) and biseriate in *A. flavus*. Probably, conidial wall ornamentation could be the best diagnostic character for separation of these two species. Conidia wall of *A. flavus* is finely to moderately roughened (Fig. 3F). Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose (Fig. 3C). Scanning Electron Micoroscopy micrographs clearly show these ornamentation differences.

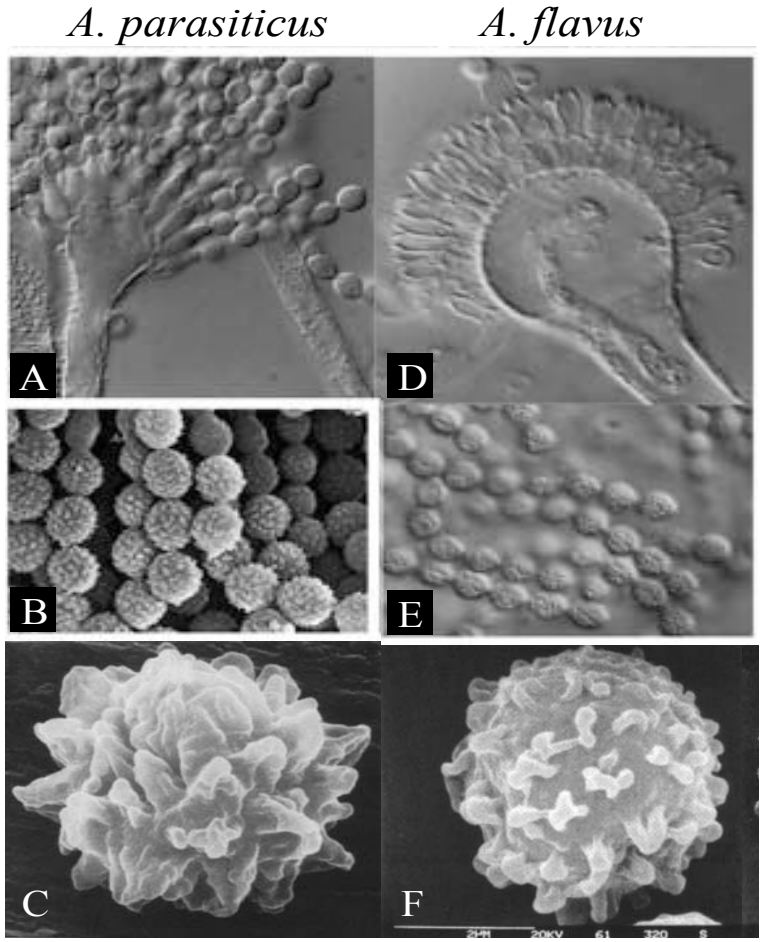


Fig. 3. *A. parasiticus* conidiospere (A), uniseriate conidia (B) and conidia ornamentation (C). *A. flavus* conidiospore (D), biseriate conidia and conidia ornamentation (E). A, B, D, E from de Hoog et al. (2000) and C, F from Rodrigues et al. (2007).

1.6 *Aspergillus flavus*

A. flavus was described by Link in 1809 and belongs to section *Flavi* (Peterson, 2008). Only recently the teleomorph form have been described and classified as *Petromyces flavus* (Horn et al., 2009).

Member of this species can differ for several characteristics such as sclerotia size and type of secondary metabolites produced, in particular mycotoxins. Sclerotia size divided the strains in L, which produce fewer and larger sclerotia (> 400 mm in diameter) and S, which produce numerous small sclerotia (< 400 mm in diameter) (Cotty, 1989). Moreover the S strains are characterized by a higher production of aflatoxins (AFs) compare to the L strains. In contrast some L strain isolates, designated atoxigenic, completely missing the ability to produce AFs (Cotty, 1989).

The infection cycle of *A. flavus* (Fig. 4) on maize is well described. Briefly, the infection start with the dissemination and germination of conidia produced by the fungus present in the soil where lives as a saprophyte (Payne, 1998; Scheidegger and Payne, 2003).

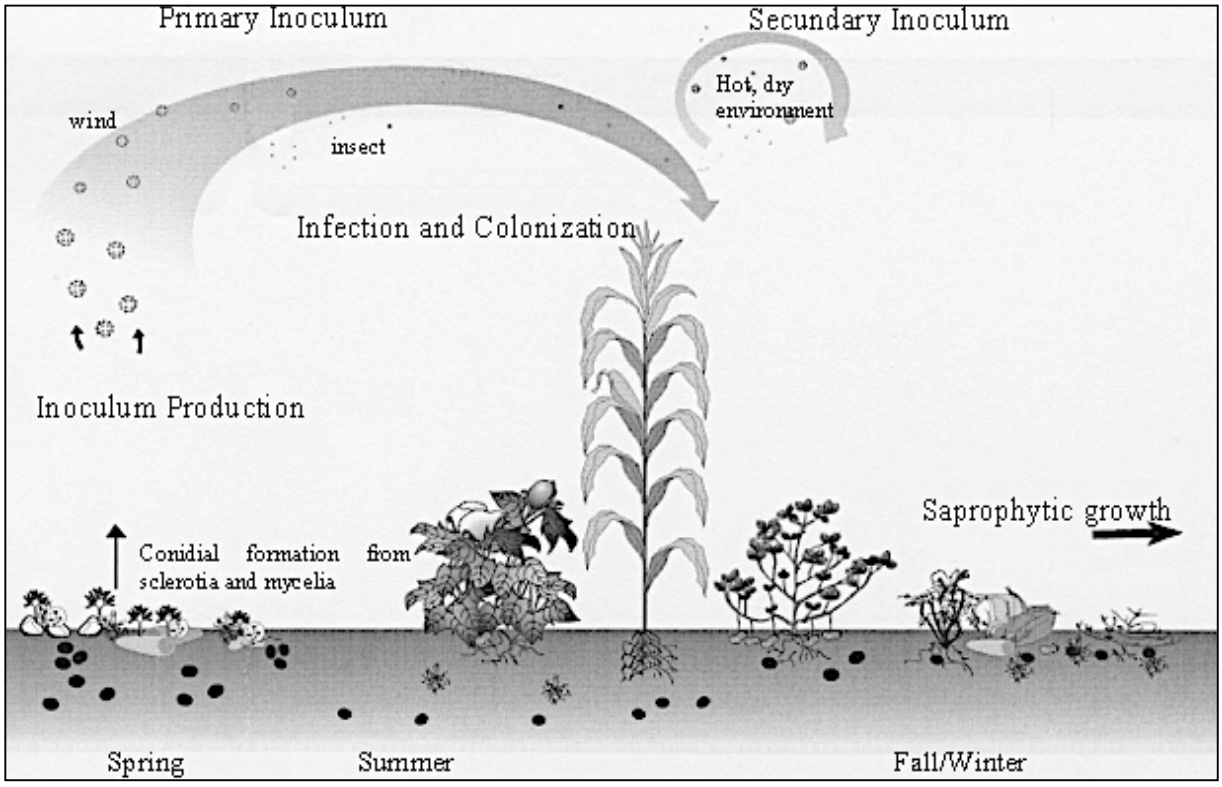


Fig. 4. The infection cycle of *A. flavus* on cotton, corn and peanuts (Scheidegger and Payne, 2003)

Primary infection is due to the conidia that carried by insect, wind or rain arrive on the corn silks were the fungus starts to colonize their tissue. After silks, the fungus can colonize the glumes (starting from the milk stage), the kernels and, infrequently, the cob (Marsh and Payne, 1984; Widstrom, 1996). Favorable environmental conditions or tissue wounds operated by insect, that may promote the infection of the seeds and cobs, are responsible of the secondary inoculum (Scheidegger and Payne, 2003). Silks and kernels surface are colonized early after silking stage and can continue and increase during the growing season. *A. flavus* colonization is principally located on the surface respect to the inside of kernel (Marsh and Payne, 1984). The minimum moisture requested for kernel infection and AFs production is around 30% and 15% respectively (Payne, 1998).

In the life cycle of *A. flavus*, saprophytic growth represent an important aspect for the persistence of the fungus in the soil. In fact plant tissue like kernels, cobs or leaf contaminated by *A. flavus* during maize growing can remain in the soil and keep the fungus until the next season when conidia arise from the mycelium or sclerotia produce the primary inoculum for a new infection cycle (Scheidegger and Payne, 2003).

How *A. flavus* can penetrate in the kernels is still not completely understood. It has been proposed that similar to other fungi also *A. flavus* can entry through the stylar canal. This could be supported by the presence of blue-greenish yellow fluorescence found near the silk scar of infected kernels. But *A. flavus* has been also found in the pedicel region of the seed. The two ways from which the fungus can enter in maize means that it can have different sites of entry influenced by environmental conditions and genotype of the maize (Payne, 1998).

Presence of *A. flavus* species and its capacity to produce mycotoxins on maize in influenced by several factors that can be divided in biological, physical and chemical (D'Mello and Macdonald, 1997) (fine VCGs).

1.6.1 Biological factors

Influence of 13 cohabiting fungi on AFs produced and growth of *A. flavus* on maize kernels was evaluated (Choudhary, 1992). All fungi tested against *A. flavus* were able to reduce AFB_1 from 34 to 100%. Inhibitions in radial growth of *A. flavus* by *F. moniliforme* (59%), *Trichoderma viride* (72%) and *Rhizopus nigricans* (42%) may be correlated to the percent inhibition of AFs production. With *A. niger*, *Cladosporium herbarum*, *F. oxysporum*

and *A. candidus* inhibition in radial growth was 63%, 33%, 30% and 26%, respectively, while percent reduction in AFs was 88%, 68%, 52% and 43%, respectively (Choudhary, 1992).

F. verticillioides can compete with *A. flavus* for the resources on the corn ear; years with temperate weather can promote *F. verticillioides* infection contrary high temperature and drought stress support *A. flavus*. In fact, years in which AFs contamination is a serious problem are characterized as having above-average temperatures and below-average rainfall (Payne, 1998).

Although *F. verticillioides* does not share the same infection site with *A. flavus*, they appear to compete on the corn ear, interfering with infection and AFs accumulation in the kernels (Wicklow et al., 1988) Also a negative correlation between the presence of the two fungi was found (Hill et al., 1985) and ears co-inoculated with *A. flavus* and *F. verticillioides* showed a reduction in the number of kernels infected by *A. flavus* (Zummo and Scott, 1992).

Wounds are not necessary for AFs formation but incidence of *A. flavus* and AFs contamination were higher in damaged kernels (Diener et al., 1987). Physical damages are principally due to invertebrate activity, farm equipment, birds and environmental factors (Bradburn et al., 1993). Insect damage activity on the kernels affords to *A. flavus* the opportunity to cross the natural protection of the integument and determine the infection in the vulnerable interior (Lillehoj et al., 1980). Moreover wounds of insects can promote the kernels drying at level of moisture that is more conducive for *A. flavus* growth and AFs production (Widstrom, 1979).

A. flavus infection of corn grain may be linked to the development of the second generation of European corn borer (ECB; *Ostrinia nubilalis*) in fact insect development period match with the period during maize kernels are susceptible to this fungus infection (Guthrie et al., 1982). Although insects take on an important role in the epidemiology of *A. flavus* (Diener et al., 1987), the fungus is also capable to infect developing kernels undamaged (Jones et al., 1980).

How insects contribute in the infection of kernels can be summarize in four points (Payne, 1998):

1. transport primary inoculum to the ears;
2. move inoculum from the silks into the ear;
3. disseminate inoculum within the ear;
4. facilitate colonization and infection of the kernels by injuring the kernels.

Contribute of insects to each processes depend on environmental conditions and insects population. In fact, *A. flavus* with favorable conditions became more aggressive infecting kernels without insect injury. Contrary, under unfavorable conditions the absence of insect damages reduces the kernels colonization by *A. flavus* (Payne, 1998).

1.6.2 Physical factors

Several physical factor are involved in *Aspergillus* spp. crops contamination. In particular temperature and water activity (a_w) are the principal factors which affect growth and AFs production (Payne, 1998).

Although *A. flavus* is adapted to live in a wide range of temperature included between 12 to 48°C (Klich et al., 1994), the optimum for fungal growth is comprise between 19 and 35°C (Northolt and van Egmond, 1981).

About AFs they may be produced in a range from 24 to 32°C (Northolt et al., 1977) but other studies reported a range of 20-35°C (Reiss, 1975). However Kheiralla et al. (1992) and Sanchis and Magan (2004) reported that 30 and 28°C are the optimum temperatures for toxin production. Differently Giorni et al. (2007) analyzing an Italian *A. flavus* population found 25°C the optimal temperature for AFs production. However no growth or AFs synthesis is observed at 5°C (Park and Bullerman, 1983).

Time of incubation of *A. flavus* on a substrate can influence the amount of AFs produced. In fact Kheiralla et al. (1992) obtained the highest concentration of AFs at 30°C after 14 days of incubation but after this period the concentration decreased probably as consequence of toxin degradation or re-adsorption by the fungus.

Production of AFs in the field seems to be like a circle were the fungus at the same time synthetize and degrade the toxins. Environmental changes, which occur within a day, could modify the way of the toxins biosynthetic pathway promoting the synthesis or the degradation influencing directly the final amount of AFs on a crop (Stutz and Krumperman, 1976).

Water availability, measured as a_w influence both fungi growth and mycotoxins production. Lee and Magan (2000) found that for *A. flavus* 0.99 was the optimal level of a_w for grown and AFs production. However, 0.77 and 0.83 a_w could be the minimum levels requested for grown and AFs biosynthesis (Sanchis and Magan, 2004). In a experiment on kernels *A. flavus* was dominant on *A. ochraceus* at high a_w levels (0.99) but not at lower a_w

levels (0.95). Contrary at 0.95 a_w AFs produced by *A. flavus* was higher than ochratoxin produced by *A. ochraceus* (Lee and Magan, 2000).

Water activity influenced also conidia germination. In particular for *A. flavus* germination was faster at a_w levels higher than 0.90 decreasing significantly when the conidia were tested at lower levels (Marin et al., 1998).

1.6.3 Chemical factors

Chemical compounds have been studied and used for the treatment of plant pathogens. In particular effect of several fungicides on growth and AFs production by *Aspergillus* spp. have been investigated. Criseo et al. (1994) tested 5 inhibitors of mycelial growth *in vitro* on strains of *A. flavus* and *A. parasiticus*. Between the compound tested mercuric chloride and cycloheximide showed the best efficiency in reducing fungal growth but they also promoted AFs production. Contrary biphenyl was able to reduce both fungal growth and AFs production at high concentration but lower concentration just delayed the AFs biosynthesis. Dichloran did not affect fungal growth but inhibited AFs production. The last compound tested, the sodium desoxycholate, was able to reduce both mycelial growth and AFs production (Criseo et al., 1994).

Reddy et al. (2009) evaluated the efficiency of 5 conventional fungicides and 5 non-conventional chemicals against *A. flavus*, *A. parasiticus*, *A. niger* and *A. ochraceus* growth and AFs production on rice. Conventional fungicides, *i.e.* bavistin (carbedazim), contaf plus (hexaconazole), folicur (tebuconazole), result (propiconazole) and saaf (carbedazim and mancozeb), were able to completely inhibited growth and AFs production of all *Aspergillus* spp. tested at 1 ml per kg of rice. About the 5 non-conventional compound tested, benzoic acid at 4 g per kg of rice reduced growth of *A. flavus* of 72% and completely inhibited grown of *A. parasiticus*, *A. niger* and *A. ochraceus*. Vanillin totally reduced AFs production and the sodium chloride also the mycelial growth (Reddy et al., 2009).

Efficacy of different fungicides against *A. flavus* growth and AFs production was evaluated on different media incubated at 20 and 30°C. Tebuconazole (25% of active ingredient) and mancozeb (80% of active ingredient) were the most effective in inhibiting growth and AFs production under the condition tested. However efficiency of each fungicides was influenced by temperature and substrate used (Santos et al., 2011).

Also two ergosterol biosynthesis inhibitors, prochloraz and imazalil, seem to be able to

reduce *A. flavus* and *A. parasiticus* growth and AFs biosynthesis. Although the fungi are adapted to these fungicides, some differences in the colony morphology were observed. In particular the molecules altered conidial formation and AFs production, determining an AFs reduction over 80% (Delen and Tosun, 1999).

The efficacy of fungicides is strain-dependent and it is influenced by temperature. Moreover, the same fungicide that was particular able to reduce fungal growth can enhance AFs production because of stress caused to the fungus. So the best fungicide should, at the same time, prevent fungi growth and AFs production (Santos et al., 2011). However, no fungicides have showed their efficacy in controlling *A. flavus* on maize when applied at concentrations that are cost-effective and environmental safe (Bhatnagar et al., 1993). Consequently, although several fungicides are available to limit AFs concentration on crops, their use could asked an unacceptable cost for the farmer (Brown et al., 1998).

In Italy, no fungicide able to reduce *A. flavus* growth and AFs contamination is allowed for in field treatment of maize destined for both food and feed.

1.6.4 Vegetative Compatibility Groups (VCGs)

Vegetative compatibility is the ability of fungi to undergo hyphal fusion to form stable heterokaryons. Heterokarions could be stable for long period of time or may be short because the haploid nuclei fuse and immediately make meiosis (Leslie, 1993). This self/nonself system limits hyphal anastomosis and consequent gene flow between individuals belonging to different VCGs (Leslie, 1993). Fungi may have kept this through the evolution, to limit transmission of hazardous viruses and plasmids (Caten, 1972; Biella et al., 2002) or parasitic nuclei (Hartl et al., 1975). Loci that regulate vegetative incompatibility are defined *vic* and ranged from 9 in *Podospora anserine* (Saupe et al., 2000), to at least 8 in *A. nidulans* (Anwar et al., 1993), 7 in *Cryphonectria parasitica* (Cortesi and Milgroom, 1998), and 11 in *Neurospora crassa* (Perkins and Davis, 2000). Stable fusion of vegetative hyphae is possible only between isolates with the same alleles at all *vic* loci while hyphal fusions of isolates with different alleles produce a programmed cell death (Glass and Dementho, 2006). Two isolates that can fuse their hyphae belongs to the same VCGs (Barros et al., 2006) and members of the same VCG are considered to be members of the same clonal lineage (Papa, 1986); sequence data confirm that isolates within a VCG are closely related and distinct from other VCGs (Grubisha and Cotty, 2009). Phenotypic characteristics (*i.e.*, size of sclerotia and aflatoxin-

producing ability) of isolates are usually conserved within VCGs more than isolates belong to different VCGs (Novas and Cabral, 2002; Mehl and Cotty, 2010).

Complementation tests between nitrate nonutilizing auxotrophs (*nit* mutants) are used to evaluate vegetative compatibility among isolates of *A. flavus* (Bayman and Cotty, 1991). Two enzymes are involved in nitrate assimilation: nitrate and nitrite reductase and the active site of nitrate reductase is a molybdenum cofactor, codified by several genes called *cnx* (cofactor nitrate reductase and xanthine dehydrogenase) (Schwarz and Mendel, 2006). *Nit* mutants differ in their ability to utilize various nitrogen sources depending on which genes, essential for nitrate assimilations, carry on the mutation. Three classes of mutants are possible: niaD (defective in the nitrate reductase genes), nirA (mutation in the nitrite reductase genes) and cnx (defective in the *cnx* genes).

Vegetative compatibility analysis (VCA) of population of *A. flavus* (Bayman and Cotty, 1991; Pildain et al., 2004; Barros et al., 2006), showed that *niaD* mutants are isolated in higher proportions than *cnx* and *nirA* mutants. But *cnx* mutants are preferred as testers for identifying VCGs because usually they provide the strongest reactions between compatible isolates when paired with *niaD* or *nirA* mutants (Correll et al., 1987) and are the most consistent for VCA among *A. flavus nit* mutants (Bayman and Cotty, 1991). Differences in proportions of the various genes involved in nitrate assimilation and use have been frequently reported. However, the mechanism because these differences occur is not yet understood. It has been suggested that either physical size of the genes may play a role or that some loci may be more susceptible to mutation than others (Klittich and Leslie, 1988).

Vegetative compatibility analysis is largely used in plant pathology to study genetic diversity of fungi populations. Liu et al. (1996) analysed 4 populations of *Cryphonectria parasistica*: 3 from USA and 1 from Italy. Each population was composed of 16, 19, 57 and 50 isolates, respectively. All 146 isolates were tested for vegetative incompatibility and assigned in 37 VCGs. In particular 1, 2, 3 and 31 VCGs were individuated in the population with 16, 19, 50 and 57 isolates, respectively.

Genetic variability was also assessed in a population of *Cercospora coffeicola* sampled from organic and conventional coffee plantings in three Brazil regions (Martins et al., 2008). Sixty-five isolates, 33 from organic and 32 from conventional coffee plants, were assigned in 28 VCGs. Several VCGs were detected in more than one region and under both agricultural system. However region and crop system did not influenced genetic structure of

the pathogen population (Martins et al., 2008).

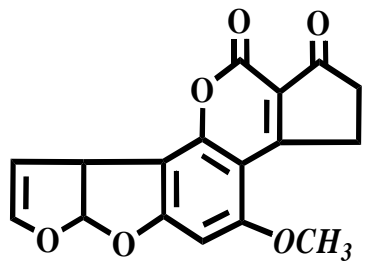
Vegetative compatibility analysis is also used to investigate diversity within *A. flavus* populations and several VCGs are commonly found in each geographic area studied. Sweany et al. (2011) identified 16 VCGs from 669 isolates of *A. flavus* from ears and soil in 11 Louisiana corn fields. Habibi and Banihashemi (2008) identified 16 VCGs from 44 sesame *A. flavus* seed isolates collected in Iran. Barros et al. (2006) identified 56 VCGs from 100 *A. flavus* isolates collected from soil in an Argentinian peanut-growing region. VCG diversity of 79, 76 and 64 isolates of *A. flavus*, *A. parasiticus* and *A. tamarii*, respectively, sampled in peanut field in Georgia were examined. Forty-one, 9 and 15 VCGs were individuated from *A. flavus*, *A. parasiticus* and *A. tamarii*, respectively (Horn and Green, 1995). Bayman and Cotty (1991) identified 13 VCGs from 61 isolates of *A. flavus* from soil and cottonseeds in an Arizona cotton field and Papa (1986) assigned 32 isolates from Georgia corn kernels in 22 VCGs.

Vegetative compatibility analysis is also important in developing biological agents (Donner et al., 2010) because the diversity of VCGs correlates negatively with the success of biological control (Anagnostakis, 1987; MacDonald and Fulbright, 1991). Ideally, the atoxigenic isolates for safe use in biological control should belong to VCG that do not have toxigenic members (Cotty, 2006). This is a precaution to ensure that atoxigenic and toxigenic isolates within a VCG will not exchange genetic material and generate progenies that produce aflatoxins (Ehrlich et al., 2007).

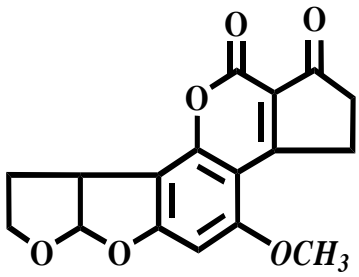
1.7 Aflatoxins and their toxicity

Aflatoxins are polyketide-derived secondary metabolites that can be produced by six species of *Aspergillus* section *Flavi*: *A. flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. pseudotamarii* and *A. tamarii* (Goto et al., 1996; Ito et al., 2001; Varga et al., 2003). However, the two most important AFs producer fungi are *A. flavus* and *A. parasiticus*, whose AFs contamination determines important economic losses (CAST, 2003).

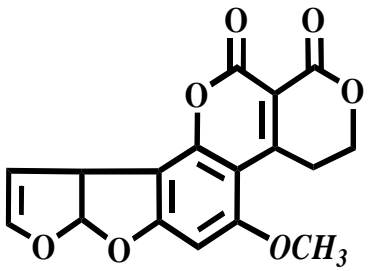
It is generally accept that *A. flavus* produces only the aflatoxin B_1 (AFB_1) and B_2 (AFB_2) (Fig. 5) and the cyclopiazonic acid (CPA) but a taxon similar to S strains, named S_{BG}, can produce also the G-aflatoxins (Fig. 5). Instead *A. parasiticus* can produce all four AFs but not the CPA (Amaike and Keller, 2011). However should take in consideration that not all the strains are toxins producer and atoxigenic strains are common (Smith and Moss, 1985).



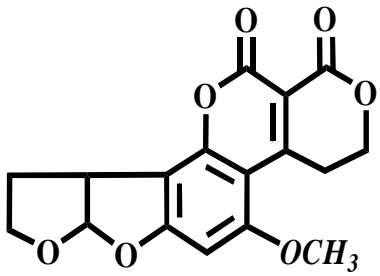
AFB_1



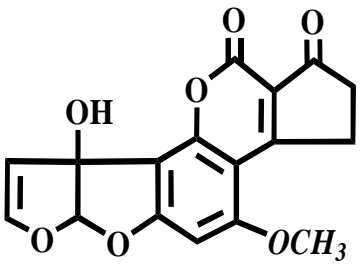
AFB_2



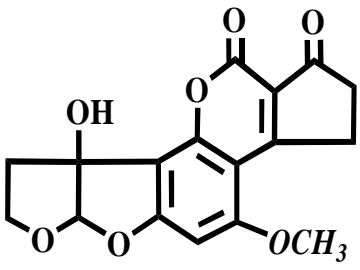
AFG_1



AFG_2



AFM_1



AFM_2

Fig. 5. Chemical structure of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2).

Aflatoxins take the name from blue (B) or green (G) fluorescence exhibit when exposed to ultraviolet light (366 nm) on silica gel thin layer chromatography (Hartley et al., 1963). In addition, in the milk of dairy cows it is possible to find the aflatoxins M_1 and M_2 (Fig. 5), as a consequence of AFB_1 and AFB_2 feed contaminated products (van Egmond, 1989).

The discovery of AFs is linked to the death of more than 100,000 turkeys in England in 1961. The cause of the death initially was unknown and for these reasons was called Turkey X disease. The subsequent investigations reveled that the groundnut meals used as feed were contaminated with a toxic metabolites produced by some strains of *A. flavus*. The compound was chemically characterized and named AFs, from the acronym Aspergillus flavus toxins (Forgacs and Carll, 1962).

Since its discovery, the dangerous effects of AFs on humans have been documented. In particular, consumptions of contaminated food can cause aflatoxicosis and/or liver cancer, but also the fungus can invade the human body and cause aspergillosis often fatal in immunocompromised patients (Amaike and Keller, 2011).

Aspergillosis is a disease caused at least by 20 species of Aspergilli through invasive mycelia grown inside the body. *A. flavus* is second only to *A. fumigatus* causing invasive and noninvasive aspergillosis (Krishnan et al., 2009). Source of infection is represented by the fungal spores breathed from contaminated foods (Adhikari et al., 2004) or smoked contaminated plant material like tobacco (Verweij et al., 2000). The symptoms caused by repeated exposures to spores are asthma or allergic bronchopulmonary aspergillosis (Hedayati et al., 2007).

Aflatoxicosis arises inhaling or ingesting high levels of food contaminated with AFs. We can distingue aflatoxicosis in chronic, determinated by the assumption of low doses of AFs for long time, and acute, caused by high concentrations of AFs in one or a limited exposures. Symptoms of chronic aflatoxicosis are stunted grown, immune suppressions and in some cases thus can lead to liver cancer development (Cardwell and Henry, 2004; Gong et al., 2004). Acute aflatoxicosis has been a big problem in developing countries especially Asia and Africa. For example in Kenya outbreaks were reported in 1981-1982, 2001, 2004-2006 and 2008 (Ngindu et al., 1982; Farombi, 2006; Probst et al., 2007). In particular, more than 150 people died between the years 2004-2005 after consuming maize contaminated with AFs (Strosnider et al., 2006).

Aflatoxins are also dangerous for animals. In additions to citied outbreaks in turkeys in England in the early 1960s, other animals followed the same end. This is the case of more than 100 dogs in the United States killed by AFs group B contained in maize used for dogs dry food (Stenske et al., 2006; Dereszynski et al., 2008). AFs contaminations represent also a problem in the dairy farm. For example, in Italy in 2003 high levels of AFM_1 and AFM_2 were detected in cow milk as consequence of elevated concentrations of AFB_1 and AFB_2 in the maize feeds (Giorni et al., 2007; Piva et al., 2006).

1.8 Aflatoxin biosynthesis and pathway gene cluster

Aflatoxins biosynthesis pathway has been described and involves at least 23 enzymatic conversions, through a series of pathway intermediates, which are summarized in Fig. 6 (Sweeney and Dobson, 1999). Briefly, acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase, which is then extended by a polyketide synthase to norsolorinic acid, the first stable precursor in the pathway. Instead penultimate precursors of AFs are the sterigmatotocystin (ST) and the dihydrosterigmatocystin (DHST). In particular the ST is the precursor of AFB_1 and AFG_1 and the DHST is the precursor of AFB_2 and AFG_2 (Yu et al., 2004).

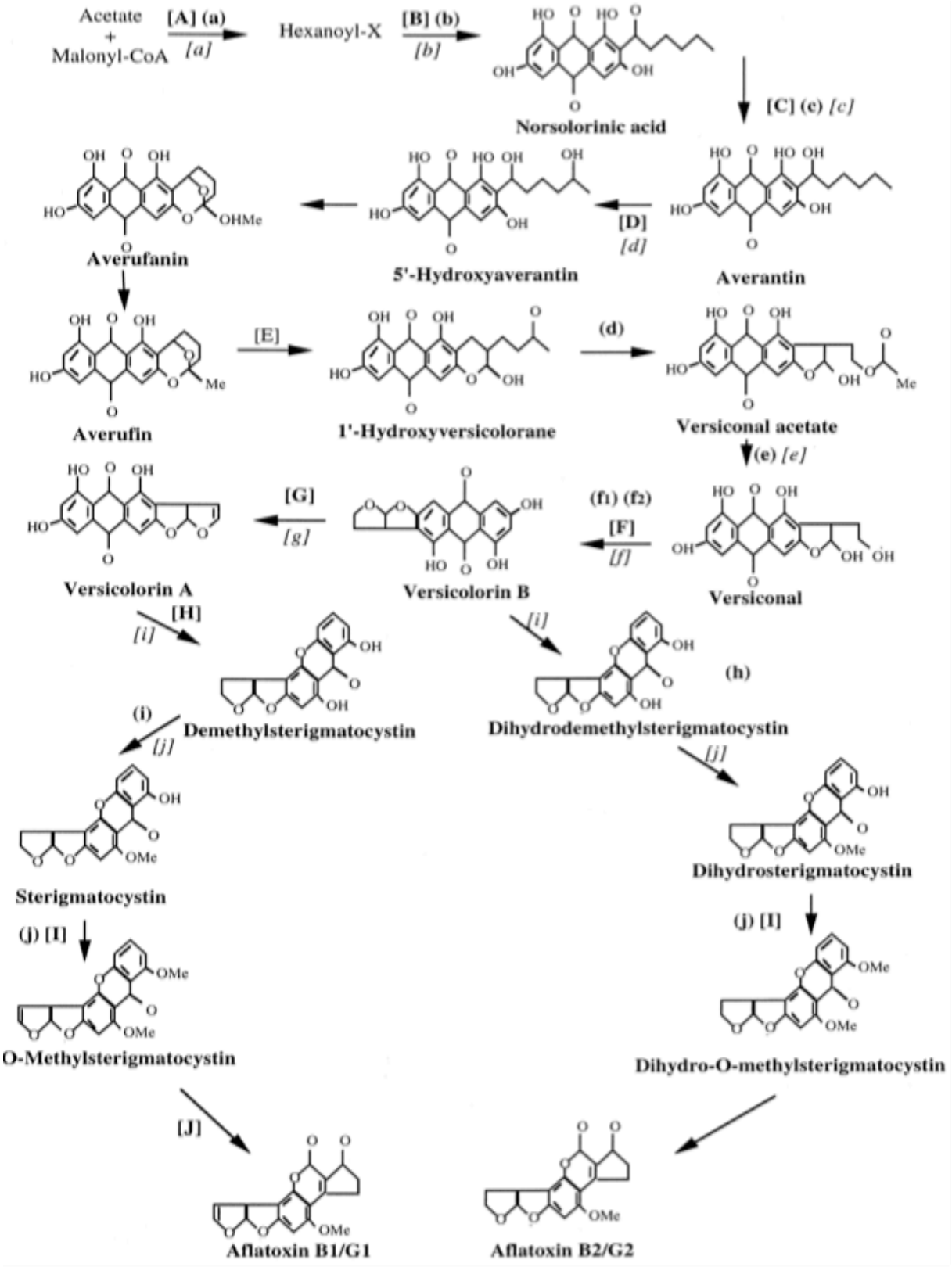


Fig. 6. Aflatoxins biosynthetic pathway. Enzymes involved: (a) fatty acid synthase, (b) polyketide synthase, (c) norsolorinic acid reductase, (d) versiconal hemiacetal acetate reductase, (e) esterase, (f1) versicolorin B synthase, (f2) versiconyl cyclase, (g) desaturase, (h) O-methyltransferase (MT-II), (i) O-methyltransferase, (j) O-methyltransferase (MT-I) (Sweeney and Dobson, 1999).

Whole genome sequences of several species of *Aspergillus* are updated and available in many web sites. In particular a 5X sequence coverage of *A. flavus* was released by The Institute for Genomic Research in 2005 (Amaike and Keller, 2011).

The genome size of *A. flavus*, distributed on 8 chromosomes, is estimated in 37 Mb and encode for more than 12,000 functional genes (Payne et al., 2006; Chang and Ehrlich, 2010). Compare to other Aspergilli, *i.e. A. terreus* (30 Mb), *A. nidulans* (31 Mb), *A. fumigatus* (30 Mb), and *A. niger* (34 Mb), genome of *A. flavus* is bigger although all species shared the same number of chromosomes (Birren et al., 2004; Galagan et al., 2005; Nierman et al., 2005; Pel et al., 2007). Probably the different size of *A. flavus* (and is twin *A. oryzae*) is due to extra copies of lineage specific genes (Machida et al., 2005) that are generally located in non-synthetic blocks (Chang and Ehrlich, 2010).

Genes responsible of AFs synthesis in *A. flavus* are well known (Fig. 7). Secondary metabolites genes involved in the synthesis of a single metabolite are generally clustered in the genome where are present the enzymatic genes and often also the transcriptional factors for compound synthesis (Hoffmeister and Keller, 2007; Turner, 2010). The cluster for AFs biosynthesis is located near one telomere of chromosome 3 and at least 25 genes are involved in the biosynthesis; the functions of 19 genes have been assigned but for 6 of them the functions are still unassigned. The average size of each gene is about 2 kb but three are bigger: the fatty acid synthase alpha (5.8 kb) and beta (5.1 kb) subunits and the polyketide synthase (6.6 kb) (Yu et al., 2005). These last three genes are required to synthesize the first stable AFs precursor, nonsolorinic acid (Amaike and Keller, 2011).

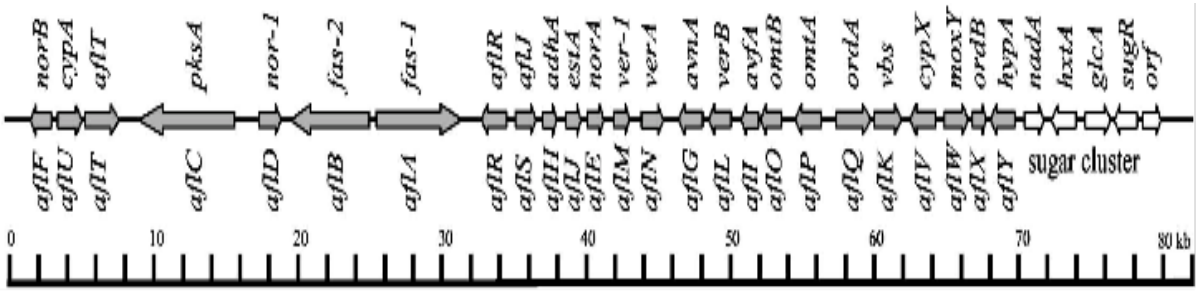


Fig. 7. Aflatoxins clustered genes. The horizontal line represents the 82 kb aflatoxins biosynthetic gene cluster in *A. flavus* and indicates the relative sizes of the genes in kilobases. The new name of each gene is shown on the top of the horizontal line and the old on the top of the arrows (Yu et al., 2004).

Many members of *Aspergillus* section *Flavi* lack AFs production. *A. sojae* and *A. oryzae* have homologues of AFs biosynthetic genes (Chang et al., 1995; Klich et al., 1995; Yu et al., 2000) but they do not produce AFs (Wei and Jong, 1986). In fact, for century, they have been largely used in food fermentation industry and are considered safe (Machida et al., 2005; Chang et al., 2007). Also *A. oryzae* strains, domesticated atoxigenic strain of *A. flavus* (Wicklow, 1984; Chang et al., 2006), have the AFs biosynthesis gene cluster but it is not functional due to deletions, frame-shift mutations and base pair substitutions (Tominaga et al., 2006).

Deletion of portions of the AFs biosynthetic gene cluster within atoxigenic *A. flavus* isolates is not rare (Chang et al., 2005) and strains of *A. flavus* with large deletions in the AFs gene cluster have been used to study the genetics of AFs biosynthesis for over a decade (Prieto et al., 1996). Also PCR detection for presence or expression of AFs biosynthetic genes have been used as diagnostic tool for searching aflatoxigenic fungi in selected food commodities (Geisen, 2007).

Chang et al. (2005) characterized deletions in the aflatoxin gene cluster of 38 atoxigenic *A. flavus* isolates. They individuated 8 different patterns of deletion named from A to H. Some isolates presented different degrees of deletions. In detail isolates in the pattern A did not miss any genes while isolates in the patter H missed all genes. Similar results were also found by Donner et al. (2010), who analyzed 23 atoxigenic isolates of *A. flavus* from Nigeria. Eight patterns were identified and although two patterns were similar to those reported by Chang et al. (2005) new type of deletion were individuated.

1.9 Aflatoxins legislation

As consequence of the high toxigenic activity of AFs, in particular AFB_1, limits in food and feed are strictly regulated worldwide. First overview of worldwide mycotoxins was presented in 1981 at International Symposium on mycotoxins in Cairo (Schuller at. al., 1983). In 2003 at least 99 countries had mycotoxin regulations for food and/or feed, an increase of approximately 30% compared to 1995 (FAO, 2003). AFB_1 limit in food differ in each country but substantially is comprised between 1 and 20 μg/kg (Fig. 8) and the total AFs limits ranging between 0 to 35 μg/kg (Fig. 9). On the other hand, limits of AFB_1 in feed for dairy cattle range from 5 to 50 μg/kg and the total AFs from 0 to 50 μg/kg. About AFM_1 in the milk

limits are comprised from 0 to 15 μg/kg (FAO, 2003). Regarding Italy, AFB_1 limit is 2 μg/kg and 5 μg/kg for processed adult food and dairy cattle feed, respectively (EC, 2006; 2010).

Although limits for AFs across country are different, an hormonised regulations have been done in countries belonging to economic communities. In particular in Europe the first harmonized regulations that setting maximum levels for certain contaminants in foodstuffs and also AFs was releade in 2001 (EC, 466/2001). This regulation was later modified with the EC 472/2002, EC 2174/2003, EC 683/2004, EC 1881/2006 and the more recently with EC 165/2010.

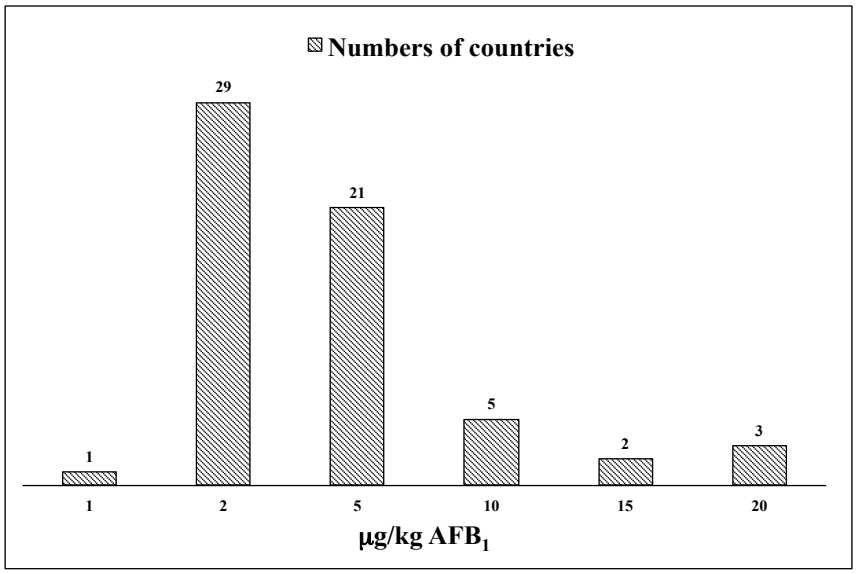


Fig. 8. Worldwide limits for aflatoxin B_1 in food

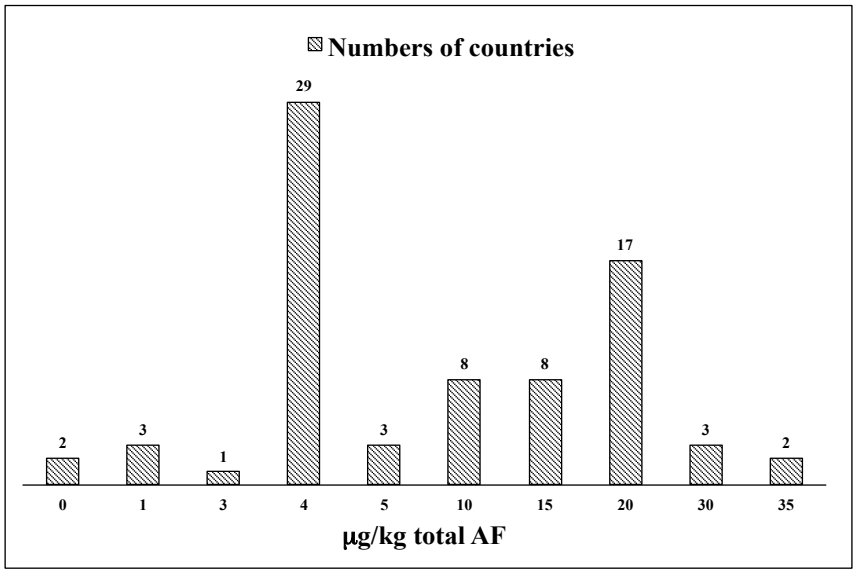


Fig. 9. Worldwide limits for total aflatoxins in food

Compared to other regions of the world, Europe has the most extensive and detailed regulations for mycotoxins in food and feeds (FAO, 2003). The current legislation in the European Union refers to the allowed limit of AFs in foodstuff (EC, 2006, 2010) (Fig. 10) and AFB_1 and others AFs (AFB_2, AFG_1 and AFG_2) in the feeds (EC, 2003) (Fig. 11).

Foodstuffs [1]		Maximum levels (µg/kg)		
'2.1.	**Aflatoxins**	B_1	Sum of B_1, B_2, G_1 and G_2	M_1
2.1.1.	Groundnuts (peanuts) and other oilseeds [40], to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs, with the exception of: — groundnuts (peanuts) and other oilseeds for crushing for refined vegetable oil production	8,0 [5]	15,0 [5]	—
2.1.2.	Almonds, pistachios and apricot kernels to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	12,0 [5]	15,0 [5]	—
2.1.3.	Hazelnuts and Brazil nuts, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8,0 [5]	15,0 [5]	
2.1.4.	Tree nuts, other than the tree nuts listed in 2.1.2 and 2.1.3, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0 [5]	10,0 [5]	—
2.1.5.	Groundnuts (peanuts) and other oilseeds [40] and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs, with the exception of: — crude vegetable oils destined for refining — refined vegetable oils	2,0 [5]	4,0 [5]	—
2.1.6.	Almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in foodstuffs [41]	8,0 [5]	10,0 [5]	—
2.1.7.	Hazelnuts and Brazil nuts, intended for direct human consumption or use as an ingredient in foodstuffs [41]	5,0 [5]	10,0 [5]	
2.1.8.	Tree nuts, other than the tree nuts listed in 2.1.6 and 2.1.7, and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0 [5]	4,0 [5]	—
2.1.9.	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
2.1.10.	Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0	4,0	—
2.1.11.	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.12, 2.1.15 and 2.1.17	2,0	4,0	—

(continued)

Foodstuffs [1]		Maximum levels (µg/kg)		
2.1.12.	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
2.1.13.	Raw milk [6], heat-treated milk and milk for the manufacture of milk-based products	—	—	0,050
2.1.14.	Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) Mixtures of spices containing one or more of the abovementioned spices	5,0	10,0	—
2.1.15.	Processed cereal-based foods and baby foods for infants and young children [3] [7]	0,10	—	—
2.1.16.	Infant formulae and follow-on formulae, including infant milk and follow-on milk [4] [8]	—	—	0,025
2.1.17.	Dietary foods for special medical purposes [9] [10] intended specifically for infants	0,10	—	0,025'

Fig. 10. Maximum levels for aflatoxin contaminants in foodstuffs (EC, 2010)

. Point 7 is replaced by the following:

Undesirable substances	Products intended for animal feed	Maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %
(1)	(2)	(3)
'7. Aflatoxin B1	All feed materials	0,02
	Complete feedingstuffs for cattle, sheep and goats with the exception of:	0,02
	— complete feedingstuffs for dairy animals	0,005
	— complete feedingstuffs for calves and lambs	0,01
	Complete feedingstuffs for pigs and poultry (except young animals)	0,02
	Other complete feedingstuffs	0,01
	Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs)	0,02
	Complementary feedingstuffs for pigs and poultry (except young animals)	0,02
	Other complementary feedingstuffs	0,005'

Fig. 11. Maximum levels for aflatoxin contaminants in feedstuffs (EC, 2003)

1.10 Preventive actions to reduce aflatoxins contamination

Due to high economic losses as consequence of AFs contamination and the toxicity of these compounds, several strategies have been studied and applied to reduce the risk in maize (Hell and Mutegi, 2011).

These strategies can be divided into:

- stopping the infection process (host plant resistance, biocontrol)
- pre-harvest crop management practices (good agricultural practices)
- post-harvest management strategies (timely harvesting, proper drying).

1.10.1 Stopping the infection process

a) Breeding for resistance

Although source of resistance to *Aspergillus* infection and AFs contamination in maize have been identified, commercial hybrids labeled as AFs resistant are not available for an economic sustainable agriculture (Henry et al., 2009). In fact it is hard to found hybrid lines that are at the same time high productive and AFs resistant in different environments (Clements and White, 2004).

Hybrids lines of maize resistant to *Aspergillus* ear rot showed lower levels of AFB_1 (Campbell and White, 1995). Similar results were found by Brown et al. (2001) analyzing 36 maize hybrids selected in West and Central Africa for moderate to high resistance to maize ear rot for their resistance to AFs; they detected in more than 50% of the lines tested lower amount of AFB_1 compare to the lines no ear rot resistant.

Scientists have also focused their studies on developing kernels with pericarp resistance (morphological and chemical) and subpericarp biochemical resistance (antifungal proteins) to fungal infection. The resistant genotypes analyzed generally seem to inhibit AFs production indirectly through inhibition of fungal growth (Brown et al., 1995; Guo et al., 1996).

One of the major factors that contribute to preharvest AFs contaminations is the drought stress. For this reasons breeders have focused their attention on drought resistant crop lines to be used in breeding programs (Guo et al., 2008).

However, new strategies that enhance host plant resistance against AFs, involving biotechnologies are explored (Brown et al., 2003). These approaches are based on individuation of maize plant that are able to reduce the incidence of fungal infection, restrict

the growth of toxigenic fungi or prevent toxin accumulation. Microarrays, identifications of Resistance-Associated Proteins (RAPs), AFs accumulation resistance quantitative loci (QTL), biochemical marker or compounds that can block AFs biosynthesis can help in develop crop resistance and AFs control on several crops (Hell and Mutegi, 2011).

b) Biological control

Control of pathogenic fungi or bacteria using nonpathogenic microorganisms is largely used in plant pathology (Lindow, 1987; Lorito et al., 2010). Many organisms have been tested for biological control of AFs contamination including bacteria, yeast and non-toxigenic strains of the pathogenic organism (Hell and Mutegi, 2011). In particular, Cotty (1990) demonstrated the efficiency of atoxigenic *A. flavus* to displace toxigenic strains and hence control AFs contaminations in Arizona cotton fields. Subsequent studies demonstrated the efficiency on other crops, including peanuts and corn (Abbas et al., 2006; Dorner, 2010). Large-scale application has been reached with two commercial biological control agents registered by the U. S. Environmental Protection Agency: Afla-Guard and AF36. The active ingredient of Afla-Guard, registered on peanuts and maize, is the atoxigenic *A. flavus* strain NRRL21882 that does not produce AFs because has a deletion in the AFs biosynthetic genes (Chang et al., 2005; Dorner, 2010). Since 1996, first year of applications, AF36 has been used in Arizona to reduce AFs contaminations in cottonseeds. AF36 atoxigenicity came from a single nucleotide polymorphism (SNP) in one gene responsible of AFs synthesis that generates a stop codon blocking the biosynthesis (Ehrlich and Cotty, 2004).

A 4 years trial showed that soil inoculated with a mixture of atoxigenic and toxigenic *A. flavus* strains had significantly lower levels of AFs contamination compared to the fields inoculated with the toxigenic *A. flavus* strain alone (Abbas et al., 2006). Level of reduction ranged between 43 to 63% for the strain CT3 and from 74 to 95% for the strain K49. Although both atoxigenic strains tested were able to reduce AFs contamination on maize the strain K49 can be preferred because it produces neither AFs nor cyclopiazonic acid (Abbas et al., 2006).

Because displacement of toxigenic isolates by atoxigenic isolates is dependent on isolate reproduction and competition in the local environment, scientists research in many countries atoxigenic strains to use as biocontrol agents (Cotty et al., 2008). Recently Probst et al. (2011) collected from maize sample associated with lethal aflatoxicosis in Kenya

atoxigenic isolates with potential value in AFs management. In particular *in vitro* experiments on living kernels shoved that some isolates reduced AFs production more than 80%. Similar results, with reduction in AFs production between 70 to 99% were obtained by Atehnkeng et al. (2008) in both laboratory and field trials analyzing atoxigenic *A. flavus* from Africa on maize and peanut. Moreover a mixture of 4 atoxigenic strains of *A. flavus* from Nigeria obtained provisional registration as AflaSafe to determine efficacy in on-farm test (Hell and Mutegi, 2011).

Preliminary studies to individuate potential biocontrol agent of toxigenic *A. flavus* isolates are ongoing in Argentina and Italy. In Argentina, Barros et al. (2006) screened 100 isolates of *A. flavus* from peanut-cropped soil for VCG analysis, sclerozial size and mycotoxins production to characterize fungi populations but also to individuate potential biocontrol agents to use on peanut.

In Italy, Degola et al. (2011) evaluated the potential of 9 isolates of *A. flavus* collected from maize kernels as biocompetitors against mycotoxin producers. Some of them were unable to reduce AFs production but a few when co-inoculated on maize kernels *in vitro* reduced AFs production close to 80%.

A. flavus biocontrol agents are commercialized on coated seeds (*i.e.* wheat, barley, sorghum) to facilitate application in the field and to offer a substrate for fungi growth, sporulation and consequent dispersion on developing plants (Antilla and Cotty, 2004). However solid preparations support both the residence in the fields and sporulation for relatively long periods and, as a result, provide a window of influence that extents considerably beyond application date (Cotty, 2006).

1.10.2 Pre-harvest crop management practices

To reduce AFs contamination in the field is indispensable control the factors that can enhance their production. Many practices, like timely planting, maintaining optimal densities, proper plant nutrition, avoiding drought stress, control other plant pathogens and insect pest are some of the principal measures that could be used to reduce AFs contamination. (CAST, 2003; Bruns, 2003; Santin, 2005).

Isolates of *A. flavus* are able to live under a wide range of conditions, especially those associated with drought in tropical agricultural crops. On the other hand plant drought stress, influencing kernels integrity and health, can increase *Aspergillus* spp. infection and AFs

contamination. Although irrigation is a valid practice to reduce water stress and probably AFs production is not always available or cost-effective for growers (Payne et al., 1986).

It is also true that irrigation can aggravate the leaching problem of nitrogen derived by reduced water-holding capacity. Also plant density or excessive weed populations, which compete for soil nutrients, can promote nitrogen deficiency (Anderson et al., 1975). To avoid nutrition stress to the plants due to irrigation practice is necessary applied well-balanced fertilizers to maintain a crop with low inoculum level. Also excessive uses of herbicides to control weeds can enhance susceptibility of maize to *A. flavus* infection and to AFs contamination (Oka and Pimentel, 1970).

Tillage systems and crop rotation can affect soil inoculum availability and root/soil interface and prevent inoculum build up. Crop rotation and management of crop residues also play an important role in the cycle and so in infection of *A. flavus* on the maize in the fields (Diener et. al., 1987).

Not only favorable environment conditions or plant stresses are able to promote AFs contaminations in pre-harvest but also insect and bird damage, which provide access sites to the fungus and increasing infection by damaging the kernel pericarp (Lillehoj et al., 1980; Horn and Pitt, 1997; Payne, 1998). In fact in Missouri and Illinois (USA) maize ears damaged by ECB (*O. nubilalis*) and corn earworm (*Heliothis zea*) had significantly higher levels of AFs than undamaged ears (Lillehoj et al., 1975).

Rodriguez-del-Bosque (1996) showed that a combination of good cultural practices (*i.e.* early planting, reduced plant population and irrigation), together with an optimal maize hybrid and insect control, reduced AFs concentrations down to 0-6 ng/g, compared to 63-167 ng/g in late-planted, non irrigated maize at a higher plant population without insect control.

Chemical treatments to control ear feeding insects are usually expensive and principally used on high-cash maize such as sweet maize or white maize destined to human alimentation, while yellow maize grown for livestock feed is seldom treated without insecticides (Bruns, 2003). Important to obtain good result and to optimize the control of insect on maize, especially for ECB, it is fundamental to determine the stage of the insect's lifecycle and timing the application of insecticides. In fact late treatment of infested fields will be ineffective once the borer enters the interior of the stalk (Bruns, 2003). In recent studies managed in Italy it was suggested to schedule ECB control as silk browning instead of based

on the insect detection in field. It seems to generate good control even limiting time for field surveys (Mazzoni et al., 2011).

Corn earworm requires numerous insecticide treatments to obtain an efficient control but, in same case, like for corn destined to animal feed, costs of applications are excessive for the value of the crops (Bruns, 2003). However this insect does not represent a relevant problem in Europe.

Insecticides applications are not the only practice to manage insect damage on corn. For example, *Bacillus turingensis* (Bt)-transformed maize hybrids, which are resistant to ear-feeding insects, present a lower level of AFs contamination of the kernels compare to the not resistant hybrids line (Dowd, 2000; Williams et al., 2002). Bt-maize influencing corn borer larval establishment and survival inside the plants reduce AFs contamination in areas where high corn borer infestations occur (Williams et al., 2005). However Bt-maize can reduce AFs contamination only under certain circumstances (Abbas et al., 2009) and in addition it planting is not admitted in most European country.

1.10.3 Post-harvest management strategies

Stored maize with high levels of moisture can increase AFs contamination 10 fold in only 3-day (Hell et al., 2008). For this reason is recommended to harvest corn early at high moisture content (26-28%) and dry as quickly as possible to safe moisture levels of 10-13% (Brown et al., 1999). Although technological solutions to dry crop are largely distributed and applied in develop country for example, in Africa these dryers are not used by farmers because large capital investments are needed to acquire them (Hell et al., 2008).

Maize mechanically harvested presented more damage that could enhance AFs contamination than those manually de-husk. In addition, seeds harvested as maize ears yielded better physiological quality than those harvested as a maize grain (Oliveira et al., 1997). Plett (1994) found a correlation between grain moisture and percentage of grain cracking. Kernels integrity is fundamental for a good and long term storage because broken kernels can promote fungal penetration in seed and, consequently, AFs production. In fact, the highest levels of AFs are produced when the fungus invades the seed embryo, where simple sugars are present in high quantities compared to other parts of the seed where complex carbohydrates are predominant (Bhatnagar et al., 2006).

It is necessary to take into consideration that in regions with little late-season rainfall

or where maturation occurs during hot periods of the year early harvesting is of limited usefulness. However harvesting at the optimum stage of maturity and rapid drying after harvesting can represent a good strategies for *A. flavus* and AFs control (Jones, 1987; Brown et al., 1999).

Several compounds, like organic acids or ammonia are used as fungi inhibitor on stored crops. These compounds change the pH of food preventing development of fungi. The use of these acids is related to the moisture content: with high moisture content more acid is needed. The dissociated form of propionic acid does not fit for use since it is corrosive, whereas the undissociated form is effective in killing fungi (Dixon and Hamilton, 1981).

1.11 Aim of the work

Aflatoxins are the most carcinogenic natural compound known in nature. These molecules, toxic for animal and humans, are produced on important economic commodities worldwide by the secondary metabolism of several fungal species of Aspergilli of the section *Flavi*. In Italy the principal responsible of aflatoxins contamination on maize is *A. flavus* and the area more exposed area to contamination is the northern of the peninsula where almost 90% of the cultivation is located. Generally, climatic conditions in this area are not favourable for aflatoxins contamination; however, reduced rainfall and increased temperature during maize developing season can occur along with associated levels of AFB_1 exceeding the legal limits, as happened in 2003. Since this year, more attention has been dedicated to characterize Italian *A. flavus* populations associated with maize cultivation and to develop a useful tool to reduce aflatoxins contamination.

To accomplish this goal the following studies were conducted:

- Vegetative compatibility analysis of a population of *A. flavus* from northern Italy
- Identification of atoxigenic isolates
- Evaluation of the ability of atoxigenic isolates to reduce *in vitro* aflatoxins produced by toxigenic isolates *in vitro*
- Selection of atoxigenic isolates potentially useful as biocontrol agents
- Individuate deletions in the aflatoxin biosynthesis gene cluster of selected atoxigenic isolates
- Biocontrol field trial with the selected potential biocontrol agents

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CHAPTER 2

Structure analysis of an *Aspergillus flavus* maize kernels population in northern Italy

2. Structure analysis of an *Aspergillus flavus* maize kernels population in northern Italy

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This study aimed to characterise an Italian populations of *Aspergillus flavus* in order to investigate on the causal agents responsible for aflatoxin contamination of maize in the most affected districts of Italy. Forty-six percent of *A. flavus* strains, isolated from maize kernels collected in 5 districts of northern Italy between 2003 and 2010, were unable to produce aflatoxin. The genetic diversity of the population was assessed by analysis of vegetative compatibility groups (VCGs) and aflatoxin biosynthesis gene cluster. Fourty-eight VCGs were identified through complementation between nitrate non-utilizing mutants. Twenty-five VCGs included only atoxigenic isolates, and the remaining 23 only aflatoxin producers. Members of the largest atoxigenic VCG (IT6) were found in 4 of the 5 districts sampled. Six deletions patterns in aflatoxin gene cluster were detected. Twelve atoxigenic isolates did not show deletions of the aflatoxin gene cluster. Conversely 10 had a deletion of the entire aflatoxin gene cluster. One isolate showed a deletion pattern that was found only once in Nigeria in a previous study. This study gives the basic knowledge for the selection of candidate to use as biological control agents in maize growing areas of Italy.

Keywords: AFB_1 cluster, VCGs.

2.1 Introduction

Maize (*Zea mays*) is a very important crop in Italy, cultivated on about one million ha with a production of about 8.5 million tons. The growing area is mainly located in 5 districts that supply 89% of the national production (Istat, 2011). These districts (Emilia Romagna, Friuli Venetia Giulia, Lombardy, Piedmont and Veneto) are situated in the north of the peninsula. Fumonisins, toxin produced by *Fusarium verticillioides* in maize, represent a very frequent problem in this area (Pietri et al., 2004; Battilani et al., 2008). Unfortunately, in 2003 for the first time, high levels of aflatoxins (AFs) were also detected (Giorni et al., 2007; Piva et al., 2006).

Aflatoxins are secondary metabolites produced by several members of *Aspergillus* section *Flavi* (Sweeney and Dobson, 1998). Giorni et al. (2007) reported that AFs contamination in northern Italy is basically due to *A. flavus* species, producers of aflatoxin B_1 (AFB_1) and B_2 (AFB_2). The International Agency for Research on Cancer (IARC, 2002) classified AFB_1 as a class 1 toxin, due to its demonstrated carcinogenic and teratogenic activity in humans (Wang and Tang, 2004).

Aspergillus flavus is a filamentous fungus that has a vegetative incompatibility system (Papa, 1986), regulated by *vic* loci (Leslie, 1993), that limits hyphal fusion and gene flow between individuals belonging to different vegetative compatibility groups (VCGs; Leslie, 1993). Isolates are assigned to VCGs with functional Vegetative Compatibility Analyses (VCAs) typically utilizing nitrate nonutilizing auxotrophs (*nit*$^-$ mutants). In VCAs, VCG membership is defined by complementation of an isolate *nit*$^-$ by one or both members of a tester pair composed of complementary auxotrophs, usually one *cnx*$^-$ (deficient in the cofactor required by both nitrate reductase and xanthine dehydrogenase) and one *niaD*$^-$ (nitrate reductase deficient) (Cove, 1976; Papa, 1986; Bayman and Cotty, 1991).

VCA is a useful tool to investigate diversity within *A. flavus* populations and several VCGs are commonly found in each geographic area studied. Sweany et al. (2011) identified 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 sesame seed isolates collected in Iran. Barros et al. (2006) identified 56 VCGs from 100 *A. flavus* isolates collected from soil in an Argentinian peanut-growing region. In these studies VCG diversity ranged from 0.02 in Louisiana corn to 0,56 in soil from Argentina.

Aflatoxin production is another character that is highly diverse within *A. flavus* populations. Isolates may produce anywhere from over 100 ppm aflatoxins to zero aflatoxins (*i.e.* are atoxigenic). The more than 25 genes involved in aflatoxin biosynthesis are contained within a 65 to 70 kb cluster (Yu et al., 2004), and several lesions within this cluster responsible for atoxigenicity in various isolates have been described (Ehrlich et al., 2004; Chang, et al., 2005; Yin et al., 2009; Donner et al., 2010). In recent years, specific primers have been designed for several aflatoxin biosynthesis genes and PCR has been applied to study expression of aflatoxin biosynthetic genes by *A. flavus* in different matrices, including food commodities (Schmidt-Heydt and Geisen, 2007).

Since 2003, special attention has been dedicated to *A. flavus* population in northern Italy; however, neither the diversity of *A. flavus* VCGs nor the distributions of lesions in the aflatoxin biosynthesis gene cluster have been examined. The present study sought to examine the characteristics of an *A. flavus* Italian population in order to gain insight on the causal agents of aflatoxin contamination of maize in the most affected area of Italy.

2.2 Materials and Methods

2.2.1 Fungal isolates and culture conditions

One hundred and thirty-eight *A. flavus* isolates from maize kernels grown in 5 districts of northern Italy were used in the current study. The geographic area studied lies between longitude 7.49° and 13.33° E and latitude 43.85° and 46.16° N (Battilani et al., 2008) (Fig.1). Fifty-four, 14, 53, 7 and 10 samples were, respectively, collected in Emilia Romagna, Friuli Venezia Giulia, Lombardy, Piedmont and Veneto during the 2003–2010 growing seasons. One strain per field was included in the collection, with the exception of a field chosen in 2008 in Lombardy; it was sampled weekly from the early dough crop stage till maize ripening, and 42 isolates were obtained. Isolation and identification methods of *A. flavus* were those described by Giorni et al. (2007). These strains are part of the culture collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza, Italy.

Isolates were transferred by single spore twice serially on malt agar (1% malt, 2% agar). After 2 days on malt, colonies were transferred to 5/2 agar (5% V8 juice, 2% agar, pH 5.2, 1,000 ml of water) and incubated at 31°C for 5-6 days. Conidial suspensions from plugs

of mature cultures were maintained in vials (4-ml) containing sterile distilled water and used as working cultures throughout the study. Long-term storage was on silica gel.

2.2.2 Aflatoxins quantification

All the isolates were tested for ability to produce AFs. To this end, 20 g of undamaged kernels in 250-ml Erlenmeyer flasks sealed with gas-permeable plugs (Bugstoppers; Whatman, Piscataway, NJ) were autoclaved at 121°C for 60 min (Probst et al., 2011). Kernels were inoculated with 100 µl of conidial suspension (10^5 to 10^6 conidia) and incubated for 7 days at 31°C in the dark.

After incubation, kernels were blended (30 s high speed) in 50 ml 80% methanol in a laboratory blender. The homogenized maize was filtered through Whatman No. 4 paper and the filtrate was spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) together with AFs standards (Aflatoxin Mix kit-M; Supelco Bellefonte, PA) containing a mixture of AFB_1, AFB_2, AFG_1 and AFG_2. Plates were developed in ethyl ether–methanol–water (96:3:1), air-dried, and aflatoxins were visualized under 365-nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc, Wilmington, NC, USA) (Probst et al., 2011). Filtrates initially negative for aflatoxins were partitioned twice with methylene chloride (25 ml) and concentrated prior to quantification (Cotty, 1997). One replicate was made for each isolate. To confirm inability to produce aflatoxins, putative atoxigenic isolates were retested with 4 replicates. Isolates that were invariably negative for aflatoxins were considered atoxigenic for the purpose of this study. The limit of detection was 20 µg/kg.

2.2.3 Vegetative Compatibility Groups

Supplemented Czapek-Dox (CZ) medium with 25 g/l of potassium chlorate and rose Bengal (50 mg/l) (Cotty, 1994) pH 7.0, was used to detect nitrate-non-utilizing mutants of each isolate. Plates were centre-point inoculated with 15 µl of conidial suspension and incubated at 31°C until chlorate-resistant sectors arose. At least 4 mutants were recovered from each plate and transferred on CZ (nitrate medium), hypoxanthine (HYP) medium (50 g sucrose, 10 g KH_2PO_4, 2 g $MgSO_4$.$7H_2O$, 0.2 g hypoxanthine, pH 5.5, 1,000 ml of water) and nitrite medium (50 g sucrose, 10 g KH_2PO_4, 2 g $MgSO_4$.$7H_2O$, 0.5 g $NaNO_2$, pH 5.5, 1,000

ml of water). CZ, HYP and nitrite media allowed identification of *niaD*, *cnx* and *nirA* mutants, respectively.

Self-compatibility was checked on the starch medium of Cotty and Taylor (2003). Briefly, the content of the medium was as follows: 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$ .7H_2O, 0.5 g KCl, 36 g dextrose, 20 g starch (Difco), 2% agar, pH 6.0. Each plate was inoculated with three mutants; the tester pair that defines the VCG in question and a nit mutant of the isolate to be tested for membership in that VCG. Mutants were placed 0.5 cm apart in a triangular pattern and incubated for 10 days at 31°C. Compatibility was identified by a line of wild-type growth where the mycelia interact. Complementation between the two mutants making up the tester pair was an internal control required for test to be valid. Each pairing was performed at least twice.

2.2.4 Diversity index

VCG diversity was estimated following the analysis proposed by Martins et al. (2008). Briefly, the Shannon-Wiener's H' index (Shannon and Weaver, 1949) was calculated as follows: $H' = -\Sigma_i [p_i \times \ln(p_i)]$ where p_i is the proportion of individuals in the ith VCG.

Evenness (E), a measure of the relative abundance of genotypes in a sample, was calculated dividing H' for the natural logarithm of expected number of VCGs (G_n). G_n was estimated using the rarefaction method (Oksanen et al., 2011). To contrast richness between atoxigenic and toxigenic isolates or among areas the expected number of VCGs was estimated for sample size of 8 (G_8) and 21 (G_{21}), the smallest sample sizes in subpopulations of atoxigenic and toxigenic isolates or in the areas, respectively.

The number of VCGs divided by the number of isolates is another measure of biological diversity used for VCG diversity (Chen et al., 2007); its value can range between 0 and 1, with 1 signalling the maximum diversity with each isolate representing a distinct VCG.

For the current study, Lombardy and Piedmont were grouped to form the West district, Veneto and Friuli Venetia Giulia were grouped to form the East district, and Emilia Romagna was defined as the Central district. Diversity indexes were calculated separately for atoxigenic and toxigenic VCGs for each of the three districts. Only self-compatible isolates were considered for the analysis of VCG diversity.

2.2.5 DNA extraction, primer sequences and polymerase chain reaction

DNA was partially purified from each isolate with the Spore Lysis method of Callicott and Cotty (personal communication). Plates of 5/2 agar, amended with 2% NaCl, were centre-point inoculated with 15 µL of conidial suspension and incubated at 31°C for 7 days. Spores were transferred by sterile cotton swab to 450 µl of lysis buffer (30 mM Tris buffer, 10 mM EDTA, 1% SDS, pH 8.0). Tubes were placed in the ThermoMixer at 60°C, 8,000 rpm for 1 hour and centrifuged for 30 min, 14,000 rpm. Supernatant (370 µl) was transferred in a new tube, combined with 370 µl of 4 M ammonium acetate pH 4.8 and 740 µl of ice-cold 100% ethanol, and placed at -20°C for 30 min. Following cooling, tubes were centrifuged for 5 min at maximum speed and the supernatant was discarded. After air drying, the pellet was suspended in 25 µl of sterile water prior and the DNA concentration was determined with a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). DNA was extracted from 48 atoxigenic and 6 toxigenic isolates. In particular, when available, DNA was extracted at least from 2 different isolates assigned to the same VCG. Among the toxigenic strains, the 6 highest producers were chosen, and DNA was extracted from only one member of each VCG.

Sequences of the nucleotide primers of the genes *pksA*, *norA* and *verB* and are reported in Donner et al. (2010). Primers for the genes *hexA*, *hexB*, *aflJ*, *verA*, *omtA*, *vbs*, *moxY*, *hypA* and *glcA* are described in Chang et al. (2005) and *norB-cypA* in Ehrlich et al. (2004). PCR conditions are described by Donner et al. (2010).

Amplifications were performed in a 50 µl reactions containing 1X HotMaster PCR kit (Eppendorf, Westbury, NY, USA), 50 pMol of each primer and 5 ng of template DNA. Amplifications were performed in a MyCycler thermocycler (Bio-Rad Laboratories, Richmond, CA, USA) and results were visualized with SIBR Gold after 1% agarose gel electrophoresis.

2.3 Results

2.3.1 Aflatoxins production and *nit* mutants

The low number of isolates available from Friuli Venetia Giulia, Piedmont and Veneto is partially due to the limited number of fields sampled; however, in these districts climate causes other fungi (mainly Fusaria) to dominate (Battilani et al., 2008).

Forty-six percent of isolates (64 isolates) were atoxigenic and the others (74 isolates) were toxin producer.

Mutants were generated from all 138 isolates within 10 days on chlorate amended medium. In total, 1656 mutant sectors were obtained and phenotyped revealing 73% to be *niaD* (1,209), 19% *nirA* (315) and 8% *cnx* (132). Only 55 (40%) of the 138 isolates evaluated produced *cnx* mutants. For VCGs lacking cnx mutants, tester pairs were developed from $nirA^-$ and $niaD^-$ mutants.

2.3.2 Vegetative compatibility groups

Fourteen isolates were not observed to complement themselves or other isolates within 10 days and were excluded from further VCA. It has not been investigated whether these isolates are in a single VCG or in multiple. Forty eight VCGs were obtained from the 138 isolates evaluated, on the basis of VCA. VCGs were designated with IT prefix for Italy and a progressive number in order of discovery. VCGs found in Italy can be divided into atoxigenic and toxigenic, because none of the studied VCGs contained both toxigenic and atoxigenic individuals.

Of 25 atoxigenic VCGs, 14 contained only 1 isolate, 5 contained 2 isolates, 2 contained 3 isolates, 2 contained 4 isolates, 1 contained 8 isolates and 1 contained 11 isolates (Table 1). Members of 4 VCGs (IT4, IT6, IT15 and IT18) were isolated at least in two different districts. Members of IT6 were isolated in all the years and in 4 of the 5 sampled districts.

Twenty-three toxigenic VCGs were identified; 18 of these had only 1 isolate, and 5 contained respectively 3, 4, 5, 7 and 31 isolates (Table 2). Only 5 VCGs (IT3, IT5, IT10, IT13 and IT25) were found in more than one district and in different years. Members of IT25 were detected in 3 of the 5 districts examined.

2.3.3 Diversity index

The overall Shannon index was 3.19. VCG diversity was lowest in the West district (H' = 1.87) compared to the Central (H' = 2.53) and East (H' = 3.20) districts (Table 3). Component for both richness, G_{21}= 8.49 and evenness, E = 0.691, were lowest in the West district. Lowest variability for West district was essentially due to the richness component (48.7 and 43.4 lower than G_{21} calculated for Central and East, respectively) rather than to evenness (27.3 and 26.0% lower than the E calculated for the Central and East districts, respectively) (Table 3).

As regards the two subpopulations, atoxigenic and toxigenic, variability was also

estimated between the areas. The highest H' value for the atoxigenic isolates was found in the Central district (2.67). This value was 40.5 and 35.2% higher than the H' for the atoxigenic isolates in the West and East areas, respectively. Richness, $G_7 = 6.73$ and evenness, $E = 0.942$ were greater in the Central district than in the other two areas. The greater variability estimated for the Central district was mainly due to the richness component (30.5 and 22.7% greater than G_8 calculated for West and East areas, respectively) rather than to evenness (5.8 and 5.6% greater than the E calculated for the West and East areas, respectively) (Table 3).

For the toxigenic subpopulations lowest variability was observed in the West area (1.27). In this case, richness (G_8) and evenness (E) contribute almost equally.

The overall VCG diversity index, expressed as the number of VCGs divided by the total number of isolates, was 0.39. In particular Central (0.64) and East (0.71) districts had higher values than the West district (0.27). Diversity of toxigenic isolates was greatest in the East district (1.00) and also smallest in the West district (0.20). For atoxigenic isolates, diversity was greater in the Central district (0.55) and East district (0.54) than in the West district (0.46).

2.3.4 Aflatoxin biosynthesis genes

Thirteen fragments of genes from across the aflatoxin biosynthesis gene cluster and nearby gene regions were amplified by PCR. All 13 fragments were amplified for all 6 aflatoxin producer isolates. However, certain fragments did not amplify from DNA of several atoxigenic isolates indicating gene deletion patterns similar to those previously described (Chang et al., 2005; Yin, et al., 2009; Donner et al., 2010) for other atoxigenic *A. flavus*.

Isolates assigned to the same VCG had identical deletion patterns, but identical deletion patterns were found in multiple VCGs. Six different profiles were obtained from atoxigenic isolates (Table 4).

Out of 48 atoxigenic isolates, 19 belonging to 8 different VCGs had the entire aflatoxin biosynthesis cluster deleted. However, for 15 atoxigenic isolates, assigned to 9 VCGs, all the genes were detected (Table 4). Members of IT34 showed only the presence of *glcA* and *hypA*, and members of IT1 and IT44 were lacking both *moxY* and a region from *verA* to the 3' end of the aflatoxin cluster. Finally for 9 isolates belonging to 4 VCGs, genes between *verB* and *cypA* could not be amplified.

2.4 Discussion

Maize cultivated in Italy is principally dedicated at animal feed, in particular for dairy animal feed (Istat, 2011). High level of AFB_1 contamination in maize leads to an increase of aflatoxin M_1 excreted into milk and, as a result, the milk may exceed the European maximum permissible level (0.050 µg/kg; EC, 2006 2010). Generally, climatic conditions in this area are not favourable for AFs contamination; however, reduced rainfall and increased temperature during maize production can occur along with associated levels of AFB_1 in excess of the legal limits (EC, 2010). This occurred in 2003 and, to a lesser extent, in a few following years (Battilani et al., 2008). Even though contamination was an economic concern during those years, detailed investigations on the causal agents of the contamination episodes have not been previously undertaken. The current work suggests that the L strain morphotype of *A. flavus* is the primary cause of aflatoxin contamination in Italy.

Nitrate non-utilizing mutants *(nit-)* were obtained with the rose Bengal-chlorate selection medium (Cotty, 1994) for all 138 isolates examined. This allowed elimination of bias caused by media that only allow selection of mutants from a subset of a fungal population. In agreement with other *A. flavus* VCG reports (Bayman and Cotty, 1991; Pildain et al., 2004; Barros et al., 2006), *niaD* mutants were isolated in higher proportions than *cnx* and *nirA* mutants. When available, *cnx* mutants were preferred as testers for identifying VCGs because usually they provide the strongest reactions between compatible isolates when paired with *niaD* or *nirA* mutants (Correll et al., 1987) and are the most consistent for VCA among *A. flavus nit* mutants (Bayman and Cotty, 1991). Differences in proportions of the various genes involved in nitrate assimilation and use have been frequently reported. However, the mechanism by these differences occur are not understood. It has been suggested that either physical size of the genes may play a role and that some loci may be more susceptible to mutation than others (Klittich and Leslie, 1988).

Out of the 138 *A. flavus* isolates tested for aflatoxin-producing ability on maize kernels, 46% did not produce AFs. Thus, in the district of Italy sampled, based on the fungal population analysed, there appears to be a type of balancing selection maintaining relatively equal proportions of atoxigenic and toxigenic *A. flavus*. Similarly, Astoreca et al. (2011) reported 38% of atoxigenic isolates of 85 *A. flavus* strains tested and isolated from poultry feed, while an higher percentage of atoxigenic *A. flavus* strains (72.5%) was found by Razzaghi-Abyaneh et al. (2006) that tested 58 Iranian isolates from soil. However, this is

contrary to other reports where aflatoxin producers dominate. For example, Barros et al. (2006) reported that the percentage of toxigenic *A. flavus* isolated from peanut soil in Argentina was 95%. Similar results were reported by Sweany et al. (2011) who analysed 867 *A. flavus* isolates from maize kernels and soil in Louisiana and found 6% atoxigenic isolates.

All VCGs were tested against both toxin producers and atoxigenic isolates and none of the studied VCGs contained both toxigenic and atoxigenic individuals. This is in contrast to the previous report by Barros et al. (2006) that indicated presence of both atoxigenic and toxigenic *A. flavus* in one VCG. In the current study, care was taken to transfer all isolates twice serially by single spores in order to avoid confusing results from mixed cultures. This practice was not reported for the previous study and mixed cultures may have resulted in association of a VCG with characters of a co-cultivated *A. flavus.* The prior paper also tested the isolates on synthetic media which support lower levels of aflatoxins than maize kernels (Probst and Cotty, unpublished results) but had a more sensitive limit of detection (1 ppb).

Diversity is influenced by richness (number of genotypes in a sample) and evenness (how genotypes are distributed in a population). Generally, with the increase of the sample size, a greater diversity is observed (Grünwald et al., 2003). In our study this trend was not seen in the West district probably due to the limited variability in genotype differences recorded among toxigenic isolates. The West district included 42 isolates sampled from a single field; this is the likely cause of lower diversity despite a large sample size. On the other hand in the Central and East districts the value of H' is influenced approximately in the same proportions by the atoxigenic and toxigenic VCGs. This indicated a good equilibrium between atoxigenic and toxigenic VCGs in Central and East districts. This is due to the smallest difference between the atoxigenic and toxigenic for the E and $g_{(8)}$ values in the Central and East districts compare to the same differences in the West district.

Atoxigenic *A. flavus* have been isolated from diverse crops and soils worldwide (Cotty, 1994, Barros et al., 2006, Probst, et al., 2011) and successfully used to reduce AFB_1 produced by toxigenic isolates (Cotty, 2006). Two atoxigenic isolates of *A. flavus*, AF36 (Ehrlich and Cotty, 2004) and NRRL 21882 (Dorner, 2004), are used commercially in the US to reduce aflatoxin contamination of maize. These two atoxigenics are registered with the US-EPA as biopesticides. However, the structure of the aflatoxin biosynthesis cluster differs significantly between these two *A. flavus*. AF36 has remnants of all the genes in the cluster, but a single nucleotide polymorphism (SNP) in the aflatoxin polyketide synthase gene (*pksA)*

generates a stop codon that causes early termination of transcripts and total inhibition of AFB_1 production (Ehlrlich and Cotty, 2004). On the other hand, NRRL 21882, the active component of Afla-Guard (Dorner, 2004), has a deletion that eliminates the entire cluster (Chang et al., 2005). The current study indicates that many atoxigenic *A. flavus* isolates from northern Italy have large deletions in the aflatoxin biosynthesis gene cluster similar to that in NRRL 21882 with almost 45% of the analysed atoxigenic VCGs lacking the entire cluster. This result, named pattern H, was first reported by Chang et al. (2005). They analysed 38 VCGs and 17 missed the entire cluster. Donner et al. (2010) found 11 of 21 atoxigenic isolates from Nigeria had deletions in the cluster but, only one of the analysed VCGs (about 5%) lacked the entire cluster. The other deletion patterns detected in the current study are comparable to deletions found in the previous studies (Chang et al., 2005; Donner et al., 2010). For example, the pattern shared by IT18, IT32, IT40 and IT48 is similar to pattern C and 9 VCGs have a pattern similar to pattern A (Chang et al., 2005). IT34 has a pattern reported by Donner et al. (2010) for 2 VCGs. Again, no deletions were detected among the 6 toxigenic VCGs analysed as is consistent with current knowledge about the aflatoxin biosynthesis cluster.

VCA is a useful tool to estimate genetic diversity (Bayman and Cotty, 1991), understand population dynamics and evaluate measures to reduce aflatoxin contamination (Pildain et al., 2004). The current work reports for the first time the VCG structure of *A. flavus* resident in the major maize producing districts of Italy. In particular the relatively wide distributions across districts and years and high frequencies of the atoxigenic VCGs IT4, IT6 and IT18 indicate that these VCGs are well adapted to environmental conditions and maize production systems of Italy and as such are good candidates for development of aflatoxin biocontrol agents targeted for this district.

Atoxigenic strains act primarily through competitive exclusion of aflatoxin producers in the environment and during crop tissue infection. Atoxigenics with superior adaptation to a district may allow for both better initial displacement of aflatoxin producers and better survival between crops and thus long-term displacement providing for both long-term (multiple year) and additive benefits. Additional information on the behaviour of these atoxigenics during crop infection and within the ecology of the target agroecosystem will be needed to choose the best atoxigenics for use as biological control agents.

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2.6 Figures and tables

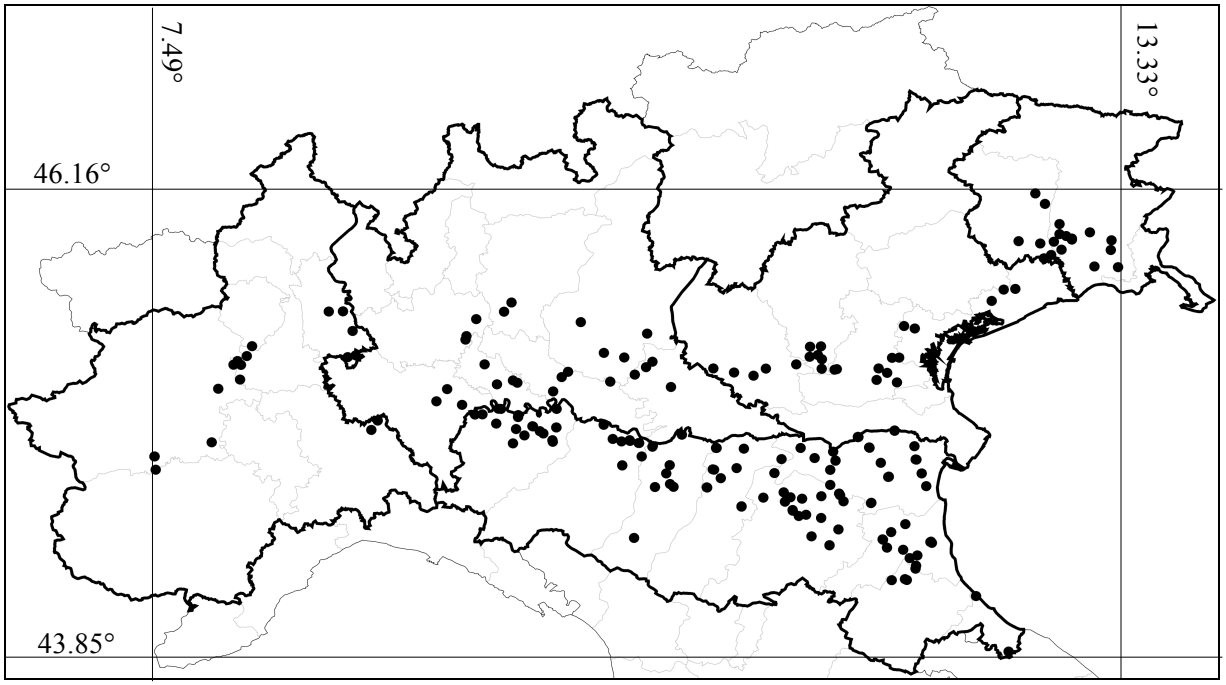

Fig. 1. Locations from which maize samples were collected from 2003-2010. *Aspergillus flavus* used in the current study were isolates from these samples.

TABLE 1. Distribution of vegetative compatibility groups (VCGs) among atoxigenic Italian isolates of *Aspergillus flavus*

VCG	Number of isolates[a]	Location[b]	District[c]	Year
IT1	2	Pordenone (2)	FVG	2003
IT4	8	Pordenone (2)	FVG	2003
		Udine (3)	FVG	2003
		Cremona (3)	L	2008
IT6	11	Torino	P	2003
		Verona	V	2003
		Bologna	ER	2004
		Modena	ER	2004
		Piacenza	ER	2004
		Reggio Emilia	ER	2004
		Cremona (3)	L	2008
		Cremona	L	2009
		Modena	ER	2010
IT8	1	Mantova	L	2003
IT9	4	Forli-Cesena	ER	2004
		Reggio Emilia (3)	ER	2004
IT12	1	Padova	V	2003
IT15	3	Vicenza	V	2003
		Piacenza	ER	2004
		Ferrara	ER	2010
IT17	2	Modena	ER	2004
		Ferrara	ER	2009
IT18	4	Pordenone	FVG	2003
		Udine	FVG	2003
		Piacenza	ER	2004
		Reggio Emilia	ER	2010
IT19	1	Parma	ER	2009
IT20	1	Bologna	ER	2004
IT21	1	Ferrara	ER	2009
IT22	2	Cremona (2)	L	2009
IT23	3	Ravenna (3)	ER	2009
IT24	1	Alessandria	P	2003
IT29	1	Cremona	L	2008
IT31	1	Bologna	ER	2010
IT32	1	Piacenza	ER	2010
IT34	2	Modena	ER	2009
		Parma	ER	2009
IT38	1	Ravenna	ER	2009
IT40	1	Ferrara	ER	2004
IT41	2	Piacenza (2)	ER	2004
IT43	1	Modena	ER	2010
IT44	1	Bologna	ER	2010
IT48	1	Udine	FVG	2003

[a] Isolates assigned to each vegetative compatibility group.

[b] Number in brackets indicates number of isolates that were collected at site. Absence of number means only one isolate was collected.

[c] ER = Emilia Romagna. FVG = Friuli Venetia Giulia. L = Lombardy. P = Piedmont. V = Veneto.

All VCGs were tested against both toxin producers and atoxigenic isolates and none of the studied VCGs contained both toxigenic and atoxigenic individuals.

TABLE 2. Distribution of vegetative compatibility groups (VCGs) among toxigenic Italian isolates of *Aspergillus flavus*

VCG	Number of isolates[a]	Location[b]	District[c]	Year
IT2	1	Alessandria	P	2003
IT3	7	Piacenza (3)	ER	2004
		Cremona (4)	L	2008
IT5	31	Vicenza	V	2003
		Cremona (30)	L	2008
IT7	1	Parma	ER	2009
IT10	2	Milano	L	2003
		Modena	ER	2004
IT11	1	Lodi	L	2003
IT13	4	Brescia	L	2003
		Mantua	L	2003
		Venice	V	2003
		Cremona	L	2008
IT14	1	Vicenza	V	2003
IT16	1	Ferrara	ER	2009
IT25	5	Vicenza	V	2003
		Ferrara	ER	2004
		Alessandria (3)	P	2010
IT26	1	Udine	FVG	2003
IT27	1	Udine	FVG	2003
IT28	1	Padua	V	2003
IT30	1	Modena	ER	2009
IT33	1	Modena	ER	2004
IT35	1	Reggio Emilia	ER	2010
IT36	1	Parma	ER	2010
IT37	1	Ferrara	ER	2004
IT42	1	Parma	ER	2010
IT45	1	Ferrara	ER	2010
IT46	1	Venice	V	2003
IT47	1	Brescia	L	2003
IT49	1	Mantua	L	2003

[a] Isolates assigned to each vegetative compatibility group.
[b] Number in brackets indicates number of isolates that were collected at each site. Absence of number means only one isolate was collected.
[c] ER = Emilia Romagna. FVG = Friuli Venetia Giulia. L = Lombardy. P = Piedmont. V = Veneto.
All VCGs were tested against both toxin producers and atoxigenic isolates and none of the studied VCGs contained both toxigenic and atoxigenic individuals.

TABLE 3. Shannon diversity index, richness and evenness of *Aspergillus flavus* populations sampled in maize in three districts (West, Central and East) of northern Italy

Districts[a]	**Sample size**	G_{obs}[b]	G_n[c]	$G_{(8)}$[d]	$G_{(21)}$[e]	**H'**[f]	**E**[g]
West	58	15	15.00	4.45	8.49	1.87	0.691
Central	45	29	29.00	7.30	16.55	3.20	0.950
East	21	15	15.00	6.77	15.00	2.53	0.934
West/atoxigenic (A)	13	6	6.00	4.68	N/A	1.59	0.887
Central/A	31	17	17.00	6.73	13.53	2.67	0.942
East/A	13	7	7.00	5.20	N/A	1.73	0.889
West/toxigenic (T)	45	9	9.00	3.35	5,98	1.27	0.578
Central/T	14	12	12.00	7.23	N/A	2.40	0.966
East/T	8	8	8.00	8.00	N/A	2.08	1.000
Overall	124	48	48.00	6.42	13,74	3.19	0.824

[a] West = Piedmont & Lombardy. Central = Emilia Romagna. East = Veneto & Friuli Venezia Giulia. Overall = Indicate all the isolates assigned into VCGs.
[b] Number of vegetative compatibility groups (VCGs) observed.
[c] Expected number of VCGs calculated for the correspondent sample size (*n*) estimated by the rarefaction method (Oksanen et al., 2011).
[d] Expected number of VCGs calculated for a sample of size $n = 8$ per subpopulation estimated by the rarefaction method.
[e] Expected number of VCGs calculated for a sample of size $n = 21$ per population estimated by the rarefaction method. This index was calculated only for subpopulations with large enough sample sizes. N/A = not applicable.
[f] Shannon value for VCG diversity.
[g] Evenness calculated by scaling the Shannon index by the expected number of VCGs $\{\ln(G_n)\}$ to adjust for sample size dependence.
Table adapted from Martins et al. (2008)

TABLE 4. Different PCR amplification in the studied *A. flavus* population

Isolate	VCG[a]	AFB_1[b]	*glcA*[c]	*hypA*[c]	*moxY*[c]	*vbs*[c]	*omtA*[c]	*verB*[c]	*verA*[c]	*norA*[c]	*adhA*[c]	*hexB*[c]	*hexA*[c]	*pksA*[c]	*cypA*[c]
A2087	n.a.	-	■												
A2330	n.a.	-	■												
A2085	IT6	-	■												
A2066	IT8	-	■												
A2090	IT9	-	■												
A2103	IT15	-	■												
A2099	IT17	-	■												
A2321	IT19	-	■												
A2318	IT21	-	■												
A2313	IT23	-	■												
A2322	IT34	-	■	■											
A2344	IT31	-	■	■	■	■	■	■	■						
A2058	IT1	-	■	■		■	■	■	■						
A2341	IT44	-	■	■		■	■	■	■						
A2044	IT48	-	■	■	■	■	■	■							■
A2047	n.a.	-	■	■	■	■	■	■							■
A2102	n.a.	-	■	■	■	■	■	■							■
A2349	n.a.	-	■	■	■	■	■	■							■
A2050	IT18	-	■	■	■	■	■	■							■
A2343	IT32	-	■	■	■	■	■	■							■
A2084	IT40	-	■	■	■	■	■	■							■
A2043	n.a.	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2072	n.a.	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2342	n.a.	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2079	IT4	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2049	IT12	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2106	IT20	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2319	IT22	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2042	IT24	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2284	IT29	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2329	IT38	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2093	IT41	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2339	IT43	-	■	■	■	■	■	■	■	■	■	■	■	■	■
A2039	IT11	+	■	■	■	■	■	■	■	■	■	■	■	■	■
A2062	IT13	+	■	■	■	■	■	■	■	■	■	■	■	■	■
A2068	IT26	+	■	■	■	■	■	■	■	■	■	■	■	■	■
A2097	IT10	+	■	■	■	■	■	■	■	■	■	■	■	■	■
A2295	IT3	+	■	■	■	■	■	■	■	■	■	■	■	■	■
A2300	IT5	+	■	■	■	■	■	■	■	■	■	■	■	■	■

[a] Vegetative Compatibility Group; n.a.= not available.

[b] AFB_1: (+/-): aflatoxin and no aflatoxin production.

[c] Presence (grey box) or absence (empty box) of PCR products of the tested genes.

CHAPTER 3

Selection of Italian endemic atoxigenic *Aspergillus flavus* isolates for biocontrol of aflatoxins producer strains on maize

3. Selection of Italian endemic atoxigenic *Aspergillus flavus* isolates for biocontrol of aflatoxins producer strains on maize.

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3.1 INTRODUCTION

Aflatoxins are highly toxic metabolites produced by several *Aspergillus* species (McKean et al., 2006; Klich, 2007). Aflatoxin B_1 is a genotoxin classified by the International Agency for Research on Cancer as a group 1A human carcinogen (IARC, 1982; 2002). Chronic or acute (hepatitis, death) effects are linked to aflatoxins, according to a dose-effect relationship. Aflatoxins cause both chronic effects (immune suppression, cancer) as a result of ingestion of multiple low doses over long periods and acute effects (hepatitis, death) from single or few exposures to high concentrations (Cardwell and Henry, 2004). In most countries regulations set strict limits on the concentrations of aflatoxin B_1 allowed in food and feed (Payne and Yu, 2010). These regulations have great influence on the value of both domestic and imported crops, as well as, meat, eggs, milk and their byproducts because aflatoxins can be transferred to animal products from feed (Wu and Khlangwiset, 2010). In Italy in 2003 high levels of aflatoxin M_1, hydroxylated metabolites of the aflatoxin B_1, were detected in milk as consequence of contaminated maize used as feed for dairy cows (Giorni et al., 2007; Piva et al., 2006).

Aflatoxin contamination of maize also is primarily caused by *A. flavus* in Italy (Giorni et al., 2007). This species is divided into many genetic groups called vegetative compatibility groups (VCGs) by a heterokaryon incompatibility system (Bayman and Cotty, 1993). Characteristics that are highly variable within the species like aflatoxin-producing ability, morphology of sclerotia and production of conidia, are less variable among isolates within the same VCG (Cotty, 1989).

Several biological control strategies based on bacteria, yeast or fungi with the intent of reducing pre-harvest aflatoxin contamination have been developed (Dorner, 2004; Yin et al., 2009; Hell and Mutegi, 2011). Among these, biological control with atoxigenic strains of *A. flavus* is the most promising (Amaike and Keller, 2011). Atoxigenic strains displace aflatoxin producers during crop development resulting in an aflatoxins contamination reduction (Cotty and Bayman, 1993; Cotty, 2006). Currently there are biopesticide registrations with the U.S. Environmental Protection Agency for use of atoxigenic *A. flavus* for reducing aflatoxin contamination of maize, peanut, cottonseed, and pistachios. The two registered atoxigenic strains were used on thousands of hectares of commercial crops in 2011 (Cotty, personal communication).

Competitive exclusion of aflatoxin producers both in the environment in which the crop is developing and in crop tissues is the main mechanism through which atoxigenic strains influence contamination. However, secondary mechanisms also exist in direct interference with aflatoxin producers through still undefined mode of action (Mehl and Cotty, 2010; Huang et al., 2011).

In northern Italy, high levels of aflatoxins can be detected in maize (Battilani et al., 2008) producing significant economic losses and safety risk. So, efficient strategies to reduce aflatoxins contaminations are strongly requested by farmers.

The current study evaluates the ability of atoxigenic isolates of *A. flavus* collected from maize ears in Italy to reduce aflatoxins contamination *in vitro*. The most efficient as candidate biocontrol agents candidates on maize in the northern Italy have been selected and the effect and the efficiency of two ratios between atoxigenic and toxigenic *A. flavus* on toxin containment were also considered.

3.2 MATERIALS AND METHODS

3.2.1 Fungal isolates and culture conditions

Seven aflatoxin-producing and 19 atoxigenic isolates of *A. flavus* were used in this study (Table 1). Isolates from Italy were previously collected in 4 districts of northern Italy from 2003 to 2009 (Giorni et al., 2007; Mauro et al., in preparation) and are included in the culture collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza, Italy. NRRL 21882, the active ingredient of the AflaGuard biopesticide (Dorner, 2004) and AF13 (Cotty, 1989) both from United States were also included in these studies for comparison with isolates from Italy. Italian *A. flavus* were isolated and identified by Giorni et al. (2007). Isolates were transferred by single conidium, cultured on 5/2 agar (5% V8 juice, 2% agar, pH 5.2, 1,000 ml of water) and incubated at 31°C for 5-6 days prior to transferring culture plugs (eight to ten 3 mm diameter) to vials containing sterile water (2.5 ml) for storage 8°C (Probst et al., 2011). The isolates from Italy were previously examined for aflatoxin production and categorized into VCGs (Mauro et al., in preparation).

3.2.2 Co-infection of viable corn kernels with atoxigenic and toxigenic isolates of *A. flavus*

Conidia from 6 days cultures (31°C, dark) were collected from the culture surfaces with cotton swabs, suspended in sterile distilled water, and spore concentrations adjusted as previously described (Probst et al., 2011). Briefly, a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY), was used to quantify concentrations of conidia in suspensions using a nephelometric turbidity unit (NTU) versus CFU curve: $Y = 49.937X$, where X = NTU and Y = conidia/ml. Conidial concentration for each isolate were adjusted to 10^5 conidia/ml. Undamaged kernels of Pioneer hybrid 33B50 were surface sterilized in hot water for 45 s at 80°C (Mehl and Cotty, 2010) and dispensed in sterilized glass flask (10 g of maize per 250-ml flask). Flasks then were sealed with gas-permeable BugStopper (Whatman, Piscataway, NJ) plugs to prevent humidity loss and allow gas exchange. After sterilization, maize moisture level was quantified with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) and the volume of water in which the atoxigenic and toxigenic spore suspensions were applied to the kernels was adjusted to bring the maize water content to 25%. Conidia of atoxigenic and toxigenic isolates were added to the flasks simultaneously and then the flasks were gently agitated to allow kernel coating. Control flasks received the same quantity of water but containing only toxigenic spores. Inoculated maize was incubated at 31°C for 7 days in the dark. Tests had a completely randomized design with four replicates. All tests were performed twice.

Influence of the italian atoxigenic strains on aflatoxin contamination of viable maize kernels was examined in three different types of co-infection studies.

In the first set of experiments, all 18 Italian atoxigenic isolates were compared with NRRL 21882 for ability to reduce contamination of kernels by *Aspergillus flavus* AF13, an *A. flavus* isolate commonly used in laboratory and field tests (Cotty, 1989).

In the second set of experiments, the ability of two atoxigenic isolates from Italy to reduce aflatoxin contamination of maize was compared among six aflatoxin producing isolates of *A. flavus* from Italy. Each aflatoxin-producer belonged to a separate VCG. One atoxigenic isolate evaluated in test two (A2085) belonged to the most prevalent VCG found in the area (IT6; Mauro, et al. in preparation) and the second (A2321) is the isolate that caused the greatest aflatoxin reduction in the initial experiments. The toxigenic isolates are the strongest producer of aflatoxins found in the population of *A. flavus* analysed in a previous work (Mauro, et al. in preparation). Viable maize kernels surface sterilized as above were

inoculated (10^5 conidia per flask) either with one of 6 aflatoxin-producing VCGs endemic to Italy alone or with both one of the aflatoxin producers and one of the two atoxigenic strains simultaneously with equal quantities (10^5) of conidia in sufficient water to bring the kernel water content to 25% and incubated as above.

In the third set of experiments, the ratio of atoxigenic to aflatoxin-producer was varied in order to see the impact of a reduced frequency of atoxigenic strain on the extent to which corn kernels become contaminated. The 5 atoxigenic isolate from Italy most effective at reducing aflatoxin contamination in the initial experiments and NRRL 21882 from North America were used in these experiment. As in the prior two sets of experiments viable maize kernels were inoculated simultaneously with both atoxigenic and aflatoxin-producing isolates at a rate of 10^5 conidia per flask. The quantity of each atoxigenic isolate used was varied so that kernels were inoculate with either equal quantities of conidia from both atoxigenic and aflatoxin-producing isolates or one quarter as many atoxigenic conidia as conidia from the aflatoxin-producer. In the third set of experiments, the aflatoxin producer was *A. flavus* AF13 and incubation was under the conditions outlined under the first set of experiments.

3.2.3 Aflatoxins quantification

Kernels infection by the *A. flavus* isolates was terminated at the end of the incubation period by blending kernels in 50 ml of 80% methanol. The homogenate was passed through Whatman No. 4 filter paper and spotted directly on thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) beside aflatoxins standards (AFB_1, AFB_2, AFG_1 and AFG_2; Aflatoxin Mix kit-M; Supelco Bellefonte, PA). Plates were developed in ethyl ether–methanol–water (96:3:1), air-dried, and aflatoxins were visualized under 365-nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc, Wilmington, NC, USA). The protocol followed had a limit of detection of 20 μg/kg. (Probst et al., 2011).

3.2.4 Statistical analyses

Randomized complete block designs with four replicates were used in all experiments. Aflatoxin concentration was log transformed and percentage reduction was arcsine transformed prior to analysis of variance (ANOVA). ANOVA was performed with the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Mean

separations were performed on data from experiments with statistically significant ($P = 0.05$) differences using Tukey's Honestly Significant difference test (Pagano and Gauvreau, 2000). Mean differences in aflatoxin levels (percent difference between inoculated maize and control maize treatments) were calculated as [1 – (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/total aflatoxin content in maize inoculated with the toxigenic isolate alone)] × 100. In the formula the toxigenic isolate is represented by AF13 in the first and third experiment and by Italian aflatoxin producers in the second experiment. The efficiency (E) of each isolate was calculated following the formula E = R/(A/A+T); where R is the percentage of aflatoxin reduction and the denominator is the percentage of the total *A. flavus* inoculum made up by the atoxigenic isolate (A). A is the quantity of atoxigenic strain and T is the quantity of aflatoxin-producer. All analyses and calculations were performed with SAS.

3.3 RESULTS

3.3.1 Efficacy of Italian atoxigenic isolates

Co-inoculation of aflatoxin-producer AF13 with any of the 18 Italian atoxigenic *A. flavus* isolates lowered maize kernel aflatoxin B_1 content compared to the aflatoxin content of kernels inoculated with AF13 alone (Table 2). Kernels inoculated with AF13 alone averaged 146 ppm aflatoxin B_1 and those inoculated with both AF13 and one of the Italian atoxigenic isolates had between 61% and 91% less AFB_1. Half (9) of the Italian isolates reduced the aflatoxin content greater than 80%. The 5 most effective isolates belonged to 5 different VCGs, originated from two districts, Lombardy (1) and Emilia Romagna (4) and were collected in 2003 (1), 2004 (3) and 2009 (1) (Table 1).

3.3.2 Behaviours of two Italian atoxigenic isolates

Both Italian atoxigenic isolates, A2085 and A2321, reduced aflatoxin B_1 contamination of viable maize kernels by all 6 aflatoxin-producing Italian isolates evaluated. The Italian aflatoxin producers contaminated the kernels with between 17 up to 98 ppm aflatoxin B_1 when inoculated onto kernels individually. The extent to which contamination of maize kernels with aflatoxin B_1 by the 6 aflatoxin producers was reduced by the atoxigenic isolates ranged from 61 to 78% for atoxigenic isolate A2085 and from 61 to 83% for atoxigenic isolate A2321. Atoxigenic isolate A2085 caused similar reductions (60% up to

68%) in aflatoxin content for kernels inoculated with 5 of the aflatoxin producers but caused significantly greater reductions (78%) in kernels inoculated with the aflatoxin producer A2039 (Table 3). The other atoxigenic isolate (A2321) also was most effective at reducing contamination caused by A2039 as compared with contamination caused by the other aflatoxin producers. However, A2321 had a broader range of efficacy (61% to 83%) than A2085 and reduce contamination by two isolates (A2097 and A2300) significantly ($P<0.05$) more than A2085. Both atoxigenic isolates were most effective at reducing contamination by A2039, the isolate that contaminated maize with the greatest quantities of aflatoxin B_1. However, the ability of aflatoxin producers to contaminate maize was correlated with the extent to which the atoxigenics reduced contamination (A2085: $r = 0.947$; $P < 0.01$; A2321: $r=0.823$; $P < 0.05$).

3.3.3 Influences of the ratio of atoxigenic strain to aflatoxin producer on contamination

Maize kernels inoculated with equal quantities of atoxigenic and aflatoxin-producing isolates became contaminated with significantly less aflatoxin B_1 than kernels inoculated with one quarter as much atoxigenic as aflatoxin producer (Table 4). In the ratio 1:4, aflatoxin B_1 ranged from 56 to 86 ppm and in the ratio 1:1 aflatoxin B_1 ranged from 20 to 44 ppm. Percent reductions ranged from 24% to 50% for the 1:4 ratio and from 61% to 83% for the 1:1 ratio. However, on average, atoxigenic isolates had greater efficiency (E = the percent reduction adjusted for the proportion of the inoculum composed by the atoxigenic) when applied at the one quarter rate than at the one to one rate (Table 4).

The efficiency of isolates tested at 1:4 ranged from 1.20 to 2.52 and at 1:1 from 1.21 and 1.65. For both ratios the lowest efficiency was expressed by the isolate A2066 and the highest by the isolate A2321. However, significant individual differences in efficiency between the two ratios occurred only for the isolates A2090, A2103 and A2321.

3.4 DISCUSSION

Aflatoxin contamination is a health and economic problem worldwide with annual losses worth over 500 million US dollars (CAST, 2003; Yu et al., 2005). As a result, considerable effort has been directed at developing strategies to prevent or reduce aflatoxin contamination especially in several key crops: groundnut, maize, cottonseed, pistachio, and pepper. Use of cultivars with reduced susceptibility to aflatoxin contamination (Henry et al.,

2009), use of agronomic practices to reduce effects of drought and heat stress and proper plant nutrition, (Bruns, 2003) can help in reducing aflatoxins in maize. In addition to these strategies biological control based on use of atoxigenic isolates of *A. flavus* able to competitively exclude aflatoxins producer has efficacy on cotton, peanuts, pistachios and maize (Cotty and Antilla, 2003; Yin et al., 2009; Doster et al., 2004; Atehnkeng et al., 2008) and in areas of the US most severely affected by contamination, biological control with atoxigenic strains has rapidly become the preferred method for aflatoxin mitigation. In the northern portion of the Italian peninsula, where aflatoxin contamination of maize was economically important during 2003, *A. flavus* communities associated with maize production have been characterized (Giorni et al., 2007; Mauro et al., in preparation) and atoxigenic strains endemic to Italy identified. However, this work is the first to quantitatively compare among the Italian atoxigenic *A. flavus* for ability to displace aflatoxin producers during infection of viable maize kernels and to contrast performance of the Italian atoxigenics with an atoxigenic strain used commercially for aflatoxin mitigation in North America.

The Italian isolates reduce aflatoxin contamination of maize with efficacy similar to those reported for atoxigenics from North America (Mehl and Cotty, 2010) and Africa (Probst et al., 2011). The extent to which 12 of the Italian isolates reduced contamination was statistically equivalent to NRRL 21882, an isolate native to North America and the active ingredient of a product with a biopesticide registration for aflatoxin mitigation on maize. However, there were consistent differences among the Italian isolates in efficacy providing a criterion for selecting among atoxigenic strains endemic in Italy for entrants into aflatoxin mitigation field trials. Ability to displace aflatoxin-producers is probably a multi-genic trait (Mehl and Cotty, 2010) and variance in competitive ability may be attributed to variability in several traits including variation in life strategy. Isolates with a predominantly sporulating life strategy (Mehl and Cotty, 2010) may provide superior displacement of aflatoxin-producers during epidemic increases in *A. flavus* populations during crop contamination epidemics, but be rated low in the viable kernel assays due to lower competitiveness during kernel invasion. Field evaluations comparing strains varying in laboratory assays are needed to confirm the value of such assays for optimal selection of aflatoxin biological control agents.

In the current study, isolates A2085 and A2321 displayed greatest efficacy in reducing aflatoxin contamination. Atoxigenics were effective against diverse aflatoxin producers independent of the quantity of aflatoxins produced in control kernels inoculated with aflatoxin

producers alone. This wide efficacy of both of the evaluated atoxigenics (Table 2) indicates that these isolates may have utility against widely differing communities of aflatoxin producing fungi. The atoxigenics originated from the most important Italian maize producing regions requiring occasional aflatoxin mitigation and they were effective against toxin producers from the same regions and a highly toxigenic one from the US. These data suggest the atoxigenics have the potential to be widely useful when needed for mitigation of aflatoxins in Italy.

A measurement of Efficiency (E), the proportional ability of a given proportion of atoxigenic strain to reduce contamination, was evaluated for two proportions of atoxigenic strain 1:1 and 1:4 in the current study with the smaller proportion of atoxigenic strain expressing a higher E. An efficiency of 1 means toxin was reduced proportionately to the percent atoxigenic strain inoculated (50% atoxigenic strain causing a 50% reduction in aflatoxin has an efficiency of 1). E>1 indicates aflatoxin is reduced by an atoxigenic strain to an extent greater than that explained the its proportion in the inoculum. Increased E indicates an improvement in the amount of aflatoxin reduction each unit of atoxigenic achieves. In the current study, reduced proportions of atoxigenic were associated with increased E. This suggests atoxigenics act efficiently even when present at low proportions in nature to modulate aflatoxin production by the overall fungal community aflatoxin production. Furthermore, low proportions of multiple (perhaps many) atoxigenics may result in better aflatoxin reductions than the same level of displacement by a single isolate.

The current study identified several potential atoxigenic strain biocontrol agents for mitigating aflatoxin contamination in northern Italy. The most effective five Italian atoxigenic isolates caused reductions in contamination of viable maize kernels similar to a commercial product registered as a biopesticide in the United States. The identified atoxigenics are endemic to the target regions in Italy. Endemic atoxigenics are thought to offer several advantages over introduced isolates including improved environmental safety and better adaptation to the target region (Probst et al., 2011). Better adaptation to the local environment may allow for both increased efficacy in the treated crop and greater carryover between crops.

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3.6 Figures and tables

Table 1. *Aspergillus flavus* isolates

Isolate[a]	VCG[b]	AFB_1[c]	Location	District[d]	Year
A2049	IT12	-	Padova	V	2003
A2066	IT8	-	Mantova	L	2003
A2085	IT6	-	Reggio Emilia	ER	2004
A2087	n.a.	-	Reggio Emilia	ER	2004
A2088	IT9	-	Reggio Emilia	ER	2004
A2090	IT9	-	Reggio Emilia	ER	2004
A2096	IT9	-	Reggio Emilia	ER	2004
A2098	IT6	-	Bologna	ER	2004
A2100	IT18	-	Piacenza	ER	2004
A2102	n.a.	-	Piacenza	ER	2004
A2103	IT15	-	Piacenza	ER	2004
A2105	IT6	-	Modena	ER	2004
A2313	IT23	-	Ravenna	ER	2009
A2319	IT22	-	Cremona	ER	2009
A2320	IT22	-	Cremona	ER	2009
A2321	IT19	-	Parma	ER	2009
A2322	IT34	-	Modena	ER	2009
A2323	IT23	-	Ravenna	ER	2009
NRRL 21882		-	Georgia (USA)		1991
A2039	IT11	+	Lodi	L	2003
A2062	IT13	+	Venezia	V	2003
A2068	IT26	+	Udine	FVG	2003
A2097	IT10	+	Modena	ER	2004
A2295	IT3	+	Cremona	ER	2008
A2300	IT5	+	Cremona	ER	2008
AF13	YV13	+	Arizona (USA)		

[a] All isolates came from Italy except the NRRL 21882 and AF13 which originated in the United States of America (USA).
[b] Vegetative Compatibility Group; n.a.= not available.
[c] AFB_1: (+/-): aflatoxin and no aflatoxin production.
[d] Italian regions: ER = Emilia Romagna, FVG = Friuli Venetia Giulia, L = Lombardy, V = Veneto.
[e] Year of isolation.

TABLE 2. Ability of 18 atoxigenic isolates of *Aspergillus flavus* to reduce aflatoxin B_1 production in maize

Isolate[a]	**VCG**[b]	**ppm AFB_1**[ce]		**R (%)**[de]	
A2321	IT19	15	H	90	A
A2103	IT15	21	GH	85	AB
A2090	IT9	22	FGH	85	ABC
A2066	IT8	23	EFGH	85	ABC
NRRL 21882		24	EFGH	84	ABC
A2085	IT6	25	EFGH	83	ABC
A2088	IT9	27	EFG	82	ABC
A2105	IT6	27	DEFG	81	ABC
A2313	IT23	28	DEFG	81	ABCD
A2098	IT22	29	CDEFG	80	ABCD
A2096	IT6	30	CDEFG	79	BCD
A2102	n.a.	32	BCDEFG	78	BCDE
A2319	IT22	33	BCDEFG	78	BCDE
A2049	IT12	39	BCDE	73	CDEF
A2320	IT9	41	BCDE	72	CDEF
A2087	n.a.	47	BCD	68	DEF
A2322	IT34	51	BC	65	EF
A2323	IT23	54	BC	62	EF
A2100	IT18	57	B	61	F
AF13	YV13	146	A	0	G

[a] Atoxigenic isolates were inoculated with AF13 at $1x10^5$ conidia/ml. NRRL 21882 is the active ingredient of the Afla-Guard biopesticide. AF13 produces aflatoxins.

[b] Vegetative compatibility groups of isolates; n.a.= not available.

[c] Aflatoxin B_1 concentration (ppm) after 7 days incubation of infected kernels co-inoculated with atoxigenic and aflatoxin-producing isolates.

[d] Percentage aflatoxin reduction (R) = [1- (total aflatoxin in co-inoculation/total aflatoxin in AF13)] x 100.

[e] Letters indicate significant differences among isolates. Combined data from two independent trials ($P<0.05$) are presented.

Table 3. Reduction of two atoxigenic isolates of *Aspergillus flavus* from Italy in maize contaminated with aflatoxins produced by 6 Italian isolates of *A. flavus*

Isolate[a]	ppm AFB_1[c]		ppm AFB_1[cf]				R (%)[df]			
			A2085[b]		A2321[b]		A2085[b]		A2321[b]	
A2039	98	A	22	A	17	A	78	A	83	A
A2062	35	BC	11	BC	8	B	68	B	78	AB*
A2068	41	B	16	AB	16	A	60	B	61	C
A2097	29	C	11	BC	8	B	61	B	71	BC
A2295	23	D	8	CD	7	BC	65	B	71	BC
A2300	17	E	7	D	5	C	60	B	72	BC*

[a] Toxigenic isolates.

[b] A2085 and A2321 atoxigenic isolates were co-inoculated at the same time of toxigenic isolates at $1x10^5$ conidia/ml.

[c] Aflatoxin B_1 concentration (ppm) after 7 days in infected kernels co-inoculated with atoxigenic and toxigenic isolates.

[d] Percentage aflatoxin reduction (R) = [1 - (total aflatoxin in co-inoculation/total aflatoxin in respective toxigenic isolate)] x 100.

[e] Efficiency (E) was calculated following the formula: E= R/(A/A+T); where R is the aflatoxin reduction and the denominator term represents the percentage of atoxigenic in each treatment.

[f] Letters indicate significant differences among isolates (columns); * indicate differences among atoxigenic isolates (rows). Combined data from two independent trials ($P<0.05$) are presented.

Table 4. Influence of atoxigenic and toxigenic ratios of *Aspergillus flavus* on reduction of aflatoxin B_1 produced by toxigenic isolates of *A. flavus*

Isolate[a]	ppm AFB_1[cf]				R (%)[df]				E[ef]			
	(1 : 4)[b]		(1 : 1)[b]		(1 : 4)[b]		(1 : 1)[b]		(1 : 4)[b]		(1 : 1)[b]	
A2066	86	B	44	B*	24	D*	61	B	1.20	C	1.21	B
A2085	70	CD	31	B*	38	BC*	72	AB	1.90	B	1.45	AB
A2090	76	BCD	37	B*	33	BCD*	67	B	1.66	BC	1.35	AB*
A2103	68	D	34	B*	39	AB*	70	AB	1.97	AB	1.39	AB*
A2321	56	E	20	C*	50	A*	83	A	2.52	A	1.65	A*
NRRL 21882	83	BC	40	B*	26	CD*	65	B	1.30	C	1.29	B
Mean	73		34	*	35	*	70		1.76		1.39	*
AF13	113	A	113	A	…		…		…		…	

[a] NRRL 21882 is the active ingredient of the Afla-Guard biopesticide. AF13 produces aflatoxins and it represents the positive control.

[b] Atoxigenic (A) and toxigenic (T) isolates were co-inoculated at $1x10^5$ conidia/ml in two ratios: (a) one fifth atoxigenic and 4 fifth toxigenic (1:4) and (b) equal proportions (1:1).

[c] Aflatoxin B_1 concentration (ppm) after 7 days in infected kernels co-inoculated with each of atoxigenic and toxigenic isolates.

[d] Percentage aflatoxin reduction (R) = [1 - (total aflatoxin in co-inoculation/total aflatoxin in AF13)] x 100.

[e] Efficiency (E) was calculated following the formula: E = R/(A/A+T); where R is the aflatoxin reduction and the denominator term represents the percentage of atoxigenic in each treatment.

[f] Letters indicate significant differences among strains (columns); * indicate differences among treatments (rows). Combined data from two independent trials ($P<0.05$) are presented.

CHAPTER 4

Biocontrol field trials to evaluate the efficiency of selected biocontrol agents

4.1 Introduction

Aflatoxins (AFs), in particular AFB_1, are among the most toxic natural compounds with demonstrated cancerogenic effect on humans (Wang and Tang, 2004). Aflatoxins are secondary metabolites produced by several species of *Aspergillus* on important commodities like maize (Klich, 2007). The principal responsible of AFs contamination on maize is *Aspergillus flavus* (Giorni et al., 2007). In Italy maize growing area is mainly located in 5 districts that supply 89% of the national production (Istat, 2011). These districts are placed in the north of the peninsula were generally, the climatic conditions are not favourable for AFs contamination; however, reduced rainfall and increased temperature during maize production can occur along with associated levels of AFs in excess of the legal limits (EC, 2010). Unfortunately, in 2003 for the first time, high levels of AFs were detected (Giorni et al., 2007; Piva et al., 2006).

Although several strategies have been applied to reduce pre-harvest AFs contamination, biological control with atoxigenic strains of *A. flavus* is the most promising (Amaike and Keller, 2011). Atoxigenic strains displace aflatoxin producers during crop development with a consequent reduction in AFs contamination (Cotty and Bayman, 1993; Cotty, 2006; Atehnkeng et al., 2008; Dorner, 2009). *Aspergillus flavus* biocontrol agents are commercialized on coated seeds (*i.e.* wheat, barley, sorghum) to facilitate the application in field and to offer a substrate for fungi growth, sporulation and consequent dispersion on developing plants (Antilla and Cotty, 2004). However solid preparations support both the residence in fields and sporulation for relatively long periods and, as a result, provide a window of influence that extents considerably beyond application date (Cotty, 2006).

Efficacy of biocontrol agents belonging to *A. flavus* to reduce AFB1 contamination on different crops in field is well documented. Dorner et al. (1998) found a reduction in AFB1 contaminations on peanuts from 74.3 to 99.9%, applying on the soil 9 or 227 kg/ha, respectively, of wheat coated with atoxigenic *A. flavus*. In an experiment on maize field in Georgia, similar results were obtained (Dorner et al., 1999). In particular a reduction in AFB1 concentration of 87% and 66% were achieved with 225 kg/ha and 22.5 kg/ha, respectively, of atoxigenic *A. flavus* grown on wheat seeds. Also Abbas et al. (2006), in a corn field, observed a reduction in AFB1 contamination between 65 and 95% applying 20 kg/ha of wheat coated with atoxigenic *A. flavus*.

AFs contamination produce significant economic losses and safety risk. So, efficient strategies to reduce aflatoxins contaminations are strongly requested by farmers. The current study sought to evaluate the ability of 5 atoxigenic isolates of *A. flavus* to reduce AFs contamination in a maize field.

4.2 Materials and Methods

4.2.1 Isolates of *A. flavus*

Five atoxigenic isolates, A2066, A2085, A2090, A2103, and A2321 of *A. flavus* were used in this study. These isolates were selected because they showed *in vitro* the highest efficacy in AFB1 reduction when co-inoculated with high toxigenic strain (Mauro et al., in preparation). VCG, location, district and year of isolation and the percentage of reduction of AFB1 are listed in Table 1.

Table 1. Characteristics of 5 atoxigenic *Aspergillus flavus* strains used for in filed trials.

Isolate[a]	VCG[b]	Location	District[c]	Year[d]	Source[e]	R (%)[f]
A2066	IT8	Mantova	L	2003	maize	85
A2085	IT6	Reggio Emilia	ER	2004	maize	83
A2090	IT9	Reggio Emilia	ER	2004	maize	85
A2103	IT15	Piacenza	ER	2004	maize	85
A2321	IT19	Parma	ER	2009	maize	90

[a] All isolates came from Italy.
[b] Vegetative Compatibility Group.
[c] Italian districts: ER = Emilia Romagna, L = Lombardy.
[d] Year of isolation.
[e] Maize kernels
[f] Percentage aflatoxin B_1 reduction

The 5 isolates were also analysed for deletion in the aflatoxin biosynthetic gene cluster and all isolated missed the entire cluster (Mauro et al., in preparation).

4.2.2 Inoculum preparation

Sorghum was used as the inoculant carrier. Almost thirty kg, divided in 5 bags, of sorghum seeds were autoclavated for 20 min at 121°C. Starter cultures of *A. flavus* were grown on Czapek agar in 9-cm Petri dishes at 28-30°C for 6 days in the dark. Conidia were dislodged from each plate with 5 ml of sterile distilled water and adjusted at 10^5 spore/ml prior to inoculation. Five ml of spore suspension of each isolates were used to inoculate

almost 6 kg of sorghum seeds. Sorghum was incubate at 28-30°C for 10 days in the dark and manually shaken every day. All sorghum aliquots were combined together prior of field applications.

4.2.2 Field study experimental design

The hybrid DeKalb 6286 was planted at a rate of 7 plants/ha on April 20th 2011 in an almost 2 ha field located in Modena, Emilia Romagna district. The treatments were: (1) distribution of infected sorghum, (2) spray against *Ostrinia nubilalis* (European Corn Borer-ECB) and (3) no treatments (Test). Inoculations were made at maize growth stage of V8-V10 by tractor spreader at rate of 35 kg/ha. The field, almost 200 m x 100 m was divided in 9 plots. The treated plots (1 and 2) were 10 m large and the non-treated plots 14 m large and both were 200 m long; shared in 3 replicates. On September 7th, 10 plants, in the middle of each plot, were sampled.

4.2.3 Evaluation of European corn borer (*O. nubilalis*)

Damages caused by ECB were evaluated on the ears sampled based on 6 different types of lesion produced (Table 2; Battilani et al., 2011).

Table 2. Scale for the European corn borer (ECB) attack assessment. The score was based on the presence of visible signs in different parts of the ear

Visible signs	Value
Symptomless ear	0
at ear apex	1
at ear apex + centre	3
at ear apex + centre + base	3
at ear apex centre	1
at ear apex centre + base	3
at ear apex + base	3
at ear base	2
at ear peduncle	4
apex + centre + base + peduncle	5

4.2.4 Aflatoxins quantification

Prior to AFs extraction, corn ears were de-husked, the kernels dried at 45°C for 5 days and milled. Aflatoxins were analyzed according to the method of Stroka et al. (1999). Aflatoxins were extracted from 25 g of sample with 250 ml methanol:water (80:20, v/v), using a rotary-shaking stirrer for 45 min. After filtration through filter paper, 5ml of the filtrate was diluted with 45 ml of distilled water and the solution was purified through an immunoaffinity column (R-Biopharm Rhône Ltd, Glasgow, UK). Aflatoxins were eluted from the column with 2.5 ml of methanol. The eluate, concentrated to 1.0 ml under a gentle stream of nitrogen, was brought to 2 ml with acetonitrile:water (25:75, v/v); the extract was then filtered (Millipore Corporation, Bedford, MA, USA; HV 0.45 mm) and injected. Analysis was performed using an HPLC instrument consisting of two PU-1580 chromatographic pumps, an AS 1555 sampling system, a FP 1520 fluorescence detector and a post-column derivatization system (Jasco Corporation, Tokyo, Japan). A Superspher RP-18 column (Merck) was used at ambient temperature with a mobile phase of water:methanol:acetonitrile (64:23:13,v/v/v) at 1.0 ml min^{-1}. The detector was set at λ_{ex} = 365nm and λ_{em} = 440nm (Pietri et al., 2009).

4.3 Consideration

The trial was conducted in an area of northern Italy were frequent contaminations of AFs have been reported (Battilani, personal communication). Weather conditions registered in the area of the field experiment are reported in Fig. 1. Date showed in first half of July close to the silking stage the presence of rain and low temperature. Silking stage has been demonstrated as the most susceptible for AFs contamination and the production is favoured by dry weather and high temperature. In our case rain registered before silking stage could have limited *A. flavus* infection and AFs production. In fact, our preliminary analysis conducted on 8 samples from the untreated plots, showed that aflatoxins were detected only in one sample. However other analysis are still on going and results are not yet available

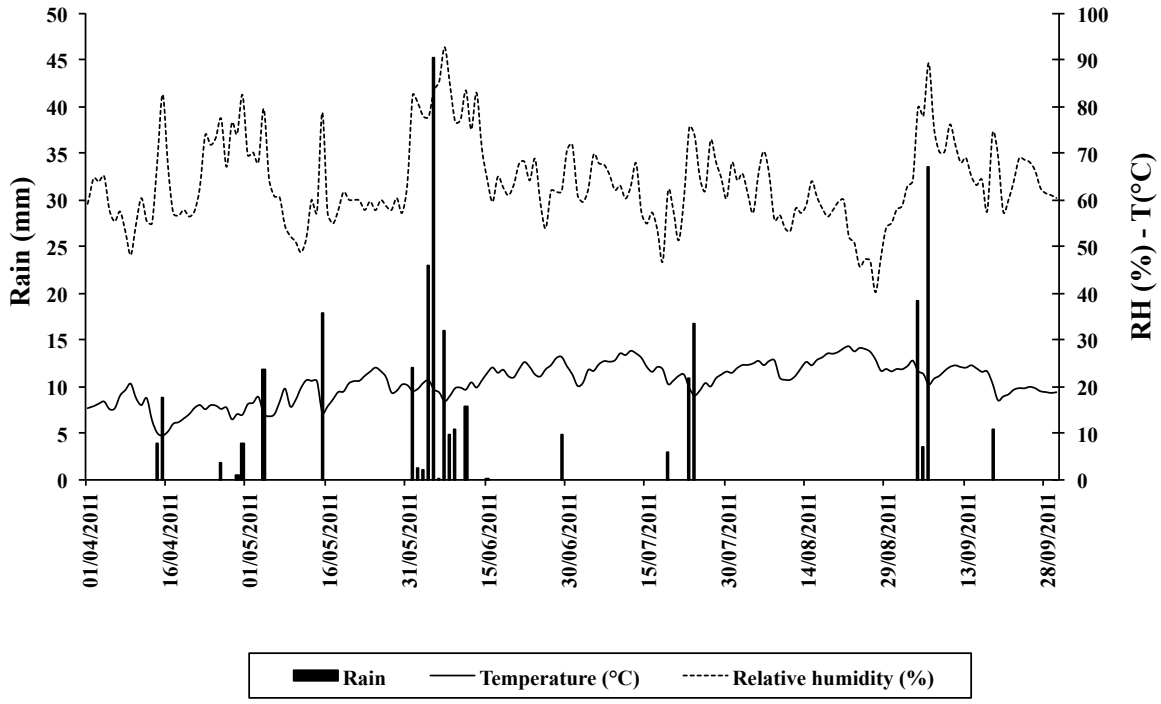

Fig. 1. Weather conditions (rain, temperature and relative humidity) of the trial field area along the maize growing season in 2011.

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CHAPTER 5

Oral communication

XVII Congress of Italian Phytopathological Society (S.I.Pa.V)
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Selection of atoxigenic Aspergillus flavus native to Italy as candidate biocontrol agents

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Maize aflatoxin contamination is economically important in Northern Italy. There are few tools for limiting aflatoxins in commercial maize production; one highly successful strategy used in the USA is biocontrol with atoxigenic *Aspergillus flavus*. The primary causal agent of maize contamination is *A. flavus*. However, some native genetic groups (called vegetative compatibility groups or VCGs), of *A. flavus* lack ability to produce aflatoxins and can competitively exclude aflatoxin producers and thereby limit crop contamination. Atoxigenic VCGs of *A. flavus* are used commercially in the USA to prevent aflatoxin contamination of several crops and similar atoxigenic VCGs may be of value in Italy. For optimal efficacy and safety, candidate VCGs must be selected from the region in which they will be used. The current study sought to select VCGs of potential use in biocontrol from northern Italy and to evaluate the capacity of atoxigenic VCGs native to that region to reduce aflatoxin in viable maize kernels. *Aspergillus flavus* was isolated from maize grown in Emilia Romagna, Friuli Venezia Giulia, Lombardia, Piemonte, Toscana and Veneto between 2003 and 2010. Isolates (139) were tested for aflatoxin producing capacity on autoclaved corn. Complementation tests between nitrate non-utilizing auxotrophs placed isolates into 49 VCGs of which 24 contained only atoxigenic isolates. The most common atoxigenic VCG composed 8% of the *A. flavus* and was isolated from 4 of 6 regions. Atoxigenics varied in capacity to reduce aflatoxin in viable maize; the five best VCGs reduced kernel aflatoxin content over 80% with the best reducing contamination 91%.

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CHAPTER 6

General conclusions

This research would contribute to develop biocontrol agents of *A. flavus* with the intent to reduce aflatoxin contamination in the principal maize-growing area of Italy.

Vegetative compatibility analysis is a useful tool to estimate genetic diversity, understand population dynamics and evaluate measures to reduce aflatoxin contamination. The current work reports for the first time the VCG structure of *A. flavus* resident in the major maize producing districts of Italy. In particular the relatively wide distributions across districts and years and high frequencies of the atoxigenic VCGs IT4, IT6 and IT18 indicate that these VCGs are well adapted to the environmental conditions and maize production systems of Italy and as such are good candidates for the development of aflatoxin biocontrol agents targeted for this district.

We identified several atoxigenic strains as potential biocontrol agents for mitigating aflatoxin contamination in northern Italy. The most effective five Italian atoxigenic isolates caused reductions in contamination of viable maize kernels similar to a commercial product registered as a biopesticide in the United States. The identified atoxigenic strains are endemic in the Italian target regions. Endemic atoxigenics are thought to offer several advantages compared to the non resident isolates including improved environmental safety and better adaptation to the target region; therefore, increased efficacy in the treated crop and greater carryover to neighbour crops is expected.

Atoxigenic strains act primarily through competitive exclusion of aflatoxin producers in the environment and during crop tissue infection. Atoxigenics with superior adaptation to a specific area may allow both better initial displacement of aflatoxin producers and better survival between crops and thus long-term displacement providing both long-term (multiple year) and additive benefits. Additional information on the behaviour of these atoxigenics during crop infection and within the ecology of the target agroecosystem will be needed to choose the best strains useful as biological control agents

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