Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN SCIENZE AGROAMBIENTALI

Ciclo XXIV

Settore Concorsuale di afferenza: 07/B1-Agronomia e Sistemi colturali erbacei ed ortofloricoli

Settore Scientifico disciplinare: AGR/02-Agronomia e Coltivazioni erbacee

PRACTICAL IMPLICATIONS OF AFLATOXIN CONTAMINATION IN CORN

Presentata da: Dr.ssa Mariangela Mencarelli

Coordinatore Dottorato **Relatore**

Prof. Giovanni Dinelli **Prof. Alberto Vicari**

Esame finale anno 2012

TABLE OF CONTENTS

ABSTRACT

Aflatoxin (AFL) contamination of corn is a serious economic and food security issue. Although a variety of technical solutions for reducing AFL contamination of corn have been proposed, only a few have produced satisfactory results. A successful approach is a biocontrol strategy consisting of using non-aflatoxigenic strains of *Aspergillus flavus* to replace indigenous AFL-producing isolates. The main objective of the present thesis was to investigate the dynamic and contamination of AFL/*A. flavus* in corn in Northern Italy. The study also investigated the role of the key-pest of corn, the European Corn Borer (ECB), on AFL contamination and dispersal of *A. flavus* propagules in corn. Finally, the study evaluated the feasibility of bioplastic-based granules entrapping a non-aflatoxigenic *A. flavus* strain for the biocontrol of this fungus in corn.

The 2-year field study demonstrated the efficacy of the bioplastic formulation to reduce AFL contamination in corn. More precisely, although AFL contamination varied among the two years, application of 15 and 30 kg ha^{-1} of granules reduced AFL contamination to up 60 and 85% in 2009 and 2010 respectively. Microbiological analysis showed that the relative abundance of non-aflatoxigenic soil isolates significantly increased after 1 month from granules application (mid-May) and throughout the corn-growing season. These findings were consistent with data obtained using a bioplastic-based bait specifically developed to selectively isolate Aspergilli from soil and other environmental samples. In addition, field and laboratory evaluations showed that the levels of damages produced by ECB larvae were not significantly correlated to *A. flavus* infestation and AFL contamination.

Taking together, these findings demonstrated that AFL contamination of corn in Northern Italy was variable, but above the EU limit for human consumption. First proposed in the USA, this study showed the practical possibility of this formulation to be used for reducing AFL contamination in corn in the EU.

Keywords: Aflatoxins, *Aspergillus flavus*, Biocontrol, Biodegradable polymers, European Corn Borer.

CHAPTER 1. INTRODUCTION

1.1. AFLATOXINS

Mycotoxins are secondary toxic metabolites produced by various genera of fungi. The principal classes of mycotoxins are the following: aflatoxins (AFL) produced by *Aspergillus spp.*, fumonisins, trichothecenes and zearalenone produced by *Fusarium* spp., ochratoxins and patulin produced by *Aspergillus spp.* and *Penicillium* spp. Although mycotoxins belong to different chemical families and have well-delineated modes of action, all mycotoxins are characterized as being toxic to higher organisms, including humans (Hussein and Brasel, 2001).

Among the different mycotoxins, AFL are the most studied for their negative impact on human and animal health. AFL were discovered in the early ‘60s, from the investigation of the cause of an epidemic called ‘‘turkey X’ disease” that killed numerous turkeys, ducks and chicks in Southern England. Investigations revealed that toxicity was associated with the occurrence of the fungus *Aspergillus flavus* in feed diet containing peanut meal coming from Brazil (Blount, 1961). Further researches revealed that extracts of cultures of the fungus isolated from the peanut meal were able of inducing the ‘‘turkey X’’ syndrome, and the toxic agent was named ‘‘aflatoxin’’ as “*A. flavus* toxin” (Kensler et al., 2011). Chemically, AFL are highly oxygenate bisfurano-cumarine compounds (Asao et al., 1965). (Fig. 1).

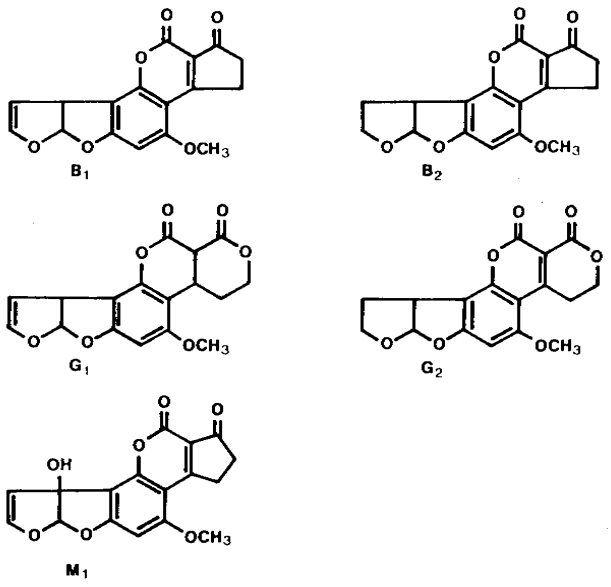

Fig. 1. Structure of AFB1 and related aflatoxins

AFL, like many other heterocyclic compounds, are distinguished by their fluorescing properties. Both aflatoxin B1 (AFB1) and B2 (AFB2) fluoresce blue and aflatoxin G1 (AFG1) and G2 (AFG2) fluoresce yellow-green under ultraviolet light (Sargeant and Carnaghan, 1963). Other important toxins are the aflatoxin M1 (AFM1) and M2 (AFM2), metabolites derived from hydroxylation of AFB1 and AFB2 respectively.

Although AFL B and G are detected in a large variety of foods and feeds (e.g., cereals, tree nuts, oil nuts, figs, pistachio, spices…) corn, peanuts and cottonseed are the most frequently contaminated products. AFM are principally detected in milk and in other animal products (meat, cheese, eggs...) following carry over (Bryden, 2007). Residues of cyclopiazonic acid (CPA), a co-contaminant with AFL, have been detected in meat, milk and eggs (Bryden et al., 2004).

Among AFL, AFB1 is the most dangerous. Studies demonstrate its carcinogenic, mutagenic and teratogenic effect and it is classified by the IARC in the Group 1 "Carcinogenic to humans". The AFM1, is also known as carcinogenic and mutagenic substances, and is classified in the group 2A "Probably carcinogenic to humans" (IARC, 2002).

1.1.1. AFL and human health

AFL are related with various human and animal diseases worldwide (Hussein and Brasel, 2001). It is estimated that up to 25% of foodstuffs in the world are contaminated by fungi (Mannon and Johnson, 1985). The chronic intake is the most widespread form of AFL exposure (Bryden, 2007). This contamination is difficult to reduce because fungi producing AFL are ubiquitous in the environment (Wicklow et al., 2003); they infect crop and other food commodities in pre and post-harvest and, under favorable environmental conditions, produce toxins (Payne, 1992). Usually heavily contaminated food is not found in the market of developed countries and health consequences for the population are low. In the opposite, AFL heath related problems are common in developing and underdeveloped countries affected by food insecurity (Wu, 2004). The symptomatology due to intoxication can be acute or chronic, both in animal and human. The acute syndrome is characterized by vomiting, abdominal pain,

pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart (IARC, 2002). Acute intoxications are relatively rare, but several epidemics occurred in undeveloped countries, mostly Asia and Africa, leading to the death of several hundred people (Wu and Khlangwiseta, 2010). For instance, since the scarcity of food determined the consumption of corn contaminated with high levels of AFL, more than 125 people died due to acute aflatoxicosis in Kenya (Lewis et al., 2005). Acute liver disease following AFL consumption has been reported also in India and Malaysia (Wu, 2004). Chronic states are the most common and are difficult to diagnose due to the unclear symptomatology and many interacting factors in their pathogenesis (Bryden, 2007). In general the ill subject shows a reduced growth and development, immunosuppression, endocrine disruption symptoms and disease linked to alterations of DNA metabolism (cancer, mutation...) (IARC, 2002). Since late sixties, studies associated the AFB1 with hepatocellular carcinoma (HCC), the actual third-leading cause of cancer death worldwide (WHO, 2008). In fact, once ingested, B1 are metabolized in their epoxide by cytochrome P450. The epoxide reacts with guanine in DNA and RNA leading to depurination. The primary effect is to inhibit protein and DNA synthesis in the most active tissues (liver, intestines and bone marrow), (Kensler et al., 2011).

AFL have a synergic effect with hepatitis B (HBV) infection determining 30-fold higher liver cancer risk in HBV-positive (Kensler et al., 2011). The World Health Organization indicates that the most part of new cases of HCC, estimated in 550.000-650.000/year, occur in East Asia and sub-Saharan Africa where there are the highest rise up of HBV infection and AFL contamination. The WHO evaluated that vaccinations against HBV can determinate a substantial reduction of HCC (Liu and Wu, 2010).

1.1.2. AFL producing fungi

AFL are produced by the filamentous fungi belonging to the Aspergillus spp., in particular to the subgenus Aspergillus section Flavi. Although *Aspergillus flavus* and *Aspergillus parasiticus* are the economical most important species, other fungi producing this metabolite were found like *A. australis, A. arachidicola, A. bombycis, A. minisclerotigenes, A. nomius, A. ochraceoroseus, A.*

pseudotamarii, *A. rambellii*, *Emericella venezuelensis* (The aspergillus website http://www.aspergillus.org.uk/). *A. parasiticus*, which produce both AFLG and B, proliferates mainly in warm climates and it is found to infect prevalently peanuts. *A. flavus*, which produces only B toxins, is diffused in temperate zones and generally infects major crops such as cottonseed, corn and other cereals, (Abbas et al., 2009). Other crops and commodities are infected by Aspergilli and contaminated with AFL, such as sorghum, rice, wheat, peanuts, soybean, sunflower, chili pepper, black pepper, coriander, almond, pistachio, walnuts, coconuts (FAO, 2004).

As reported above *A. flavus* has a worldwide distribution and is ubiquitous in the environment, it has been isolated from air, soil, plant debris and living tissue, decaying wood, food commodities, insect, animal carcasses, animal and human patients (Abbas et al., 2009; Hedayati et al., 2007).

A. flavus is a common component of soil, generally it is associated with the soil organic matter and subsists as mycelium, conidia, or sclerotia. It can grow at temperature ranging between 12 and 48°C but its optimal range is at 28-37° (Hedayati et al., 2007). Fungal invasion of crops before harvest is of prime importance for the AFB1 contamination and the *A. flavus* ear rot infection in corn is the most studied process (Sheidegger and Payne, 2003). *A. flavus* life cycle in corn field is summarized in Fig. 2.

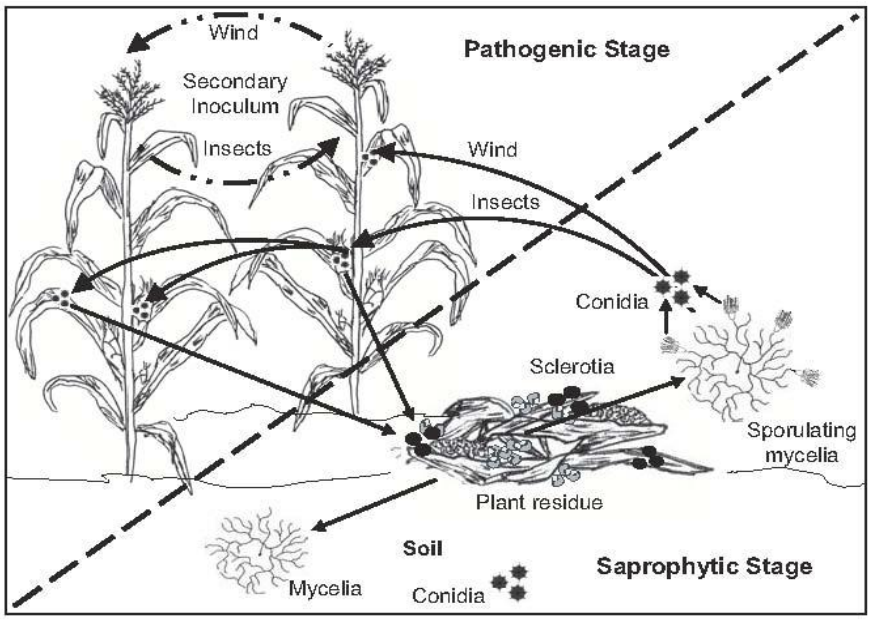

Fig. 2. Life cycle of *A. flavus* in a corn cropping system.
From Abbas et al.**, 2009**

A. flavus sclerotia or conidia germinate and, under favorable environmental conditions, colonize corn, especially stressed and damaged plants. Aflatoxigenic aspergilli strains produce AFL especially in drought conditions. Infected plant tissues can serve as source of secondary inoculum during the growing season or as reservoir for the next season (Wicklow et al., 1993). Studies have shown that *A. flavus* is most present in no-till soil surface compared to conventional soils (Zablotowicz et al., 2007). Other studies demonstrate the expression of several AFB1 biosynthesis genes in soil thus further indicating that corn residues may serve also as source of AFL contaminations (Accinelli et al., 2008 b).

A. flavus is not only a plant pathogen, but can also cause diseases in animals and humans by tissue invasion. This specie is the second leading cause of invasive and noninvasive aspergillosis, a pathology involving the upper respiratory tract in humans and animals, next to *A. fumigatus* (Denning et al., 1998). This pathology is diffused in immunocompromised patients, moreover people affected by AIDS, cancer and recently transplanted patients. *A. flavus* is also an allergen; it is a common etiological agent of fungal sinusitis and cutaneous infections and is rarely involved in allergic bronchopulmonary aspergilliosis. *A. flavus* rarely was also associated with osteoarticular infections and urinary tract infections. *A. flavus* seems to be more virulent and more resistant to antifungal drugs than the most part of other *Aspergillus spp*. (Hedayati et al., 2007)

1.1.3. AFL biosynthesis

The *A. flavus* and *A. parasiticus* AFL biosynthesis is one of most studied fungi biosynthetic pathways (Yu et al., 2004). Researcher found that at least 23 enzymatic reactions are involved for the AFL biosynthesis and 15 structurally defined AFL precursors have been isolated (Yu et al., 2004). AFL are formed after a series of oxidation-reduction reactions starting from the acetate (Yu et al., 2008). The AFL biosynthetic pathway is reported in Fig. 3. In the AFL biosynthesis the Norsolorinic acid (NOR) is the first stable intermediate. After the VER B step, there is a branch point in the pathway that leads to AFL B2 and G2 formation.

Twenty-nine genes (or open reading frame) clustered within a 75 KB DNA region have been identified, cloned and characterized in the AFL formation. Particularly important are the genes:

- *aflA(fas-2), afiB(fas-1* and *afiC(pksA)* are directly involved in the backbone formation for the conversion from acetate to NOR in AFL synthesis;
- *aflD (nor-1),* identified for first, encoding for a ketoreductase in *A. parasiticus* for the conversion of NOR to averantin (AVN). Deletion or disruption of this gene determines losses of AFL pathway;
- *aflM* encoding for a ketoreductase, which is required for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST) and versicolorin B (VERB) to demethyldihydrosterigmatocystin (DMDHST) in *A. parasiticus;*
- *aflP P (omtA)* encoding an O-methyltransferase for the conversion of STto OMST and DMST to dihydro-O-methylsterigmatocystin (DHOMST) in *A. parasiricus;*
- *aflR* is a positive regulatory gene needed for the transcriptional activation of most part of structural genes in *A. flavus* and *A. parasiticus;*
- *aflS (omtA)* is a gene involved in the regulation of transcription*;*
- *aflQ (ordA)* encoding a cytochrome P-450 monooxygenase, determine the conversion of O-methylsterigmatocystin (OMST) to AFB, and AFG1, and demethyldihydrosterigmatocystin (DMDHST) to AF1 32 and AFG 246'59 in *A. parasiticus* and in *A. flavus;*
- *aflU (cypA),* encoding a cytochrome P450 monooxygenase, is responsible for the conversion from OMST to AFG1 and DHOMST to AFG2 in *A. parasiticus.*

Between the Microbial Genome Sequencing Project promoted by the USDA National Research, in 2003 started "The *Aspergillus flavus* Genome Sequencing Project" with the aim of sequence the entire genome of this fungus. The sequencing project of *A. flavus* NRRL 3357 was directed by Gary Payne and Ralph Dean at North Carolina State University in collaboration with USDA/ARS/SRRC (New Orleans) the Institute for Genomic Research (TIGR) and the J. Craig Venter Institute. The annotated genome was released in October 2005. Preliminary data indicated that the *A. flavus* genome consists in 8

chromosomes. The genome size is about 36.3 Mb (Yu and Keller, 2005). Updated annotated scaffolds were posted to GenBank by the J. Craig Venter Institute in January 2009. The knowledge of the whole *A. flavus* genome would be useful for future studies to better understand the pathogenicity factors involved in human and animal infections, as well as identify the mechanisms of fungus-crop interaction and of mycotoxins formation (Cleveland et al., 2009).

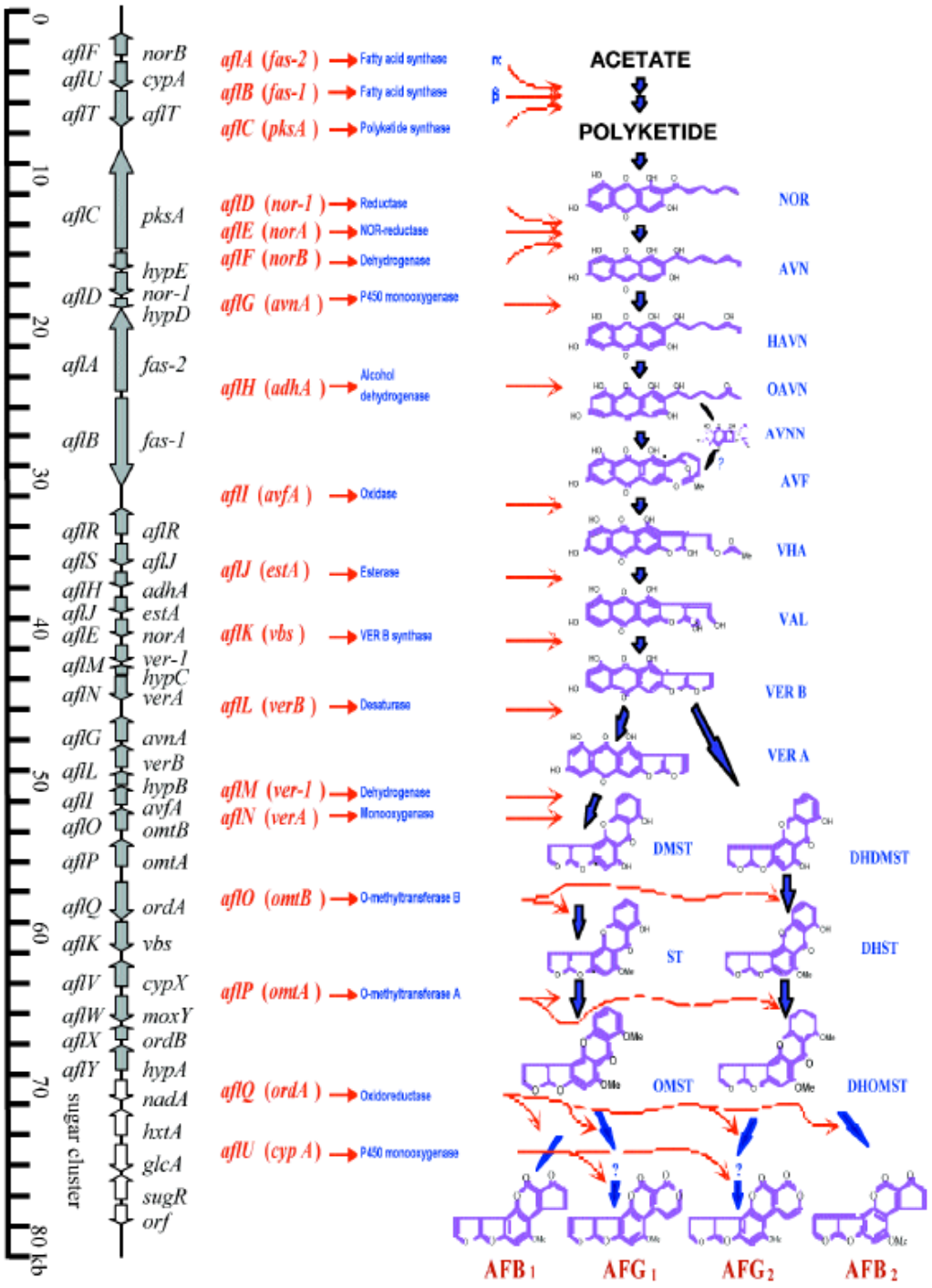

Fig. 3. AFL biosynthesis pathway. From Yu et al., 2004.

1.1.4. AFL regulation

Since the discovery of AFL and their harmful effect on human and animal health, many countries established regulations on the maximum levels of these contaminants in food and in feed. In general Maximum Limits are determined considering toxicological data, data on the occurrence of mycotoxins in various commodities and their distribution in lots, the availability of analytical methods, the need for sufficient food supply and the regulation of other countries (FAO, 2004).

Internationally, the Codex Alimentarious Commission (CAC), which among other activities establish advisory standards on natural and environmental toxins such as mycotoxins, has set two AFL standards: one for peanuts destined for further processing (15 ng/g) and one for AFL M1 in milk (0.5 ng/g), (CAC, 1997 and 2004). Even so this standard is accepted within the regulatory framework of the World Trade Organization (WTO) and the Joint Expert Committee on Food Additives (JECFA), and the Food and Agriculture Organization (FAO) invite countries to follow the codex directives, nowadays national or transnational regulations are the most diffused.

The last official survey filed by FAO (2004) indicated that approximately 80 countries have developed specific limits for AFL. The number of countries significantly increased from the previous survey on 1995 (with a slight increase in Latin America and Europe, and more significant increases in Africa and Asia/Oceania). Subsequent data indicate that the number of countries adopting mycotoxin regulation is still growing, while tolerance limits generally remain the same or tend to decrease (Fig. 4).

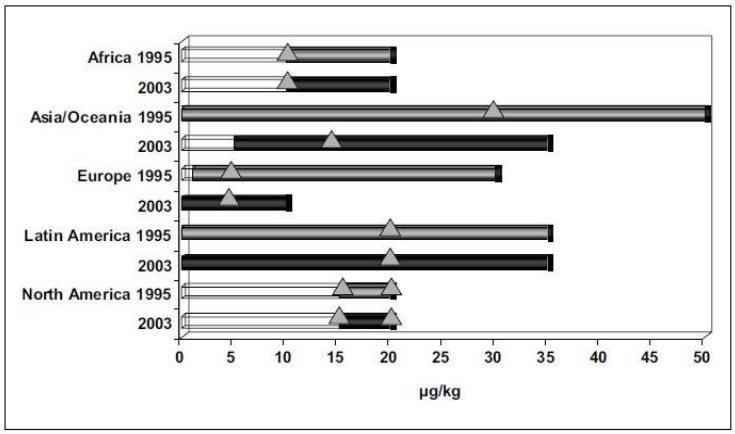

Fig. 4. Maximum level tolerated of AFLT worldwide. From FAO, 2004.

At the meantime several regulations have been or are being harmonized between "economic regions" (Australia/New Zealand, EU, and MERCOSUR). In general we can still see very big differences in the regulatory requirement among these areas. For example the USA, the first country that have regulated AFL in 1968, have standards only for the sum of AFL (AFLT). The maximum limit is 20 ppb for food and fed for immature animal and from 100 to 300 ppb for fed for mature animal. The limit of AFM1 in diary product is 0.5 ppb. These standards are used also in Canada. Australia/New Zealand have common limits applied for total AFL in peanuts and tree nuts. Countries member of MERCOSUR (Argentina, Brazil, Paraguay and Uruguay) apply common limits for total AFL in peanuts, maize and products thereof (5 ppb for AFB1 and 20 for AFLT), and for AFL M1 in fluid and powdered milk (0.5 ppb). In Africa fifteen countries are known to have specific AFL regulations, and six to have not these. Data about specific mycotoxin regulations in other African countries are not available. The European Union has the strictest AFL standards. The actual maximum levels of AFL (AF B1, B2, G1, G2 and M1) are written in Commission Regulation (EC) No 1881/2006. The standard in cereals, peanuts and tree nuts ready to eat is 4 ppb for AFLT and 2 ppb for AFB1. AFB1 have a limit of 0.01 ppb in baby and dietary foods. The limit of AFM1 in diary product is 0.05 ppb. The AFL maximum level of tree nuts, oilseeds (not for crushing), and cereals were amended from 4 to 10 ppb in the Commission Regulation No. 165/2010 (Siegel and Babuscio, 2011). Table 1.

Products	EC 1881/2006		EU 165/2010	
	AFB1 (ppb)	AFLT (ppb)	AFB1 (ppb)	AFLT (ppb)
Tree nuts ready to eat	2.00	4.00	02-12	4-15
Tree nuts for further processing	5.00	10.00	12.00	15.00
Oilseeds ready for eat	2.00-8.00	4.00-15.00	2.00-8.00	4.00-15.00
Oilseeds for further processing	8.00	15.00	8.00	15.00
Cereals ready for eat	2.00	4.00	2.00	4.00
Cereals ready for further processing	5.00	10.00	5.00	10.00
Baby food	0.10	0.05	0.10	0.05

Tab. 1. Variation of Maximum levels of aflatoxin B1 (AFB1) and total aflatoxins (AFLT) tolerated in the UE from 2006 to 2010.

In general regulations indicate also official procedures for sampling and analytical methodology. In particular, since aflatoxigenic fungi and AFL have an inhomogeneous distribution in commodity, the sampling phase is the major source of uncertainty in AFL analysis. Therefore to minimize the error it is important to adopt harmonized sampling schema. Analytical procedures are important in determining and quantifying AFL. Actually various techniques are available, some chemical, such thin layer chromatography and liquid chromatography, other immunochemical, such enzyme-linked immune-sorbent assay (ELISA) and immune-affinity column assay (ICA). Although chemical analyses are the most accurate, the immune-based kits are often preferred for their rapidity and cost.

The MERCOSUR have established common sampling procedures and analytical methods. In the USA, the US department of Agriculture in collaboration with the Federal Grain Inspection Service published the "Aflatoxins handbook" with the indication of sampling plan and analytical methods for grains following the United States Grain Standards Act (USGSA). The Canadian Grain Commission (CGC) has issued an Official Grain Grading Guide, which contains Standard Procedures for Grain Inspection. In the EU methods of sampling and analysis for the official control of mycotoxins, including AFL, are laid down in Commission Regulation (EC) No 401/2006 as amended by Commission Regulation (EU) No 178/2010.

Since AFL regulation has not only the purpose of increase the safeguard of public's health, but also to preserve the economic interests of producer and traders, the existence of different standards can be considered a trade and socio-economic concern because can determinate trade limitations or can lead in international controversial or trade disruption (Wu, 2004). For example the strictness of UE regulation and the adoption of principle of precaution have been highly criticized by international economists (Dohlman, 2003). Authors accused the EU regulation to have affected international trade and have had a negative impact in economies of third country, including African (Kellerhals, 2000; Otsuki et al., 2001), Asiatic countries (Dohlman, 2003) and USA (Wu, 2008).

Main obstacles to have a harmonized regulation are the wide variation in AFL levels in commodities worldwide, and the relative ability of different countries to reduce contaminations in a cost-effective way (Wu, 2004). In fact, differences in tolerated levels can be attributed more to specific environmental

and economic conditions than to different perception of danger. In general the highest levels tolerated are found in countries where AFL are more diffuse in nature and/or poor subsistence farming is not able to prevent contamination. On the opposite more strict standards are found in some developed countries, where farming and health conditions are optimal and AFL contaminations are episodic (Wu, 2008). Considering the worldwide heterogeneity of climatic and environmental conditions and also the non-reduction of economic and technological gap between developed and undeveloped counties is difficult to imagine the adoption of worldwide harmonized standards.

1.1.5. The impact of AFL in corn

Corn is the most produced cereal in the world (FAOSTAT). Differently from the other two major cereals, rice and wheat, which have principally a food destination, corn grains have different uses: human food, animal feed, energy and industrial products. Although corn is used primarily to feed livestock (60%), in developed countries it is also processed into a huge amount of food and industrial products, including sweeteners, oil, beverage and industrial alcohol, starches, adhesives, chemicals, explosives and fuel ethanol (USDA Statistics 2011). As show in Fig. 5., corn is also the staple food for more than 900 million people in 94 developing countries in tropical and subtropical areas of Africa, Asia, and Latin America (CYMMIT 2011).

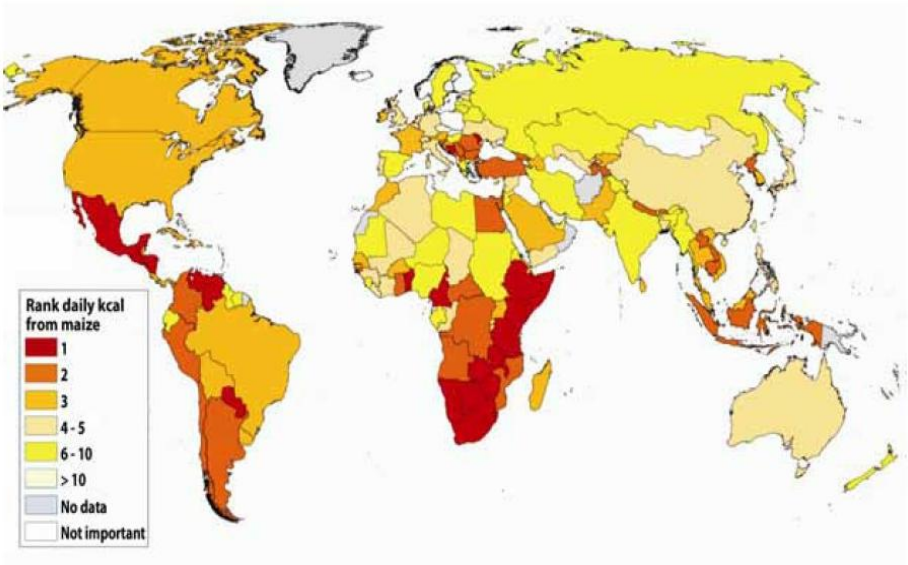

Fig. 5. Relative rank of corn as food crop worldwide. From CIMMYT 2011

Statistics indicate that U.S.A. are the first producer of corn, in 2010 they grew 40 percent of the world's corn, producing 317 million metric tons. Other major corn producing countries in 2010 included: China (177 million metric tons), Brazil (57.5 million metric tons), European Union (56 million metric tons), Argentina (22.5 million metric tons), India (21.2 million metric tons) and Mexico (20.6 million metric tons) (USDA Statistics 2011). In 2003–08 maize production increased annually by 6.0% in Asia, 5.0% in Latin America, and 2.3% in sub-Saharan Africa (FAOSTAT 2011), (Fig. 6). The U.S.A. is also the largest market of corn and is the first exporter. In 2010, the per capita consumption of corn in the U.S.A. was 2,074 pounds per person and the export was of 45.5 million metric tons accounting for 53.8 percent of world corn exports. In the same year other major corn exporters were Argentina (15.0 million metric tons) and Brazil (11.0 million metric tons). Major corn importer are Japan 15.5 million metric, Mexico, Korea, EU 27, Taiwan, Egypt, Colombia and China. (USDA Statistics, 2011).

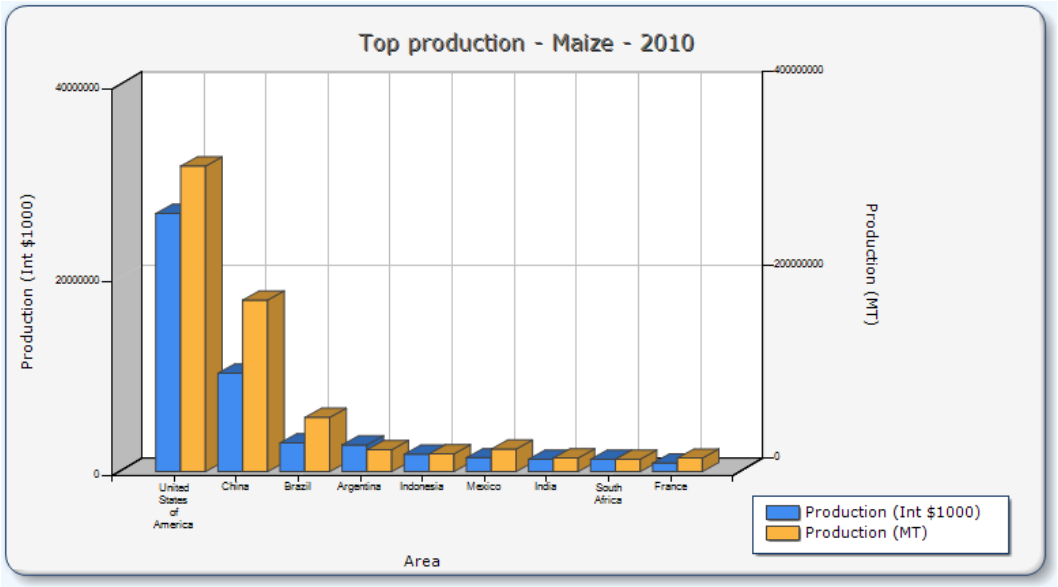

Fig. 6. Corn top production 2010. From FAOSTAT 2010

Corn cultivation is really important in Europe. In 2008/2009, grain corn was the third cereal crop produced in Europe 27 with 57.78 million of tones, following wheat (138.95 Mt) and barley (62.06 Mt). In Europe 27 grain corn is principally used as animal feed but it is also employed for industrial products and little have human destination. Due to the climatic and physiological needs corn is cultivated only in few EU geographical regions and is almost absent in northern

States as Finland, Sweden, Ireland, the United Kingdom, Denmark, Estonia, Latvia. France is the largest EU-27 grain corn producer and in 2008/2009 accounted for 26 % of EU corn production. Romania is specialized in corn production and has the largest surface area dedicated to corn. Yield in this region used to be low, but between the 2007 and 2008 its production doubled and in 2008/09 become the second producer with 14 % of production. The other two major corn producing countries are Italy (13%) and Hungary (12%)(EUROSTAT, 2011).

Corn constitutes the 25% of cereals cultivated in Italy. Corn productions are concentrated mainly in the northern regions of Veneto, Lombardy, Piedmont and Friuli, which together produce more than 60% of all Italian corn. More than 85 % of Italy's corn supplies are consumed by the livestock sector, remnants are utilized to make starch (8-9%), food products (around 3%), seeds (0.3 percent) and is used in biogas plants (0-3%). Italy imports about 2 MMT of corn annually from other EU-27 Member States, mainly Hungary, and from other Eastern European countries (ISTAT, 2011).

Corn is susceptible to many pests and diseases which can cause significant yield and quality losses. Particularly concerns are ear rots diseases caused by *A. flavus* and *Fusarium verticillioides*. Is estimated that up to 44% of corn cultivated in tropical and subtropical area is lost due to ear rot. Ear rot cause grain quality reduction and mycotoxin contamination, especially of AFL and fumonisins (CYMMIT, 2011). Above all, AFL contamination of corn is one of greatest worldwide concern since these compounds are toxic, carcinogen and teratogen for humans and animals (IARC, 2002). Ingestion of contaminated corn is the first cause of acute and chronic aflatoxicosis both in humans and animals.

AFL contamination of crops has a huge socio economic impact and several study tried to calculate the cost of AFL management worldwide. According to Wu (2008), factors determining the AFL impact can be separated in two main groups: marked-related and health-related. Marked related factors are all element directly and indirectly related with trade and affect developed, developing and underdeveloped countries. For example the diminishing returns linked with the depreciation of a product containing exceeding AFL or the total miss of profit due to the calling in are both direct marked related factors. Between the indirect factors there are, for example, these due to AFL prevention and management

(research, agricultural and storage practice, food inspector, the sampling operations, the creation public and private laboratories to do analysis, analysis costs). Clearly, as described above, the final economic impact is strictly linked with the domestic AFL incidence and national and international AFL regulations. For example in the United States the total annual costs of AFL is estimated to be up to $1 billion (Vardon et al., 2003). Robens and Cardwell (2003) estimated that the costs of mycotoxins management in US, including research and testing, are between $500 million and $1.5 billion a year. AFL costs U.S. corn growers over $200 million annually (Abbas et al., 2009). Otsuki et al. (2001) estimated that the adoption of 4 ng/g AFL standard determined a $670 million annual loss to African nations exporting to the EU. Furthermore it was estimated a loss on 63% of trade between Africa to Europe due to the observance of AFB1 EU limits in peanuts instead codex limits (World Bank, 2005). Ten millions of US dollars are annually invested by USA for research on AFL management in underdeveloped countries (CYMMIT, 2011). Differently, health-related costs affect mostly developing and undeveloped nations and are more difficult to evaluate (Wu, 2008). One of the principal problems is the difficulty to diagnose aflatoxicosis. Only in main acute episode a direct connection can clearly be established and much chronic exposure determine "suspect aflatoxicosis" (Kensler et al., 2011). In addition direct and indirect health related costs are difficult to quantify. For example stunting and immune dysfunctions affecting the working population have evident brief term consequences, but the development of poor immune-system in young children occurring in many sub-Saharan nations has long term dramatic economic consequences which are difficult to forecast. A study of Lubulwa and Davis (1994) calculated that the total social costs of AFL in Philippines, Thailand and Indonesia are $900 million. Authors estimated $200 million the combined market losses, the livestock losses in $200 million, and the human health losses in $500 million.

1.2. PRE-HARVEST STRATEGIES TO REDUCE AFL CONTAMINATIONS

Since it was point out the carcinogenicity and immunosuppressive action of AFL, the concern for their occurrence in human foods, as well as in animal feeds, caused the increase efforts to prevent AFL contamination. AFL become subject of multidisciplinary scientific investigation: in one side studies about human pathology and epidemiology increased to better understand the health effects linked to AFL contaminations, in the other side studies concerning the fungal control and analysis of AFL were intensified. Numerous approaches have been used to minimize AFL contaminations, including regulatory strategy, and a variety of pre harvest and post-harvest management techniques.

1.2.1. Agronomic management

Since the studies conducted by Anderson et al., in 1975, which demonstrate that the infections of *Aspergillus flavus* occurred principally in field and not in post-harvest, numerous investigations started with the objective to find the crop management practices which can diminish *A. flavus* infection and the consequent mycotoxin production. In Table 2 are summarized the principal crop management investigated, as reviewed by Abbas et al., 2009.

Strategy	Method	Rationale
Avoidance	Early planting, irrigation and short season hybrids	Reduce heat and moisture stress
Fertility management	Provide adequate nutrition	Mineral deficient corn more susceptible
Insecticide application	Appropriate timing of application	Insect can favorite the ingress of fungi into corn kernels
Hybrids resistant to insect	Select hybrid for natural resistance and produce engineered Bt hybrids	Improve resistance to insect attack
Biological control	Use of non-aflatoxigenic isolates of *A. flavus*	Competitive exclusion
Fungicides	Control phyllosphere fungi	Reduce inoculum density
Soil management	In corporation of crop residues	Reduce inoculum density

Tab. 2. Pre-harvest management practices to reduce AFL contamination. Adapted from Abbas et al., 2009

Avoidance. Drought and high ambient temperatures during kernel filling have been recognized as the most favorable environmental conditions to AFL contaminations in corn since, in one side, they favor growth, conidiation, and dispersal of *A. flavus* and, in the other side, they determine growth and development stress in corn (Wu et al., 2010). Furthermore recently has been demonstrated that the soil temperature has a great influence in *A. flavus* community structure: warm conditions determine higher *A. flavus* population size and higher incidence of the aflatoxigenic strains than cold conditions (Jaime-Garcia and Cotty 2010). Irrigation and early planting are the most studied and adopted agricultural practice to reduce drought stress in corn. Research has demonstrated that corn pollination under adequate humidity and temperatures determine less risk of high AFL contamination. Early planting generally is favorable to these conditions and has determined the decrease of AFL contamination in Corn Belt (Lillehoj, 1978; Jones and Duncan, 1981; Jones et al., 1981). However, as indicated by Bruns (2003), "the key is to time the onset of reproductive growth in corn to environmental conditions that are less stressful to the plant and less favorable to fungal development regardless of the time of the year". The application of irrigation or other means of reducing drought stress, particularly during maturation, is important to reduce AFL accumulation (Jones et al., 1981). However late irrigation can increase AFL infections (Cotty and Jaime-Garcia, 2007). CYMMIT corn geneticists are studying to release drought and poor soils tolerant hybrids. Since control of drought tolerance in corn is complex and polygenic, it is really important to study methods reducing breeding-cycle time. These drought resistant hybrids would be very important globally and not only for the tropical and subtropical Countries where corn is cultivated, (Wu et al., 2010, CYMMIT 2011).

Fertility management. Another important factor which influences AFL contamination of corn is mineral nutrition. Several studies have indicated that nutritional stress may increases plant susceptibility to pathogens. Adequate micro and macronutrients, above all sufficient levels of nitrogen (N), are important in reducing risks of fungal infections in corn and development of mycotoxins (Abbas et al. 2009). However studies about N rate are contrasting. For example Bruns and Abbas (2005) in south US, observed no differences among irrigated early-season corn hybrids grown in the mid-South with respect to AF contamination with N

112 kg ha^{-1} and 224kg ha^{-1}. Differently Blandino et al. (2008) in northern Italy found a negative correlation between N rate and AFB1 contamination in corn in season with conductive climate conditions.

Soil management. Further important elements are the crop rotation and the soil management. Notoriously corn mono-cropping and the no tillage practices increase the *A. flavus* presence in soil since corn debris act as reservoir of propagules (Zablotowch et al., 2007). Recently Jaime-Garcia et al showed also that the corn precession with other culture susceptible to *Aspergillus flavus* influences the size population and community composition of this pathogen: in field experiment conduct in Texas, despite corn was find to favor higher soil populations of *A. flavus* compared to cotton and sorghum, these two crops increased the amount of aflatoxigenic strains (Jaime-Garcia and Cotty 2010).

1.2.2. Insect management

One of the approaches experimented for controlling AFL was the application of insecticides. Although, in some cases, this method was successful for controlling fumonisins and other mycotoxins (Burns, 2003; Blandino et al., 2008; Dowd et al., 1999; De Curtis et al., 2011; Folcher et al., 2009), its efficiency on controlling AFL was mainly inconsistent (Lillehoj et al., 1976.; Anderson et al., 1975; Widstrom et al., 1976; McMillian, 1987; Smith and Riley, 1992; Dowd, 1998 and 2003; Wu, 2007). Generally to have an effective control of AFL several applications of insecticide are needed (Smith and Riley, 1992; Dowd, 2003), this makes this solution not only uneconomic, but also potentially dangerous for the environment. Another problem is the onset of resistant insects: in some Country like Mexico where this practice is vastly used, insect resistance to some insecticides emerged (Rodriguez-del-Bosque, 1996)

Another approach was the use of corn hybrids resistant to ear feeding insects. The identification of natural resistant varieties and the study of insect resistance mechanisms conduct in the last ten years result in the identification of some natural products as indicators of insect resistance in corn. Guo et al. (2001) identified genetic markers and controlling factors of maysin and its analogs. Hemicelluloses and cysteine protease (Penchant et al., 1999), corn ribosomal

inactivating protein (RIP) (Dowd et al., 1998; Williams, 1998) were identified as other important insect resistance mechanisms of some corn inbreds.

Among the resistant hybrid the *Bacillus thuringensis* (Bt) transformed hybrids, which contain a gene encoding for a protein toxic to some insect (mainly Lepidoptera), were the most studied. The hybrids currently available are effective against the ECB (*Ostrinia nubilalis*), SWCB (*Diatraea grandiosella*) and CRW (*Diabrotica spp.)* and CEW (*Helicoverpa zea*) (Wu, 2007). Field tests studying the AFL reduction in Bt corn showed dissimilar results. Windham (1999) reported that Bt hybrids have lower AFL levels than non-Bt isolines in the southern US in years when AFL levels were high, but there was no significant difference when AFL levels were low. Other studies showed a significant effect of Bt corn on reducing insect damages but not in reducing AFL levels (Buntin et al., 2001; Ovoid et al., 2000). Studies in central USA have not found differences in AFL levels between Bt and non-Bt hybrids (Munkvold et al., 2000; Maupin et al., 2001). In contrast studies conducted in Mississippi Delta, where natural *A. flavus* infection is common, have demonstrated some reductions in AFL in BT hybrids, but not in a consistent way (Windham et al., 1999). Williams et al (2005), however, to explain this variability, highlighted the importance of inoculation technique in testing the resistance of Bt hybrids to AFL. Authors reported that using a non-wounding technique Bt hybrids showed a lower infestation of SWCB and a significant reduction on AFL accumulation, in the opposite, using the wounding technique Bt hybrids showed AFL content like isolines. Authors indicate that wounding technique can mask benefit of Bt hybrids (Williams et al., 2005).

Considering the high complexity of factors involved in *A. flavus* infection and AFL production, it is not surprising that the use of insect control techniques to mitigate AFL contamination has given often contrasting results. All studies reported above, indicate that also for insect resistant hybrids other factors, as temperature, humidity and drought stress, are important in determining AFL contamination. In addition, although scientists focused on the ear-feeding Lepidoptera insects considering them one of the important factors for AFL contamination in corn fields at pre-harvest (Widstrom et al., 1975; Magg et al., 2001), some studies showed that other insect uncontrolled by the current insect resistant hybrid had a higher correlation with AFL still in field (Barry et al., 1985,

Mac Millian, 1987; Xinzhi et al., 2011). Current researches with the aim of contrasting AFL occurrence in corn are focused in developing hybrids resistant to corn weevil and other coleopteran (CYMMIT 2011).

1.2.3. Aspergillus flavus control

Various control techniques have been proposed over the years for controlling *A. flavus* infections in crops, including the use of fungicide, resistant hybrids and biological control.

The use of fungicide in vitro and in field conditions has shown variable/inconsistent results. For instance, in vitro applications of the synthetic fungicide Chlorobenthiazone showed high inhibition of AF biosynthesis in *A. flavus* but not in *A. parasiticus* (Abbas et al., 2009). In a 3-year study conducted in the Mississippi Delta, none of five fungicides, namely azoxystrobin, pyraclostrobin, propiconazole, teraconazole, dithiocarbamate were effective in reducing AF contamination (Abbas et al., 2009). Since benefits from fungicide use are not certain, and because for Aspergillus field treatments are used the same active ingredient used in human and animal pharmaceuticals the use of foliar application of fungicide to prevent AFL it is not recommended (CYMMIT, 2011)

Traditional plant breading methods and molecular biology approaches have been used to develop hybrids resistant to aflatoxigenic fungi. Since resistance to AFL contamination involves multiple chromosome regions and several genes, the selection of resistant hybrids with good agronomic characteristics has been slow due to difficulty in identifying coordinately expressed genes and proteins involved in resistance (Brown et al., 1991).

Among the different strategies that have been adopted to manage AFL contamination in corn, one of most interesting is based on a biological control approach. Biological control is defined as the use of living being (plant, animals, insects, bacteria, fungi, and yeasts) or their metabolite to manage or destruct undesired pathogens or pests. Biological control agents can be natural enemy of the undesired organisms acting as predator, parasite or super parasite, can produce substances toxic to the target organism or can compete for the same ecological niche determining the competitive exclusion of the undesired living being (Cotty, 1990). Since late eighty numerous microorganisms have been tested for their

ability on reducing AFL contaminations. The most studied were bacteria, yeast and non-toxigenic strains of Aspergillus spp. (Dorner, 2004)

Although in several laboratory experiments antagonistic bacteria and epiphytic yeast have shown promising results (Smith et al., 1990; Misaghi et al., 1995; Hua, 2002) field applications were mostly ineffective (Dorner and Cole, 2002). Authors attributed the scarce effectiveness of bacteria and yeast in field to their sensitivity to the environmental conditions related with AFL contaminations (hot and dry) and to the application system (Dorner, 2004). The most successful technique in reducing AFL contaminations is the application of non-toxigenic strains of *A. flavus* and *A. parasiticus* to soil of susceptible crops. As mentioned above, even though most *A. flavus* and *A. parasiticus* isolates produce AFL or other toxics, there are naturally occurring isolates that produce none. Following the discovery that AFL are not implicated in Aspergilli pathogenicity first investigation about the use of non-toxigenic strains of *A. parasiticus* and *A. flavus* as biocontrol agents started (Cotty, 1994). Since the initial studies, the potential of non-toxigenic isolates of *A. flavus* and *A. parasiticus* in reducing AFL was demonstrate both in vitro and in semi field conditions. Ehrlich (1987) in laboratory showed that co-inoculation of a mixture of aflatoxigenic and no aflatoxigenic mutant in corn cob significantly reduced the AFL contaminations compared with the inoculation of aflatoxigenic strain alone. Similarly, experiments conducted under controlled conditions indicated that co-inoculation of a mixture of aflatoxigenic and non-aflatoxigenic strains in cotton bolls resulted in a lower content of B1 at maturity compared with the treatment in which the AFL-producing strain was inoculated alone. The inoculation of non-aflatoxigenic strain 24 hrs before the application of the toxigenic one resulted in no detectable AFL at maturity (Cotty, 1990 and 1994).

Application of *A. flavus* and *A. parasiticus* aflatoxigenic strains showed good results also in field studies reducing significantly AFL contamination.

Field experiments conducted in Southern USA, demonstrated that spray application of a non-aflatoxigenic strain of *A. parasiticus* to soil reduced pre harvest AFL contamination in season favorable to AF synthesis in peanuts. In south US subsequently was demonstrated that the application of grains coated with non-toxigenic strains of *A. flavus* in field peanuts determine the competitive exclusion of toxigenic ones and the consequent reduction of AFL both in pre and

post-harvest conditions (Dorner et al., 1992; Dorner and Cole, 2002; Dorner et al.,2003; Dorner, 2008). In Australia the biocontrol technique in peanuts has been evaluated since late 90's (Dietzgen, 1999). Recently application of non-toxigenic strains reduced the AFL contamination of peanuts up to 95% (Pitt and Hocking, 2006).

Working with corn, Brown et al. (1991) demonstrated that the co-inoculation either the inoculation of non-aflatoxigenic strain 24 hrs before the application of the toxigenic one resulted in a significant reduction of AFL levels. Dorner et al. (1999), tested non-aflatoxigenic strains of *Aspergillus flavus* and A, *parasiticus* during tree crop years to determine the best biocontrol agent. Inoculation produced a significant increase in the total *A. flavus/parasiticus* soil population in treated plots. In the years when weather conditions favored AFL contamination corn was predominately colonized by *A. flavus* which determinate an AFL reduction up to 66%. Recent studies conducted in Southern USA showed that grain colonized with non-aflatoxigenic strains of *A. flavus* reduced the AFL contamination in corn up to 80% (Abbas et al., 2006 and 2008). In Africa Atehnkeng et al. (2008) in field experiments evaluated the ability of eleven Nigerian *Aspergillus flavus* isolates to reduce AFL contamination in corn. B1 and B2 concentrations in corn grains were significantly lower in the co-inoculation treatments compared with the inoculation of aflatoxigenic isolate alone, with relative levels of AFL B1 and B2 reduction ranging from 70.1% to 99.9%.

Although the competitive exclusion is a promising approach in controlling AFL contaminations, there are some important factors which can influence the effectiveness of this technique.

A critical factor is the selection of a stable non toxigenic strain which is also competitive in field, and preferably persistent (Pitt and Hocking, 2006). Other important factors are the method of application and the formulation. In the earliest investigations suspensions of homogenized culture of *A. parasiticus* and *A. flavus* were applied or sprayed on soil or to plants (Brown et al., 1991, Dorner, 1992). Even though this method was effective, because *A. flavus* is a human and animal pathogen, these solution are considerate not safe (Pitt, 2000). Then, since in 1994 Cotty demonstrate that the application to soil of wheat seeds inoculated with non-aflatoxigenic fungi was more effective for deliver *Aspergilli* propagules, several studies started to find the most effective carrier/substrate. The delivery system

using a carrier /substrate is indeed the best way to distribute Aspergilli propagules on soil: the carrier /substrate supply nutrient to the hexogen strain, and favor its growth, sporulation and dissemination giving it a competitive advantage among native aspergilli. A suitable carrier /substrate formulation should be easy to make and apply, cost effective, safe for humans and animals, resistant to transport and have a long shelf life. Grains inoculated using the solid state fermentation are at present the most used kind of biocontrol formulation. The most used grains are wheat (Bock and Cotty, 1999, Abbas et al., 2006) but in some studies were used also rice (Dorner, 2004) and millet (Pitt and Hocking, 2006). This technique has the advantage to be cost effective and doesn't need high technology supply, but has the problem that can deliver undesired pathogen with seeds. Other studied technologies to make biocontrol formulations were the alginate process and a modified process for making pasta. In the first mycelium was entrapped in pellets of sodium alginate enriched with nutrient (Daigle and Cotty, 1995). In the second a gluten matrix inoculated with fungi propagules was extruded and dried. This last granular product was called Pesta (Daigle et al., 1997 and 1998). Both formulations were effective, but the production process was too expensive and discouraged their field use (Dorner, 2004).

A more successful formulation was done by coating grain with non-toxigenic strain of *A. parasiticus* and *A. flavus* by suspending conidia in vegetable oil and diatomaceous earth (Dorner, 2002). This process has the advantage respect to the solid state fermentation that the preparation process, which doesn't require sterilization and incubation of the grain, can be made using seed coater. Then granules can be distributed using the traditional granular application equipment. Among the different seeds, the use of coated hulled barley was the most effective (Dorner, 2002), this formulation received the EPA registration as biopesticide for use in peanuts in 2003 (Dorner, 2004) and sooner an American private company started the commercialization of this product under the commercial name of Aflaguard. In 2009 this product received the EPA authorization for being used in corn and in 2010 and its license has been acquired by Syngenta Crop Protection (Accinelli and Abbas, 2011). In more recent years, other formulation have been proposed, including a clay based liquid formulation (Lyn et al., 2009) and a bioplastic based granular formulation (Accinelli et al., 2009 and 2011). This latter formulation consists of bioplastic granules entrapping *spores* of the non-toxigenic

strain NRRL 30797(Accinelli et al., 2009; Accinelli and Abbas, 2011). In this formulation spores of the biocontrol isolate NRR30797 are entrapped into the commercial starch-based bioplastic Mater-Bi Novamont spa). Once hydrated spore rapidly germinate and fungus grow rapidly using the bioplastic material as source of nutrient by using its ability to hydrolyze starch, and then start to colonize the soil (Accinelli 2009). Laboratory studies showed also the safety of this formulation for human and animals and a shelf life comparable whit Pesta (Accinelli 2009). The bioplastic matrix then gives the advantage to this formulation to not contain hexogen DNA. Bioplastic formulation was also tested during 2 years field studies showing a rapid and intense replacement of indigenous Aspergilli and satisfactory control of AFL contamination (Accinelli et al., 2012).

1.3. THE ROLE OF INSECTS IN SPREADING *ASPERGILLUS FLAVUS* INFECTION AND AFL CONTAMINATION

Insects can reduce corn yields by feeding roots (i.e. rootworms, wireworms), leaves (e.g. aphids, armyworm, stem borers, grasshoppers), stalks (e.g. stem borers, termites), ears and tassels (e.g. stem borers, earworms, adult rootworms and armyworm), and grain (e.g. grain weevils, grain borers) (CYMMIT 2011). In addition they can cause also qualitative damages by spreading fungal infection. As reported above, several studies have demonstrated the role of insect, and especially of ear feeding insect, on contributing to spreading Fumonisins and other toxins, however the role of insect on contributing to AFL contaminations is unclear.

1.3.1. The European Corn Borer *Ostrinia nubilalis* Hübner

Ostrinia nubilalis (Hübner), a noctuide Lepidoptera native of Europe known also as European Corn Borer (ECB), is the most studied corn borer. Although ECB have a huge plant host range, in literature are reported more than 223 plant host including sorghum, cotton, potato and pepper, corn is its main host (Magg et al., 2001). ECB is the most damaging corn insect in Europe producing an estimated annual loss ranging from 5 to 30% (Magg et al., 2001). It is

estimated that the ECB cause annually economic losses for more than $1 billion throughout the North America (Mason et al., 1996).

O. nubilalis is diffused through all the European Continent, in North Africa and in Northern America, following its accidental introduction in early 900. Although the presence of this insect have been reported also in some Asiatic regions, we have not idea about its real diffusion in Asia because of this species can be easily misidentified with the native Ostrinia, *O. furnacalis* (Asian corn borer).

ECB has a variable voltinism; the number of generations in a year can vary from one to six depending on strains and climatic zones. Other a variable voltinism ECB has also a variable pheromone sex communication. ECB females' sexual pheromone is a blend of Z11- and E11-tetradecenyl acetate (14: Ac). Two separate sex pheromone races have been identified: ECB (Z) which produces a mix of Z11-14: Ac: E11-14: Ac 97:3 and ECB (E) which produce a 1:99 ratio of the Z and E isomers. Males respond specifically to their race mix. A hybrid race, the EZ 50:50, have been also identified (Klun and cooperators, 1975).

As other Lepidoptera ECB life cycle go through four development stage: egg, larva (borer), pupa, and adult (moth). Larva has five different growth stage or instars. ECB larvae produce damages by feeding and tunneling on epigean corn tissue (leaves, tassel, ear, ear shank and stalk). Tunnels reduce the stalk mechanical resistance, interfere with the translocation system and make the plant susceptible to breakage. Late-stage larvae produce also cavities into the ear shank determining sometimes the fall of the ear to the ground before harvest. The borer overwinters as a full grown fifth instar larva in corn stalks, cobs, and plant debris. Temperature and humidity are critical factor on ECB population dynamics. Temperatures influence the ECB development and are the principal factor to determine the voltinism. In Italian corn grown regions ECB is present as the ECB E and also Z race and shows a bivoltine life cycle whit, sometimes, a partial third generation (Krumm et al.,2008; Pena et al.,1988). The borer overwinters as a full grown fifth instar larva in corn plant stalk and debris. Mature larvae break diapause in spring and adults appear on the end of April-beginning of May. Moths fly during night when temperatures of 13-14 °C occur. Mates occur in about 10 days in field edge. Usually adults mate between the night of emergence and the 2-3 subsequent nights of adult life. Eggs are usually laid on the lower leaf surfaces

in egg masses, and after an incubation of 6-10 days eggs hatch. The sex ratio is 1:1 and have been recognized a slight protandry. The duration of cycle development is influenced by temperatures. First brood ECB characteristic injuries are leaf "shot hole" injury in mid-whorl stage corn. Usually the second generation of adult flays the first decade of July. The mate and the egg deposition occur until the end of July and at the end of summer 5th larval instar diapauses in stalk tunnel. In years characterized with a high temperatures and humidity a third generation can occur at the end of August- beginning of September. The feeding and tunneling activity of the second generation larvae can cause heavy plant damages and are responsible of yield losses (Maini et al., 1978). For managing ECB are used different techniques ranging from cultural methods, distribution of insecticides and biocontrol agent (Vasileiadis et al., 2011). The removal of corn debris by shredding or ploughing in autumn or early spring is used to reduce the number of emerging adults in spring; however adult moths can arrive from neighboring areas. Insecticides are worldwide used. It is estimate that in the European Union, between 0.7 and 0.9 million hectares are treated against corn borers (Vasileiadis et al., 2011). The main problems linked with the insecticide use are the timing of application, the high cost of treatment and the environmental adverse effects. Chemical treatments against ECB larvae are effective only if applied before those larvae enter in corn stalk. Since the ECB cycle is strictly influenced by environmental conditions, the treatment is influenced by weather and require special devices which are owned by few operators, often is difficult effectuate well timed treatments. Insecticide treatments are also expansive, in Emilia Romagna Region the machine rent cost is about 30-40 €/ha and chemicals costs 30-50 €/ha (CAIP, personal information). Furthermore insecticide used belonging from oxadiazine, pyrethroid and organophosphates chemicals, products known to have adverse effects on non-target organisms including pollinators and natural enemies.

The main microorganisms used in the biological control are: the fungus *Beauveria bassiana*, the hymenoptera parasitoid Trichogramma spp., the protoza *Nosema pyrausta*, the *Bacillus thuringiensis-kurstaki* crystal-like proteins (Cry).

Corn variety have been selected for the resistance to ECB, in particular transgenic corn variety expressing the *Bt Cry proteins (*Cry1Ab, Cry1Ac, Cry9C) have shown a god resistance to ECB feeding activity.

In Italy field treatment are authorized only against the 2nd generation of ECB. To destroy overwinter larvae it is recommended the stalk shredding or burying. The Emilia Romagna IPM regulation permit only a treatment/year with pyrethroids (alpha cypermethrin, cypermethrin, deltamethrin, cyfluthrin, lambda cyalothrine, cypermethrin, indoxacarb) and the biological control using *B. bassiana* formulations (Emilia Romagna Region, 2011).

1.3.2. Could *Ostrinia nubilalis* and other ear feeding insects contribute to spread *Aspergillus flavus* infections and AFL contaminations in corn fields?

As indicated above, *A. flavus* is an opportunistic pathogen. Adaptation and restriction to a specific host is in some extend related with lack of a biosynthetic pathway which is supplied by the host (Scully and Bidochka, 2006). In addition, several fungi became specialized pathogens by acquiring the capability to produce host-selective toxins (Scheffer, 1991; Wicklow, 1988). Despite the genetic diversity within the *A. flavus* group, populations of *A. flavus* have not diverged into separate pathogenicity types and commonly strains have preserved the biosynthetic patterns which enable them to utilize nutrients from different hosts (Scully and Bidochka, 2006). In addition, although *A. flavus* produces a variety of toxins, including aflatoxins, which have no a direct implication in the infection process and are not host specific (Leger et al., 2000): in fact *A. flavus* -producing - AFL strains isolated from animal tissues are able to infect plants and strains isolated from plant are able to infect animal tissues (Gupta and Gopal, 2002; Murakoshi, 1977; Drummond and Pinnock, 1990; Leger et al., 2000).

In 1920, when AFL were still unknown, Taubenhaus observed a higher presence of insects, specifically *Helotes zea*, the Corn Ear Worm (CEW), in corn infected by black and yellow mold. As it was demonstrated that the most part of insect-isolates of *A. flavus* are able to infect plants the author hypothesized that insect can contribute to spread ear mold disease. Then, since AFL have been described and recognized as public health concern, the attempt to determine the role of insects in AFL contamination increased.

Despite the association of *A. flavus* with ear feeding Lepidoptera has been demonstrated (Anderson et al., 1975; Fennel et al., 1977; Lillehoj et al., 1978;

Accinelli et al., 2008a), the relationship between ear-feeding insect and AFL contamination of crops remains uncertain. For instance, Anderson et al. (1975), in an experiment conduct in the south of Georgia, noted that AFL incidence was higher on areas where ears have been damaged by insects. Similarly, Lillehoj et al. (1976) noticed that occurrence of *A. flavus* and AFL was associated with damages of 2nd generation of the European corn borer (*Ostrinia nubilalis)*. Successively Whindam et al. (1999) and Williams et al. (2005) in two similar experiment found that corn cobs artificially infested with South Western Corn Borer (*Diatraea grandiosella)* larvae and inoculated with *Aspergillus flavus*, showed a significantly increase of AFL levels compared with corn cobs inoculated with the fungus alone. Differently Widstrom et al. (1975) found no evidence of a significant relationship between *H. zea* injury and AFL B1. Furthermore Widstrom in an experiment found contrasting data: in some cases, AFL were detected more in earworm-damaged samples than in insect free ones; by contrast the highest presence of AFL was found in ears with low insect damages (Widstromet al., 1976). As extensively reported in literature, this variability is partly due, to the high dependence of AFL biosynthesis to abiotic factors and plant stress conditions (Fennel et al., 1975; Anderson et al. 1975; Williams 2002, Xinzhi et al.2011). Furthermore, despite for years *A. flavus* was considered exclusively a "wound pathogen", the AFL producing fungi can penetrate actively into corn trough the silk. (Lee et al., 1980; Jones et al., 1980; Marsh and Payne, 1985). In addition, although scientists focused on the ear-feeding Lepidoptera insects considering them one of the most important factors for AFL contamination in corn fields before harvesting (Widstrom et al., 1975, Magg et al., 2001), some studies showed that other insects had a higher correlation with AFL still in field. For example Barry et al. in 1985 found that the corn weevil, *Sitophilus zeamais* (dusted with *A. flavus* spores) can be an effective vector of *A. flavus* in corn fields under warm climatic conditions. Similarly, Mac Millian (1987) showed that corn weevils are more important in causing kernel infection and AFL contamination than Lepidoptera. Wicklow et al. (1998) found that *Carpophilus lugubris* (nitulides beetles) can spread *A. flavus* and contribute to AFL contamination. Rodriguez-del-Bosque et al (1998) -reported that infection by *A. flavus* and AFL contamination of corn were enhanced by ear wounding and incidence of sap beetles like *Carpophilus freemani*, *Cathartus quadricollis*, and

Sitophilus zeamais. More recently Xinzhi et al. (2011) in a multiple-year studies utilizing high resolution grid-sampling technique found that corn weevil infestation were positively correlated with AFL levels in corn grains.

Other studies have been conducted to investigate the direct interaction between *A. flavus* -corn borer and *A. flavus*- AFL. *A. flavus*.is also a generic enthomopathogen fungus: it was found in termites, housefly, mosquito, silkworm, ear feeding insects (ECB, SWCB...), locusts, aphids, coleopteran. Some strains of *A. flavus* are symptomless, while others heavily damage or kill insects. The insect mortality is linked with the ability of the *A. flavus* strain on producing toxins, manly AFL. Aflatoxins indeed are not only toxic to human and animals, but are also a potent natural insecticide (Wicklow et al., 1994; Wright et al., 1982). Due to this characteristic some scientist have hypnotized that AFL can have a negative effect to natural ECB and corn ear insect populations.

In vitro experiments B1 and G1 toxins and a G1 precursor were high toxic on newly hatched larvae and less toxic for 2^{nd} and 4^{th} instars (Jarvis et al., 1984 a and b). The addition of aflatoxigenic and not-aflatoxigenic spores on ECB third instar larvae diet not influence larval mortality and growth, but larvae feed with AFB1≥5 ppm show reduction of weight and an increase of mortality (Accinelli et al., 2008a). Dowd (1998) using *Spodoptera fugiperda and H. zee* as model demonstrated that kojic acid synergizes the toxicity of AFL B1 to Lepidopterian corn pests by inhibiting oxidative enzymes probably involved in aflatoxin B1 detoxification. More recently, Zeng et al. (2006) testing the toxicity of AFL on 1^{st}, 3^{rd}, 5^{th} larvae instars of *Heliotis zea* found that the cytochrome P450 monoxygenase was involved in the AFL metabolism. The changing of P450s activity among instars can explain the major sensitivity of younger larvae. The ability of insects to tolerate *A. flavus* infection and AFL can be interpreted in different ways. Some authors consider insects infected internally but asymptomatically the most important candidates as carriers (Widstrom et al., 1975; Niu et al., 2006) other, however, consider the death of insect a crucial event for the *A. flavus* dissemination: the fungus grow on insect cadavers increasing its inoculum potency (Xinzhi et al., 2011).

The state of the art shows ambiguous result in set up a relationship between occurrence of insects and AFL in corn. As suggested by Xinzhi et al. (2011) it probably brings out the fragmentary approach used in most part of

studies. It would be very important, to better understand the role of insect in carrying *A. flavus* and contribute to AFL contaminations, to considerate altogether the ecological interactions between plant, insects, and *Aspergillus flavus.*

1.4. AIM OF THESIS

As discussed above, aflatoxin (AFL) contamination of corn is either an economical and a food security issue. Although more solutions have been proposed for reducing and to manage AFL contamination, a few of them have produced satisfactory results. One of the most successful pre–harvest control techniques is a biocontrol strategy consisting of using non-aflatoxigenic strains of *Aspergillus flavus*. This approach has been successfully used in the United States. More recently, this approach has been extended in Africa and Australia, but it has never been applied in Europe.

The aim of this work was to investigate the ecology and dynamic of the AFL producing fungus *Aspergillus flavus* and AFL in corn fields of Bologna (Italy). I also investigated the feasibility of a novel biocontrol approach to control AFL contamination of corn. More specifically, the efficacy of a bioplastic-based formulation carrying propagules of the biological control strain *A. flavus* NRLL3077 was evaluated. Other than evaluating the efficacy of this biocontrol approach, I tracked the biocontrol strain over the crop season using a conventional culturing method and a novel bioplastic-based bait system.

The study also included laboratory and field investigations concerning the role of the key-pest of corn, *Ostrinia nubilalis* (European corn borer; ECB) in AFL contamination of corn. We first monitored flights of the ECB, and damages. We also conducted insect bioassay to investigate the toxicity of AFL and *A. flavus* spore on 4^{th} instar larvae of the ECB.

CHAPTER 2. MATERIALS AND METHODS

2.1. BIOLOGICAL CONTROL AND MONITORING OF A. FLAVUS USING BIOPLASTIC-BASED FORMULATIONS

2.1.1. Preparation of bioplastic based formulation delivering non – aflatoxigenic strain of *A. flavus*

The non-aflatoxigenic strain *A. flavus* NRRL 30797, isolated in the Mississippi Delta in 2001, was selected for this study. This biocontrol strain, characterized by producing large sclerotia (diameter > 400 µm), does not produce AFL and cyclopropiazonic acid. Laboratory and field studies conducted in the Delta have shown its efficacy as bio-competitor. (Abbas et al., 2006; Abbas et al., 2008). The fungus was cultured on acidified potato dextrose agar (PDA) and incubated for two weeks at 37 °C. Spores were gently scraped from PDA plates and suspended in aqueous Tween 20 (0.2%) to achieve a spore density ranging from log 5.7 to log 8.7 conidia mL^{-1}. Granules of Mater-Bi grade PE01S (diameter 3 mm) (Novamont S.p.A., Novara, Italy) were inoculated with spores as described by Accinelli et al. (2009). Briefly, bioplastic granules were autoclaved for 20 min at 120 and then transferred to a 500-mL flask containing 300 mL of a spore suspension. After shaking for 4 hours at 300 rpm, spore were further entrapped into granules by applying a pressure of 60 kPa using a piston –like device (Riff98, Italy). Inoculated granules were then dried at 40 °C for 2 hours, cleaned on surface using an air compressor and stored at room temperature (Fig. 7).

Fig. 7. Bioplastic based formulation delivering non –aflatoxigenic strain *A. flavus 30797*

Formulation potency, expressed as number of viable spores entrapped in granules, was determined by plate counting. Briefly, three granules were transferred to a centrifuge tube containing 10 mL of phosphate buffer saline (PBS) and glass beads. Tubes were vortexed for 3 min and then shaken at 300 rpm for 1 h. Suspensions were decimally diluted in PBS and 100-µL aliquots were plated onto modified dichloronitroaniline rose bengal agar (MDRBA) (Abbas et al., 2004). Colonies were enumerated after 7-10 days of incubation at 37 °C.

2.1.2. Field description and experimental design

The field study was conducted at the experimental farm of the University of Bologna (Cadriano, Bologna, Italy) during two consecutive years (2009 and 2010). For each year, experiments were performed in single adjacent 1.5-ha corn fields. Some physico-chemical properties of soil of the two selected fields are reported in Table 3 from Accinelli et al. (2002). Experiments were performed according to a completely randomized block design with three replicates. Each experimental unit consisted of a 600-m^2 area (30 m x 20 m) surrounded by a 10-m wide buffer zone (Fig. 8). Experimental treatments were the following: inoculated bioplastic granules at rate of 15 kg ha^{-1}, 30 kg ha^{-1} and an untreated control (Fig. 9). The corn hybrid Pioneer Hi-Bred PR31K18 was planted on 16 April 2009 and on 12 April 2010. Fields were managed according to ordinary practices of the region. Granules were manually applied on each plot surface at corn growth stage V4 (Ritchie and Hanway, 1982). Corn was harvested on 25 August 2009 and 27 August 2010.

Fig. 8. Experimental field of Cadriano 2010

Fig. 9. Bioplastic based formulation applied on Cadriano soil

Tab. 3. Soil properties of the Cadriano experimental site

Soil depth	Textural class [a]	Particle size (%) [b]			pH [c]	Organic C [d]	CEC [e]	*Ksat* [f]
cm		Sand	Silt	Clay	1:2.5	%	meq 100·g^{-1}	mm·h^{-1}
0– 50	Silty loam	38.0	37.5	24.5	7.96	0.85	17.75	42.2
50-75	Clay silty loam	31.2	40.3	28.5	7.86	0.35	20.02	n.d [h]
74-135	Silty loam	21.2	56.8	22.0	8.38	0.27	17.34	n.d [h]

a According to the classification established by the US Department of Agriculture.
b Determined by the hydrometer method.
c Determined in a 1:2.5 soil: water suspension.
d Determined by dichromate oxidation.
e Determined in accordance with Rhoades (Accinelli et al., 2002).
f Determined by porous plate/pressure apparatus.
h n. d. = not determined.

2.1.3. A. flavus NRRL 30937 soil and kernel colonization analysis

The size of the *Aspergillus flavus* soil and corn kernel population and the relative abundance of non-aflatoxigenic isolates were measured during the two corn-growing seasons. At each sampling time, three surface (0-10 cm) soil samples of about 500 g were randomly collected from each plot. Samples were sieved through a 4-mm sieve and stored at 4 °C until be processed. Soil moisture was determined gravimetrically. *A. flavus* propagules in soil were enumerated by dilution plate technique following the procedure described in Abbas et al. (2004) with minor modifications. Briefly, 10 g of soil were transferred in a 150-mL bottle containing 90-mL water agar solution (0.2%) and glass beads. Bottles were vortexed for 3 min, and shaken for 1 h at 300 rpm. Suspensions were decimally diluted in PBS. One-hundred-ml aliquots were plated onto MDRBA and incubated at 37 ° C for 7-10 days. Ten colonies were randomly selected, sub-cultured on PDA and incubated in the dark at 28 °C for seven days. After incubation, plates were exposed to UV light (365 nm) and AFL-producing isolates were identified as colonies displaying blue fluorescence.

Ten corn ears were randomly collected from each plot for microbiological analysis. After drying at 50° C for 72 h, ears were shelled and a randomly selected number of kernels were surface sterilized and plated onto MDRB agar. After incubation for 7-10 days at 37 °C, kernels showing *A. flavus* infection were

recorded. A selected number of isolates were isolated and exposed to UV light to assess their potential to produce AFL (Abbas et al., 2004).

2.1.4. Chemical analysis for quantifying AFL concentration in corn kernel

At crop maturity, 60 ears were randomly collected from each plot, dried at 50 °C for 72 h and shelled. Kernels were milled (<1 mm) for chemical analysis.

Samples of 30 g of ground corn were used to extract AFL. One-hundred milliliters of methanol/water (70:30) were/was add to the corn samples and shacked overnight. Samples were then centrifuged at 5000 g for 10 min. A 5-mL aliquot of the supernatant was evaporated under N_2 and the residue was reconstituted in methanol/water (70:30). Sample clean-up was done following the method of Sobolev and Dorner (2002) with little modifications. Briefly, an aliquot (800 mL) of reconstituted sample was cleaned by passing-through a 1.5 mL extract-clean reservoir mini-column packed with aluminum oxide (Alltech Co., Deerfield, IL). Twenty microliter of the purified eluate was injected on a HPLC system equipped with a Nova-Pak C18 column (150x 3.9 mm, 4 mm) and a 2475 multi-wavelength fluorescence detector (Waters Inc., Macclesfield, UK). Separation was carried out at 30°C, with a mobile phase made up of water: methanol: 1-butanol (60:25:1) and a flow rate of 0.9 ml min1. Detection of AFL was achieved by setting the detector of wavelength at 365 nm (excitation) and 440 nm (emission). Data are expressed as total of aflatoxins (sum of AFB1, B2, G1 and G2).

2.1.5. Preparation of a bioplastic-based bait formulation

In the present study for facilitating isolation and quantification of Aspergilli, a bioplastic-based bait was developed. More details of this novel approach are described in Accinelli and Abbas (2011). Autoclaved bioplastic rods (diameter 2 mm, length 40 mm) made with MB type ZF03U/A and granules (diameter 3 mm) made with MB type PE01S were used for preparing baits. Briefly sterilized rods and granules were impregnated of sterile MDRB for 3 hrs at 300 rpm and 40°C. Rods and granules were then dried under a laminar flow hood for 2 h and stored at 4 °C until use (Fig. 10)

Fig. 10. Bioplastic granules before (left) and after imbibition with MDRB

2.1.6. Soil incubation studies

A portion of each sieved- (4 mm) –soil-sample collected in 2010 from the field of the biocontrol experiment was used for the soil incubation studies. Twenty five grams (air-dried weight equivalents) were weighed in 50-ml sterilized screw-top tubes and moisture adjusted to the gravimetric water content of -33 kPa. Four rods were placed into each soil sample and tubes were incubated in the dark at 28 °C. After 14 days rods were sterilely removed and treated to quantify DNA of Aspergilli by quantitative PCR (qPCR). Rods were cut in four equal parts and DNA was isolated following the procedure described in Accinelli et al. (2009). Briefly, rod pieces were dried at 40 °C for 2 h under a laminar flow hood, transferred to 2-ml centrifuge tubes, vortexed for 5 min and air- flushed to remove sticking soil particles. A dried rod portion was transferred to a 2-ml micro-centrifuge tube containing 500 ml of CTAB buffer and glass beads and vortexed for 2 min. Micro tubes were incubated at 65 °C for 15 min, after that an equivalent volume of chloroform: isoamyl alcohol (24:1, v:v) was added to tubes. Tubes were gently shaken and centrifuged at 10,000g for 5 min, then 2/3 volume of isopropanol/7.5 M ammonium acetate was added to precipitate the DNA. A selected number of isolates recovered from the surface of bioplastic rods were transferred to PDA plates and used for DNA sequencing.

2.1.7. Kernel incubation studies

The effectiveness of bioplastic baits on selectively isolate *A. flavus* was evaluated by analyzing samples coming from the corn field biocontrol experiment conducted in 2010 with additional 25 corn kernel samples provided by two private laboratories (AGER Bologna and Caip Bologna-Modena), (Fig.11). Twenty grams of dried kernels were weighed in 50-mL sterilized screw-top tubes containing 10 MDRB-imbibed bioplastic granules and vortexed for 5 s. Tubes were then incubated at 28 °C in the dark for 10 days. Six granules were aseptically removed and used for DNA isolation to conduct qPCR analysis. Some isolates (42) recovered from the granule surface were transferred to PDA plates for DNA sequencing as described in the paragraph 2.1.8.

Others granules were used to evaluate the potential of baited isolates to produce AFL. A single granule was transferred to a test tube containing 2 ml of yeast extract sucrose broth and incubated at 30 °C for seven days in the dark without shaking (Fig.12). AFL were extracted by adding an equal volume of chloroform to the culture. Chloroform was then evaporated to dryness under vacuum and residues re-dissolved in 0.6 ml methanol/H2O (70:30, v:v). The mycelia mats were drayed for 48 h at 70 °C to determinate the dry weight of mycelium. The AFL concentration of these samples was measured by using the HPLC method described in the paragraph 2.1.9.

Fig. 11. Corn samples provided by CAIP for incubation studies (left).
Fig. 12. Bioplastic granule in YES tube for determination of ability to produce AFL of baited Aspergilli (right).

2.1.8. DNA analysis of bioplastic granules and rods after baiting

DNA amplification was carried out using the two following primer pair: omtB-F and omtb-R, targeting the AFLcluster gene omtB (aflO) (Kim, 2008). The 25-µL volume reaction contained the following: 2 µL of DNA, 12.5 µL of 2xTaqMan Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA), and 0.2 µM of each primer pair. Thermocycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The resulting samples were analyzed using an ABI Prism 7700 Sequence Detection System (Applied Biosystem Inc.). After quantification, amplified fragment samples were subjected to melting-curve analysis. A standard curve was generated by plotting cycle threshold values (Ct) against logarithmic-transformed amounts of target DNA obtained from 10-fold dilutions of DNA isolated from soil treated with spore dispersions of A. flavus NRRL 30797. A randomly selected number of fungal isolates recovered from the surface of rods and granules used to bait Aspergilli from soil and corn kernels, respectively, were used for sequencing of the internal transcribed spacer (ITS) region of rDNA. After subculturing on PDA, and extracting total DNA, the ITS region was amplified following the procedure described in Accinelli et al. (2008 b) ITS1/NL4. After clean-up with ExoSAP-IT (USB Co., Cleveland, OH), PCR products were sequenced using the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit and analyzed using an ABI 3730XL automated sequencer (Applied Biosystems Inc.). Sequences were aligned using the software DNAMAN (Lynnon Co., Quebec, Canada) and deposited in the NCBI GenBank with accession numbers from HQ844675 to HQ844716.

2.1.9. Chemical analysis for quantifying the potential AFL production of isolated Aspergillus spp.

Chemical analysis were performed using a HPLC Shimadzu (Asia Pacific) equipped with a Phenomenex Synergy C18 column (150x 4,6 mm, 4µ), a post column derivative Kobra cell (Biopharm Rhône, Ltd) and a spectrofluorophotometer RF-5301PC Shimadzu (Asia Pacific). Separation was

achieved at room temperature in 18 min by isocratic elution with a mobile phase of water: acetic acid: methanol (6:2:3) containing 350 µl Nitric Acid 4 M and 120 mg KBr in each liter, with a flow of 0.8 ml/min. Detection of AFL was carried out by setting the post column derivatizer at 100 µA and the detector wave length at 365 nm (excitation) and 440 nm (emission). Quantification was obtained by calibration curves made using 5 standard concentrations (0.2-5 ppb) of B1, B2, G1 e G2 AFL (Supelco). Detection limits were 0.2 ppb for B1 and G1 toxins and 0.6 ppb for B2 and G2. Recovery rate was calculated using a 5 point calibration curve. Analyses were made in triplicate; data were collected separately for each plot and analyzed by ANOVA.

2.1.10. Statistical analysis

Data were subjected to analysis of variance. Mean values were compared using Tukey's HSD test and significant differences were detected at the P= 0.05 level. Soil *A. flavus* populations are presented as log (10) transformed colony forming units (cfu) g^{-1} dry weight.

2.2. STUDY OF RELATION BETWEEN A. FLAVUS AND THE EUROPEAN CORN BORER OSTRINIA NUBILALIS.

2.2.1. Field monitoring of *O. nubilalis* moth population

Size and dynamic of the European Corn Borer moth (*Ostrinia nubilalis* Hübner) was monitored during the two years field studies. Cone traps (baited whit sex-pheromone (ISAGRO S.p.A., Milano, Italy) and phenylacetaldeide PAA (ISAGRO S.p.A) were placed in each corn field edge in May in order to capture both male and female, respectively (Fig.13). From May until September, pheromone was replaced monthly, PAA was changed every two weeks and basket cylinders were monitored weekly. Moths were collected, counted and frozen.

Fig. 13. Cone trap baited with sex pheromones and Phenilacetaldeide

2.2.2. Monitoring of 1st and 2nd generation of ECB larvae and evaluating crop damages

Injuries due to feed activity of 1st and 2nd generation of ECB larvae on corn plant were evaluated in fields. On July and September of each year 15 groups of 5 corn plants were randomly chosen in each plot and were evaluated for the damages due to first and second generation respectively. For the first generation

the damage-incidence was evaluated considering the presence/absence (1/ 0) of characteristic foliar ragged holes (FRH) in each plant. The average of incidence was calculated for each group of plant and data were transformed in percentage. The damage-severity was determined attributing a number to each plant using the following schedule: 0= no injuries, 1= 1–5 FRH /plant, 2 = 6–10 FRH /plant; 3= 11–20 FRH /plant, 4= 21–35 FRH /plant, 5= > 35 FRH /plant. The average of severity was calculated for each group of plant. For the second generation damages incidence was evaluated considering the presence/absence (1/0) of bore on stalks and ears of corn plant. The average of incidence was calculated for each group of plant and data were transformed in percentage. The ECB damage severity was determined considering the number of bore per plant and the percentage of stalk damaged or broken in each plant group due to larvae activity. A scale of 0–5 was used to evaluate each plant following schedule: 0 = no injuries; 1 = 1-3 tunnel /cob, stalk undamaged; 2= more than 3 tunnel/cob, stalk undamaged; 3 = presence of tunnel in cob and stalk, 1-30% plant broken in half or lodged; 4 = presence of tunnel in cob and stalk, 30-60% plant broken in half or lodged; 5 = presence of tunnel in cob and stalk, > 60% plant broken in half or lodged; the average of severity was calculated for each group of plant. Data were collected separately for each plot and were statistically analyzed.

2.2.3. Evaluation of ECB damages on corn cob

At harvest twenty-five corn cobs were taken from each plot and evaluated visually for ECB damages. After drying at 50° C for 72 h, a portion of 10 corn cobs was randomly chosen from each plot to be shelled. We divided each corn cob in 3 portions: apical, median and basal. We considered the apical part as the first 5 rows of kernels from the apex and the basal part as the last 5 rows of kernels (Fig.14). The middle part was the rest of corn cob. Apical, median and basal grains were shelled, counted and weighted separately, dividing sound grains from injured by ECB. These data were used to calculate the damage due to ECB feeding activity (express as loss of weight of corn cob). A portion of corn kernel was evaluated for infection by *A. flavus*. A group of ten grains from the apical, median and basal part of each cob was separately plated onto MDRB dishes and incubated upside-down at 38°C for 7-10 days. Then, the number of kernels

infected on each plate was recorded and the infection of the apical, median and basal part of each corn cob was calculated. Data were statistically analyzed and the correlation between ECB injures and *A. flavus* infections were calculated.

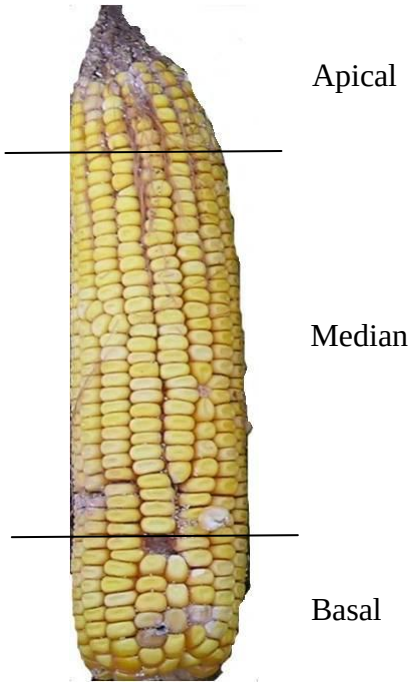

Fig. 14. Corn cob divided in apical, median, and basal part, respectively.

2.2.4. Evaluation of potential toxic effects of A. flavus and AFL on ECB larvae.

The toxicity of AFB1 and *A. flavus* conidia suspension against ECB larvae was evaluated by performing ingestion and indirect contact bioassay. All experiments were conduct in triplicate using forth instars larvae coming from the permanent artificial rearing of our department.

To evaluate the AFB1 toxicity was carried out an ingestion bioassay using a diet surface contamination method. Eight concentrations ranging from 0.125 to 30 ppm (0.125, 0.250, 0.500, 1, 2, 10, 20 and 30 ppm) were used to calculate the LD 50. A 100-µl volume of AFL solution was spread over the diet surface in plastic tubes containing about 1 g of a meridic diet prepared according with Maini et al. (1978). The same volume of EtOH was used in control. One larva was placed in each tube; tubes were sealed with a cotton cover, and incubated at 25°C. The experimental unit was made of 15 tubes. Mortality was recorded at 14 days after treatment (Jarvis et al., 1984). Mortality data were subjected to probit

analysis (Finney, 1971) after correction of mortality with Schneider-Orelli formula (1947).

The aflatoxigenic *A. flavus* strain NRLL 30796 and non-aflatoxigenic *A. flavus* strains NRLL 30797 were tested for their effect on ECB larvae performing ingestion and indirect contact bioassay. The conidia ingestion bioassays were carried out using the same surface contamination method used for the AFB1 test, using aqueous spore suspensions (log 5.7 conidia ml^{-1}) belonging from the aflatoxigenic and non-aflatoxigenic strains. For the indirect contact test three group of 30 of 4^{th} instar ECB Larvae were collected. Each group was left for eight hours into PDA Petri dishes containing a culture of toxic strain of *A. flavus* NRRL 30796, a culture of non-toxic strains of *A. flavus* NRRL 30797, respectively, and an empty Petri dish. Larvae were then transferred into plastic tubes contained untreated meridic diet. The experiment design was a completely randomized bock with 3 replicates. The mortality of larvae was recorded at 14 days after treatment; the larvae that died within the first day were excluded from the test. Data collected were corrected with the Schneider-Orelli formula (1947) and statistically analyzed.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. BIOLOGICAL CONTROL AND MONITORING OF A. FLAVUS USING BIOPLASTIC GRANULES

3.1.1. A. flavus population in soil following the application of biocontrol formulation

Results obtained using the plate-count approach for describing the size and dynamic of the *A .flavus* soil population over the two corn seasons are summarized in Table 4. Data from untreated plots are indicative about the size, composition and trend of *A. flavus* native population during the two crop seasons. A nearly constant level of *A. flavus* propagules during 2009, with an average value of 3.1 log cfu g^{-1} was observed. Similar values were observed during the second experimental year, from May to July. Then, differently to 2009-season, *A. flavus* propagules significantly increased in August 2010. Approximately 50% of the indigenous *A. flavus* isolates were capable of producing AFL in both years, without considerable changes throughout each corn-growing season (Table 4).

	Propagule density (log10 cfu g^{-1})				Aflatoxigenic isolates (%)			
	May[+]	June	July	August	May[+]	June	July	August
2009								
Untreated	3.11 a[*]	3.13 a	3.10 a	3.17 a	38 a	43 a	45 a	47 a
15 kg ha^{-1}	3.09 a	3.35 b	3.64 b	3.69 b	41 a	35 b	27 b	20 b
30 kg ha^{-1}	3.12 a	3.38 b	3.46 c	3.58 c	43 a	37 b	25 b	12 c
2010								
Untreated	3.10 a[*]	3.09 a	3.11 a	3.41 b	38 a	41 a	39 a	37 a
15 kg ha^{-1}	3.12 a	3.39 b	3.42 b	3.67 b	43 a	26 b	23 b	16 b
30 kg ha^{-1}	3.18 a	3.41 b	3.65 c	3.79 c	36 a	29 b	21 b	9 c

[+] Soil samples were collected one day before granules application.
[*] Values within a column followed by the same letter are not significantly different (P > 0.05).

Tab. 4. *Aspergillus flavus* population in soil and percentage of aflatoxigenic isolates recovered from untreated plots and plots receiving 15 and 30 kg ha^{-1} of bioplastic based biocontrol formulation.

Although the contamination of corn with AFL is becoming a serious problem in the Mediterranean areas, little information on the *A. flavus* soil population and the abundance of aflatoxigenic strains in these regions and moreover in Italian soils is available (Accinelli and Abbas, 2011). Investigations carried out in Southern USA showed that the *A. flavus* population level in soils cultivated with corn fluctuate from 2.1 to 5.7 log cfu g $^{-1}$ soil (Abbas et al., 2004, 2009; Zablotowicz et al., 2007; Jaime-Garcia and Cotty, 2010). Our findings are somewhat lower than values found in Delta Mississippi areas (Abbas et al., 2006 and 2008), where AFL contamination are endemic. Some authors reported that the size of the *A. flavus* soil population is influenced by the previous crop, by tillage practices and moreover by climatic conditions (Abbas et al., 2008; Jaime-Garcia and Cotty, 2010). Conditions of high temperature and drought during seed development are considered favorable for AFL contamination (Horn and Dorner, 1998; Scheidegger and Payne, 2003; Abbas et al., 2007). Rainfall near harvest is considered conducive to the growth of the fungus and to the increase of AFL contamination at harvest (Cotty and Jaime-Garcia, 2007). In this study, temperatures and rainfall registered through the 2009 corn-growing season would not stimulate mold growth (Fig. 15). In contrast, rains that occurred in mid-August 2010 are expected to produce the opposite effect. This is consistent with the observed dynamic of the *A. flavus* population in untreated soil during the first year and with its raising recorded at corn maturity in 2010. Research indicated that the abundance of toxigenic strains in soil can vary from region to region and it is influenced by climatic and agronomic conditions. For instance, studies conducted in Southern USA, have indicated that the percentage of aflatoxigenic strains would varying from 25% to 60% (Abbas et al., 2004 and 2008). In South Texas, Jaime-Garcia and Cotty (2010) found that the percentage of *A. flavus* communities belonging to the strain producing AFL was highest in summer. In a survey conducted in the Mississippi Delta area during winter, the percentages of *A. flavus* producing AFL changed over the seasons depending on air temperatures (Abbas et al., 2008).

Back to this study, data from plots treated with bioplastic granules carrying spores of the non-aflatoxigenic strain *A. flavus* NRRL 30797 (potency log 7.0 cfu g^{-1}) (Table 4) indicated the effectiveness of bioplastic granules as carrier, the soil colonization ability of this hexogen strain and the influence of the inoculum rate.

We observed a similar trend in both the experimental years. More precisely, granules application led to a rapid increase of *A. flavus* propagules. In plots treated with 15 kg ha^{-1}, the population, after being increased, was almost stable between June and August. Differently in plot receiving 30 kg ha^{-1} the population increased also during July and was significantly higher in July and August compared with the others treatments.

Furthermore the application of bioplastic granules determined the diminution of aflatoxigenic isolates with an effect of the inoculum rate. At harvest 2009, the mean level of soil aflatoxigenic isolates recovered from plots that received 15 kg ha^{-1} was 20% and the 12% from plot treated with 30 kg ha^{-1}. At harvest 2010 it was 16% and 9% respectively (Table 4). The effectiveness of a bioplastic based formulation in delivering and promote the soil colonization of the strain NRRL 30797 was demonstrated in previous laboratory studies (Accinelli et al., 2009).

These field results indicated that bioplastic granules were an effective carrier for the biocontrol strain NRRL 30797. Moreover our findings are consistent with the efficacy of *A. flavus* NRRL 30797 in reducing AFL contamination of corn (Abbas et al., 2006; Chang et al., 2012).

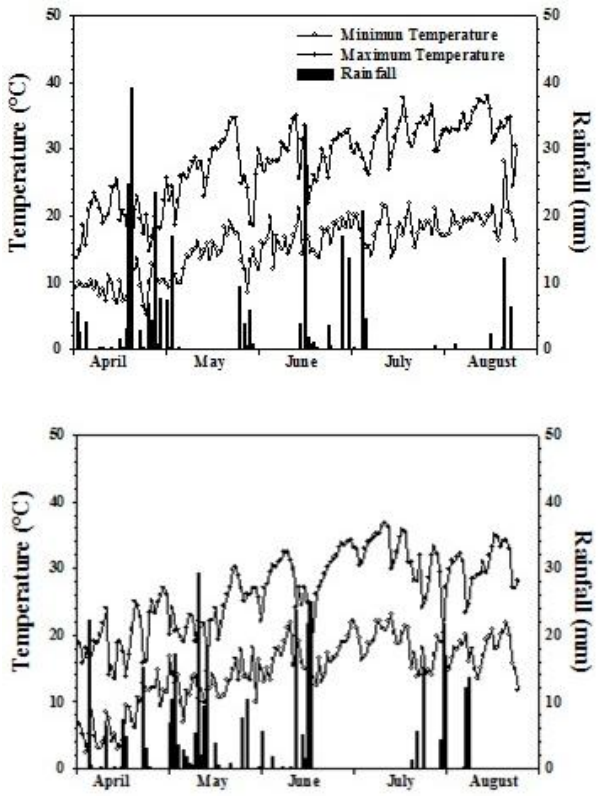

Fig. 15. Meteorological data at the experimental field during the 2009 and 2010 corn-growing seasons.

3.1.2. A. flavus kernel infection

A. flavus infection of corn kernels of ears collected from each plot during 2009 and 2010 harvest is reported in Fig.16. Data show that more than 10% of kernels from each plot were infested with the fungus in both years. In the first year, infection was about 10%, regardless of the treatment. In the second year, infection was higher in plots which received the biocontrol treatments. The infection of corn kernels by *A. flavus* can vary tremendously depending from region, years, and climate conditions. It can range from 0% to 100% (Abbas et al., 2006 and 2009).

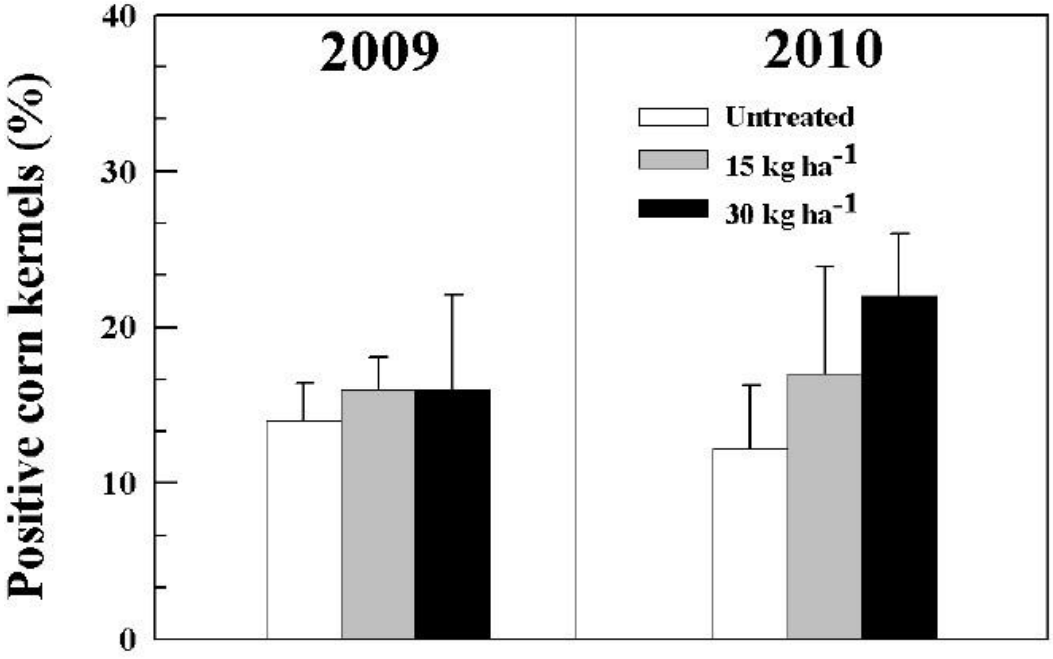

Fig. 16. Percentage of corn kernels infected by A.flavus in 2009 and 2010. Each point represents mean ±STD

Strains isolated from each plot during the two years field experiment were evaluated for their potential to produce AFL. Considering the untreated plots about 75% of *A. flavus* kernel isolates had the potential to produce AFL, with no differences between the two years (Fig. 17).

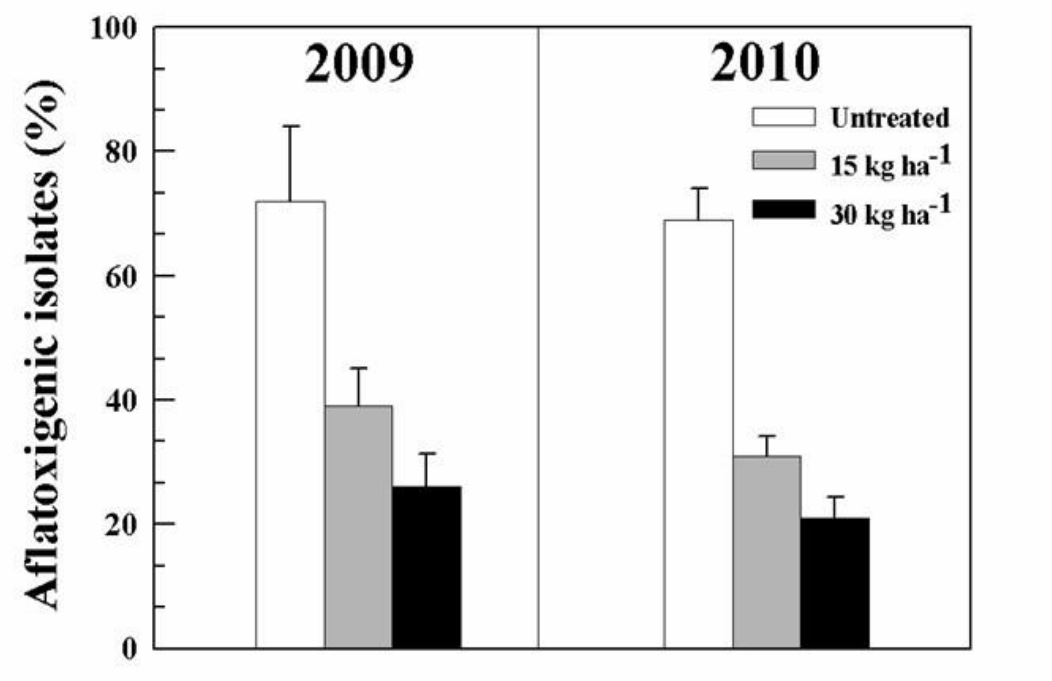

Fig. 17. Relative abundance of isolates able to produce AFL. Each point represents mean ± STD

Recently in a survey conducted in representative corn areas of Northern Italy was found that about the 70% of *A. flavus* strains recovered from kernels can produce AFL (Giorni et al., 2007).

The higher percentage of aflatoxigenic strains recovered from kernels than from soil is in accord to findings of other authors (Abbas et al., 2004 and 2006).

A. flavus NRRL 30797 was effective on replacing indigenous toxigenic strains. In plot treated with 15 kg ha^{-1} of biocontrol formulation this value decreased to 39% in 2009 and to 31% in 2010. In plot receiving the higher application rate of the biocontrol formulation, a decrease of 26% and 21% in 2009 and 2010, respectively, was observed. Our data are consistent with findings of other authors that recorded that this biocontrol strain can reduce significantly the percentage of aflatoxigenic strains isolates from kernels in natural condition and when co-inoculated with a toxigenic strain (Abbas et al., 2006).

3.1.3. AFL contamination of corn

The efficacy of this new biocontrol formulation in supporting the growth and the soil colonization of the biocontrol strain *A. flavus* NRRL 30797, consequently to the competition with indigenous aflatoxigenic isolate, resulted in reduction of AFL contamination in corn. AFL contamination is related with environmental conditions. Fig. 18 shows that the natural AFL contamination was widely different in the two years, with average values of 4.4 and 28.9 ng g^{-1} in 2009 and 2010, respectively. The higher contamination resulting in 2010 is likely explained considering the higher rainfall registered during corn maturation which favorite the mold growth. (Fig.15).

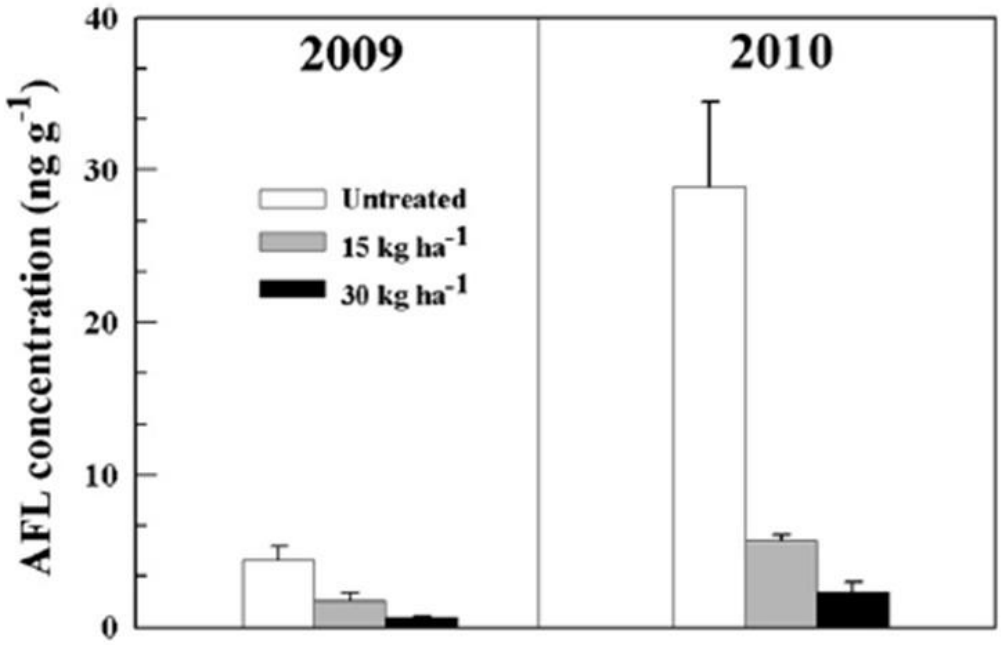

Fig. 18. Aflatoxin (AFL) contamination of corn from untreated plots and plots receiving 15 and 30 kg ha^{-1} of bioplastic based biocontrol formulation. Each point represents mean ± STD

Considering data from treated plot, the first year the average AFL contamination of corn kernels from plots received 15 and 30 kg ha^{-1} of bioplastic granules were 1.8 and 0.6 ng g^{-1}, respectively, the following year were 5.7 and 2.3 ng g^{-1}, respectively. Data shown that this formulation was effective in both years but the contamination reduction was more evident in 2010, when the natural contamination of AFL was higher Data also indicated that the treatment with 30 kg ha^{-1} was more effective on reducing AFL contamination than the treatment with the half rate. The first year we obtained a reduction of AFL of 58% using 15

kg ha^{-1} and 86% using the double rate. The following year the reduction was 80, 2% and 92%, respectively.

Although in literature we find limited information about AFL contamination occurring in Italian corn fields, an high variability between years has been documented (Pietri et al., 2004, Blandino et al., 2008, and Covarelli et al., 2011). As discussed in the introduction, among the numerous strategies that have been proposed to control AFL contamination in corn fields one of more interesting is the biological control achieved by field application of a large number of propagules of non-aflatoxigenic strains of *A. flavus.*

Numerous field studies have demonstrated the effectiveness of the use of small grain seeds inoculated with non-aflatoxigenic strains in reducing AFL contamination in corn and other crops (Cotty 2004; Abbas 2006 and 2009). Nowadays seeds inoculated or coated with fungal spore are the most used biocontrol formulations. Laboratory studies demonstrated that a bioplastic based formulation entrapped spores of the atoxigenic strain NRRL 30797 was effective on carrying *A. flavus* propagules and introduce a stable population of the biocontrol strain in soil (Accinelli et al., 2009). This two years field study was performed to evaluate the effectiveness this bioplastic formulation in managing AFL in corn fields. Results obtained from our study demonstrate that the bioplastic material can be used for making efficacies biocontrol formulation for field application of *A. flavus* strain NRRL 30797. Bioplastic granules are completely biodegradable, compostable and are produced by using renewable sources (mainly corn starch). The production of the formulate, described in materials and methods, is simple, economic and easy to industrialize. A bioplastic based formulation, in addition to their favorable environmental profile, is easy handling and can be applied on field using the conventional spreaders. Furthermore bioplastic is a DNA-free matrix; this characteristic can be useful to assess the quality of formulation during the production phase.

3.1.4. Monitoring of Aspergilli using bioplastic-based baits

Data obtained by using the conventional microbiological methods were confirmed with the novel baiting system proposed. For baiting Aspergilli from soil we used rods made of the bioplastic MB ZFU/A, a more persistent bioplastic

that maintained the physic characteristic after 14 days of incubation, thus facilitating the sample processing. As expected rods imbibed with MDRB were highly selective in recovering Aspergilli: using this system the DNA of *A. flavus* was isolated more in sample coming from treated soil than untreated control. Fig.19

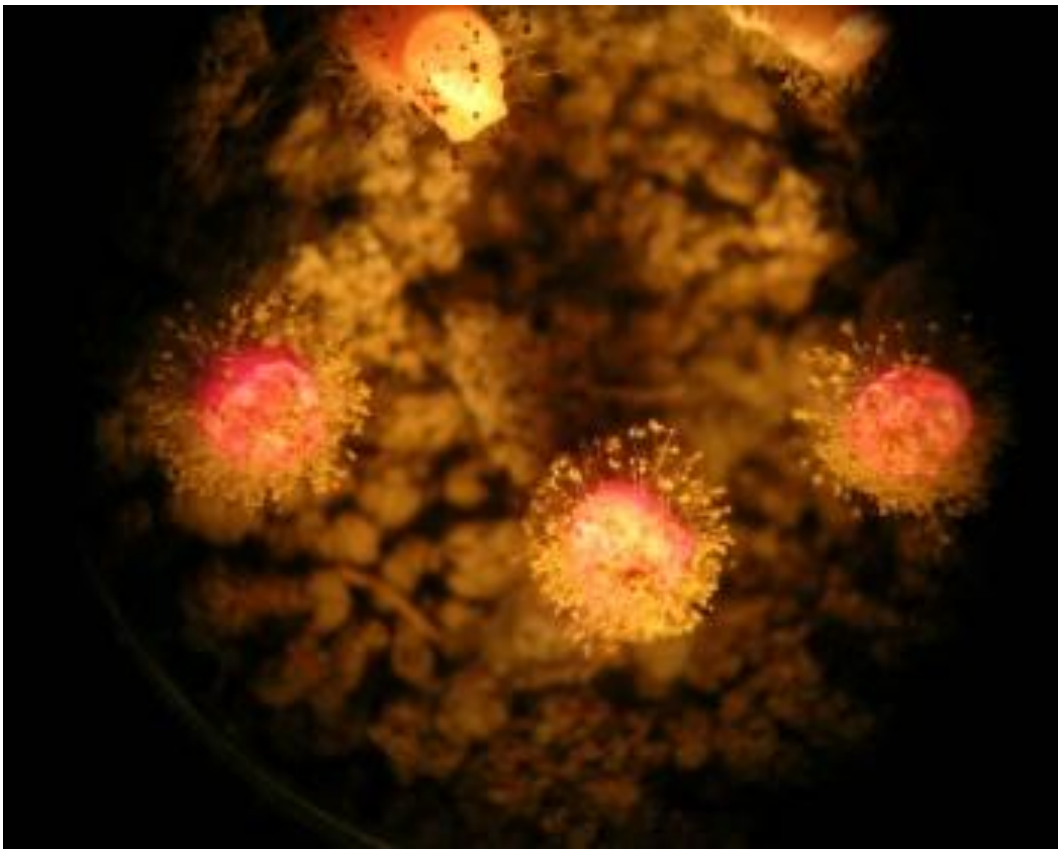

Fig. 19. Bioplastic rods baiting *A. flavus* in a sample of soil treated with 30 kg ha^{-1} of bioplastic based biocontrol formulation.

MB granules treated with selective medium were used to isolate Aspergilli from corn samples. Granular baits were effective in isolating A. flavus from kernels as shown in Fig. 20 the efficacy of this approach was further confirmed by DNA sequences of baited fungal isolates (GenBank accession number from HQ844675 to HQ844716). After basic local alignment search tool (BLAST), more than 80% of the total isolates showed high identity (>98%) to *A. flavus*. Furthermore, from the analysis of all kernel samples we obtained interesting results. Indeed, although the amount of *A. flavus* DNA recovered from granules was weakly correlated with AFL contamination of corn (r= 0.68; P= 0.05), we found a significant correlation (r= 0.89; P= 0.05) between the AFL contamination of corn kernels and the amount of AFL produced by baited fungi (Fig.21).

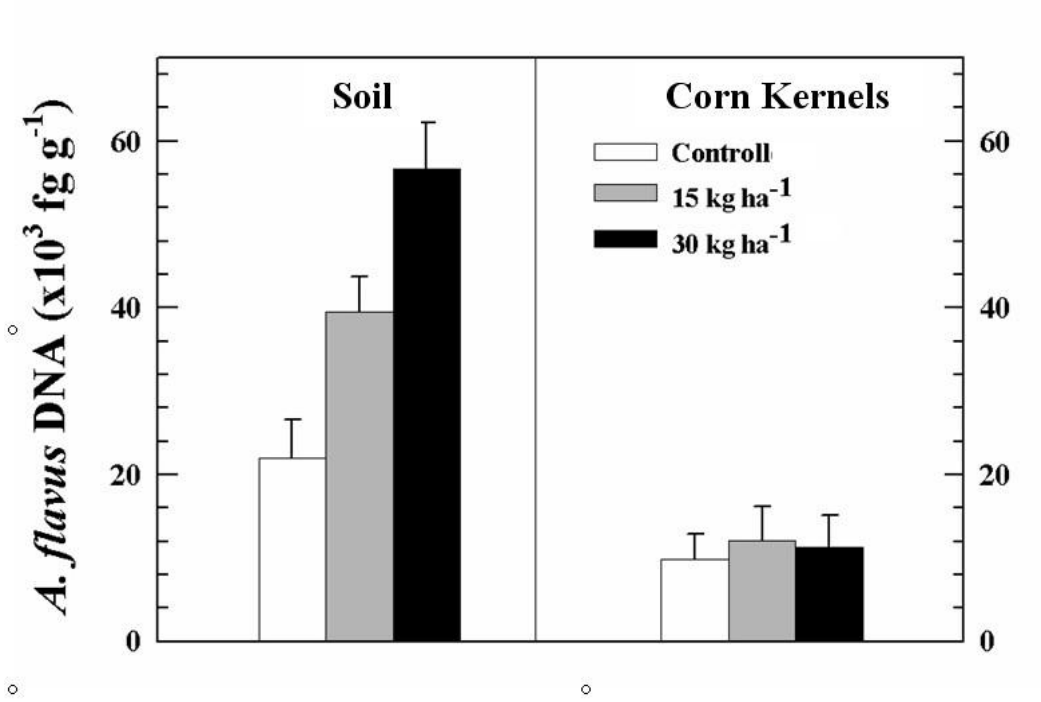

Fig. 20. DNA of *A. flavus* recovered from surface of bioplastic rods and granules incubated with soil (left) and kernel samples (right) collected on August 2010. Each point represents mean ± STD.

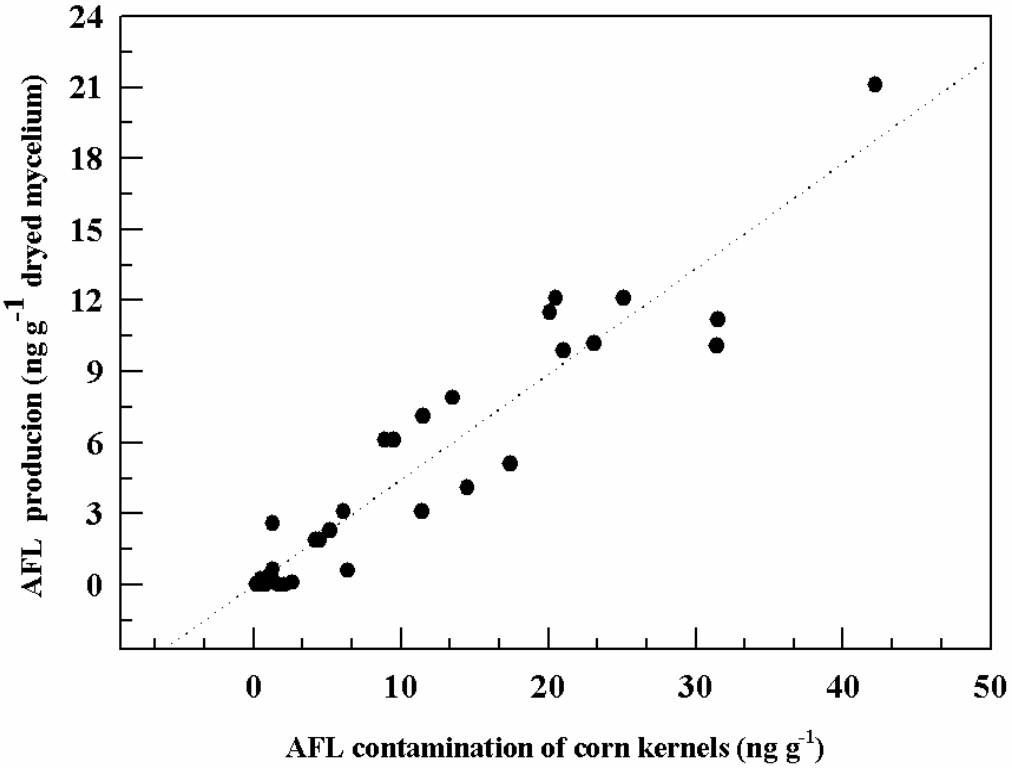

Fig. 21. Scatter plot showing the linear relationship between aflatoxin (AFL) contamination of corn kernels and aflatoxin produced by *A. flavus* isolates baited on bioplastic granules. The linear regression gives a correlation coefficient of 0.89.

Numerous baiting approaches have been proposed for monitoring plant fungal pathogens in soil and other media (Paulitz and Schroeder, 2005; Eguchi et al., 2009). The majority of these methods are based on tracking the target fungus and enumerating its propagules using conventional plate count methods. Some authors proposed some DNA-based method using endpoint PCR approaches (Nechwatal et al., 2001; Matsumoto et al., 2003). Obviously, these approaches would not provide any quantification of the target microorganism. However, in most cases, it is important to know the level of a specific plant pathogen fungus, in this experiment we focused on the development of a DNA quantify protocol. Preliminary results obtained using the baiting approach coupled with qPCR and HPLC analysis, indicate that this system would help with monitoring Aspergilli from soil and corn kernels. AFL level of corn remains almost stable in post-harvest if applied proper storage conditions. Conversely, corn stored inadequately, as often happen in developing countries, can result in additional fungal proliferation and AFL production (Shier et al., 2006). In these cases, the availability of a rapid device for monitoring the AFL risk would be helpful.

It is widely demonstrated that conventional baiting materials, such as autoclaved cereal seeds, are commonly contaminated by a large number of microorganisms which can result in false-positive samples. Differently our bioplastic rods or granules are composed of DNA-free matrices. After extrusion at high temperatures (>160 °C), rods and granules were subjected to a prolonged autoclaving process thus further reducing the occurrence of accidental contamination.

Moreover, the correlation found between AFL contamination of corn kernels and the amount of AFL produced by the baited fungal isolates suggested that this system can be used to predict AFL contamination. Due to this characteristic it could be used to improve the actual AFL forecasting models which are based on weather data .

3.2. ASPERGILLUS FLAVUS AND OSTRINIA NUBILALIS

3.2.1. Monitoring of *Ostrinia nubilalis* adult population

In this study we were also interested in monitoring the field population of *Ostrinia nubilalis* (European Corn Borer, ECB) moths using cone trap baited with phenylacethaldeide (PAA) and sex pheromones in order to catch both females and males. Captures of ECB moths provide useful information on the dynamic of the whole ECB population. However, female catches provide more reliable predictive data on potential larval infestation damages (Pena et al., 1988). In Fig. 22 and 23 is shown the dynamic of ECB males during the two corn crop season. In 2009, from the end of May to mid–July, only a few adults were captured, then a little peak of capture in June and in the end of July was observed, followed by other two peaks at mid August and mid September. In 2010, three peaks were observed, the first at mid June, the second at middle of July and the last one at the end of August. In 2010, first generation captures were greater than in 2009. Our findings showed a clear bivoltine behavior of the ECB population as indicated in previous surveys (Maini and Burgio, 1990). Although the cone traps were baited with PAA, a limited number of females were captured (data not shown).

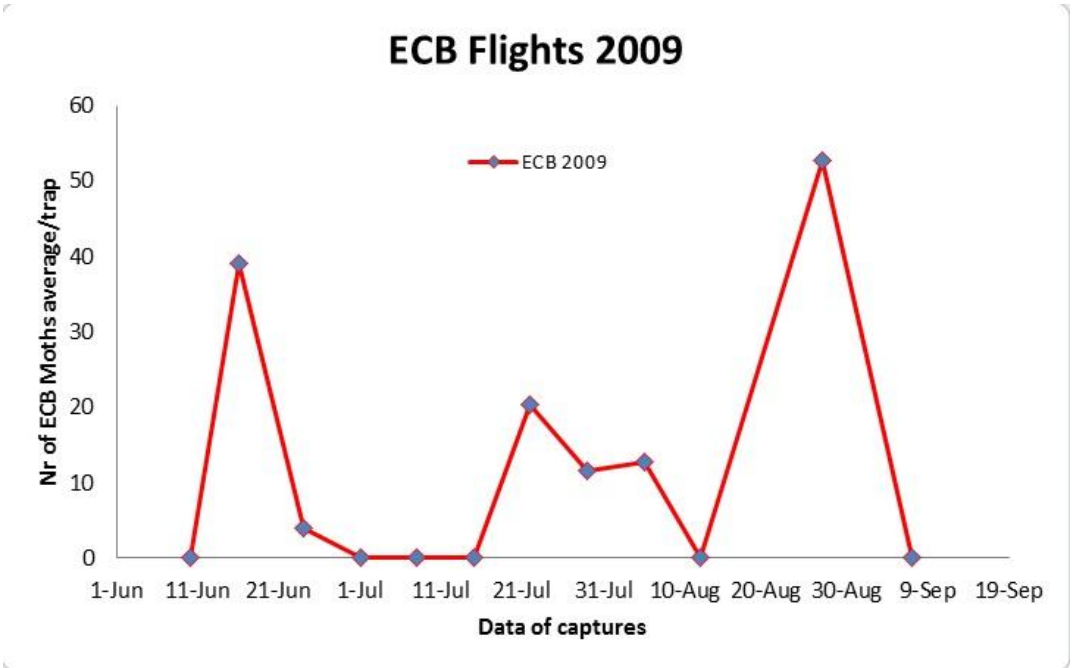

Fig. 22. Flights of ECB during the 2009 corn-growing season.

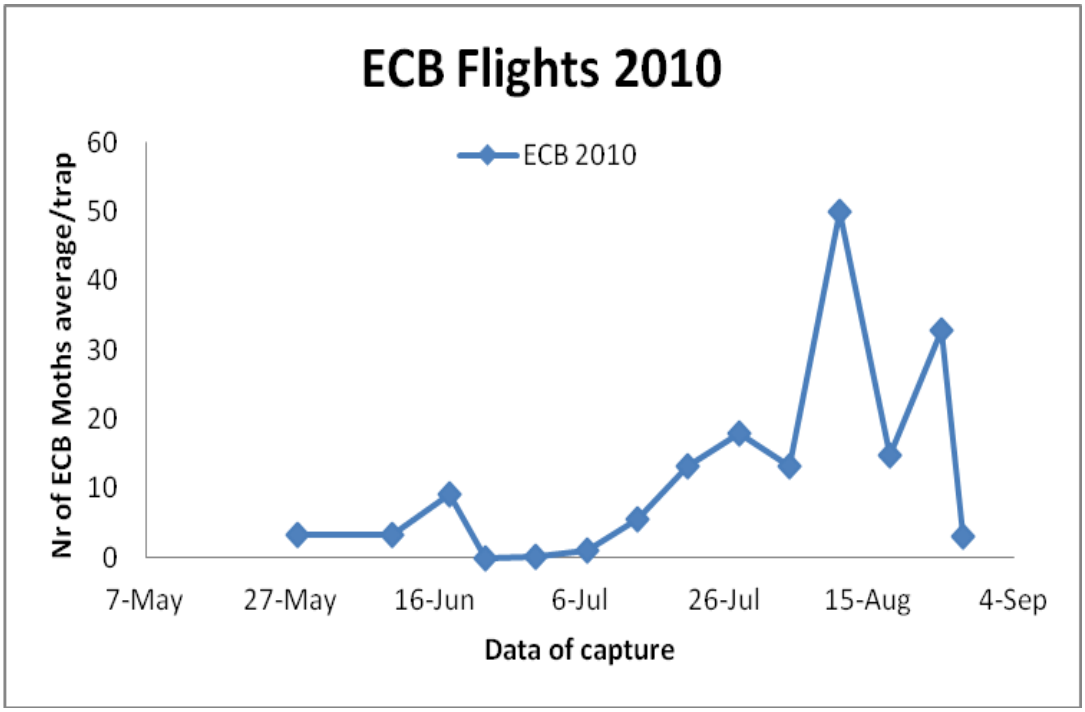

Fig. 23. Flights of ECB during the 2010 corn-growing season.

3.2.2. Field Damages of 1st and 2nd generation of ECB larvae

Feeding activity of 1st and 2nd generation ECB larvae during the two corn crop season was evaluated. Concerning damages due to feeding activity of 1st generation of ECB larvae in corn plant, no significant differences on the incidence and severity during the two crop seasons were observed (Table 5). Conversely, considering the 2nd generation damages we found a significant difference on damages severity, rather than on the incidence of the insect attack. In 2009, damages produced by first generation larvae were estimated of 5-10 ragged leaf holes per single plant and the second generation injuries were less than 3 bore per ears, with little damages on corn stalk. In 2010, more than 5 ECB ragged leaf holes/plant were counted in July. The second generation of ECB, other than produce about 3 bore/ear, damaged more than the 90 % of corn stalks determining their apical break (Fig. 24). However, ECB infestation did not significantly affect the final corn yield. Yield in 2009 was 10.4 t ha^{-1}, and in 2010 was 11.0 t ha^{-1}. The higher yield recorded in 2010 is likely associated to the severity/incidence of ECB second generation injuries which were in the apical part of plant and did not determine the ear drop and the plant lodge.

Fig. 24. Corn damages due to the 2nd generation of ECB in 2010.

Tab 5 Damages due to feeding activity of 1st and 2nd generation of ECB larvae.

Area	Year 2009				Year 2010			
	1st generation ECB [a]		2nd generation ECB [a]		1st generation ECB [a]		2nd generation ECB [a]	
	Incidence [b]	Severity [c]	Incidence [d]	Severity [e]	Incidence [b]	Severity [c]	Incidence [d]	Severity [e]
A	66.7 a	2	100	2.5	86.6 a	2	100	5
B	80.0 a	2	100	2.5	82.0a	2	100	5
C	73.3 a	2	100	2.5	76.3 a	2	100	5

Values followed by the same letter are not significantly different (P>0.05)**.**

a) Damages were evaluated considering 15 groups of 5 corn plants randomly chosen in each plot, each plant group was considered as a sample.

b) For the first generation damage incidence was evaluated considering the presence/absence (1/ 0) of characteristic foliar ragged holes (FRH) in each plant. Data were then expressed as percentage.

c) The damage severity was determined attributing a number to each plant group using the following schedule: 0 = no injuries, 1 = 1–5 FRH /plant, 2 = 6–10 FRH /plant; 3 = 11–20 FRH /plant, 4 = 21–35 FRH /plant, 5 = > 35 FRH /plant.

d) For the second generation damages incidence was evaluated considering the presence/absence (1/0) of bore on stalks and ears of corn plant. Data were then transformed in percentage.

e) The ECB damage severity was determined considering the number of bore/plant and the percentage of stalk damaged or broken in each plant group due to larvae activity. A scale of 0–5 was used to evaluate each plant group following schedule: 0 = no injuries, 1 = 1-3 tunnel /cob, stalk undamaged, 2=more than 3 tunnel/cob, stalk undamaged, 3 = presence of tunnel in cob and stalk, 1-30% plant broken in half or lodged, 4 = presence of tunnel in cob and stalk, 30-60% plant broken in half or lodged, 5 = presence of tunnel in cob and stalk, > 60% plant broken or lodged.

Fig. 25. Corn cobs infested by ECB on field.

By visual analysis of the sample of 25 cobs taken from each plot at harvest, some differences between the two years on presence of ear bore were observed. However, these differences among treatments were not statistically significant (Table 6). A portion of 10 corn cobs for each plot was used to evaluate the ECB damages and the *A. flavus* infection on kernels considering the influence of some factor (year, microbiological treatment and kernel position on the corn cob). The number of kernel damaged and the decrease of weight due to ECB feeding activity were accounted as index of ECB damage. Each parameter was considered either in relation to each portion of corn cob (kernel damages and decrease of weight) and relatively to the whole corn cob (relative kernel damages and relative decrease of weight). The formulas used for these calculations are the following:

$$Kd = (nkd * 100) / Nk$$
$$Ra = ((nkd / Nk) / (nk / Nk)) / 100$$
$$Wd = 100 - (TW * 100) / (NK * aWud)$$
$$RWd = 100 - (TW * 100) / ndW$$

Where Kd= kernel damaged; nkd= number of kernel damaged in each cob portion; Nk= total number of kernel; Ra =relative attack, nk= number of kernel of the corn cob portion, Wd= weight decrease, TW= total weight; aWnD= average weight of undamaged kernel; RWd=relative weight decrease; RndW=relative non decreased weight. In Table 8 are summarized the statistical results (ANOVA).

ANOVA for Nr. bore/corn cob					
	SS	**Df**	**MS**	**F**	**p**
Intercept	616,1067	1	616,1067	724,8314	0,000000
YEAR	9,6267	1	9,6267	11,3255	0,000980
TRT	2,8933	2	1,4467	1,7020	0,185972
YEAR*TRT	0,9733	2	0,4867	0,5725	0,565365
Error	122,4000	144	0,8500		

Tab. 6. Analysis of variance (ANOVA) for number of ECB bore in corn cobs.

Nr ECB bore/corn cob		
TRT	**2009**	**2010**
Untreated Control	1.56 a	2.13 a
15 kg ha^{-1},	1.88 a	2.60 a
30 kg ha^{-1},	1.88 a	2.13 a
Total	1.77 a	2.25 a

Tab. 7. Number of ECB bore in corn cobs from untreated plots and plots receiving 15 and 30 kg ha^{-1} of bioplastic based biocontrol formulation. Values followed by the same letter are not significantly different (P>0.05).

Factors	Kernel Damages	Relative Kernel Damages	Decrease of weight	Relative decrease of weight	Kernel infection
Year 09/10	0,012	0,000	0,173	0,013	0,769
Trt C/15/30	0,722	0,309	0,788	0,724	0,000
Kernel position	0,000	0,000	0,000	0,000	0,958
Year 09/10*Trt C/15/30	0,259	0,383	0,296	0,412	0,037
Year 09/10*Kernel position	0,526	0,074	0,333	0,035	0,264
Trt C/15/30*Kernel position	0,775	0,396	0,823	0,716	0,619
Year 09/10*Trt C/15/30*Kernel position	0,495	0,217	0,436	0,359	0,986

Tab. 8. Analysis of variance (ANOVA) for ECB damages and *Aspergillus flavus* infection on corn kernel. Data reported in table are P value.

ANOVA show a significant difference on ECB damages among the two experimental years. The percentage of kernel damaged by ECB was of 6.0% on 2009 and 11.2% on 2010. Differently from expected, the greater number of kernel attacked in 2010 did not cause a significant increase of weight loss on corn cobs (Table 8 and 9). This was in accord with the corn yields recorded during the two crop season. A weight loss due to ECB feeding activity ranging from 2.04-3.30 % was estimated.

The insect damage was significantly related to kernel position. Commonly is reported that the apical portion is the most attacked corn part by ECB and other ear feeding insect. Applying the Tucky's HDS test we found that the most attacked corn cob part was the apical, followed by the median part. If we consider that the apical part represent about the 15% in terms of number of corn kernels and about the 12% in terms of weight of the whole corn cob, the ECB damage on the apical part of corn cob caused a low relative damage as shown in Table 9.

The analysis of corn cob confirmed that the biocontrol treatments did not influenced the ECB feeding activity.

Tab. 9. (From *A.* to *E.*) Damages due to ECB feeding activity on cob kernels cobs from untreated plots (*B.*) and plots receiving 15 kg ha^{-1} (*C.*) and 30 kg ha^{-1} (*D.*) of bioplastic based biocontrol formulation. In *A.* are reported the average values of the three plot. In *E.* are reported the average value for each plot. From *A.* to *E.* values followed by the same letter are not significantly different (P>0.05). Kd= kernel damaged; Wd= weight decrease; Ra= relative attack; RWd=relative weight decrease.

Corn cob portion	Year 2009				Year 2010			
	Kd (%)	Wd (%)	**Ra (%)**	**Rwd (%)**	**Kd (%)**	Wd (%)	**Ra (%)**	**Rwd (%)**
UP	26.92 a	7.48 a	6.04 a	0.68 a	34.56 a	9.28 a	5.60 a	1.22 a
MID	3.54 b	0.80 b	6.9 a	0.64 a	6.91 b	0.69 b	4.83 a	0.51 b
BAS	2.32 b	0.81 b	4.55 a	0.07 a	5.36 b	0.87 b	0.77b	0.22 c

A.

Corn cob portion	Year 2009				Year 2010			
	Kd (%)	Wd (%)	**Ra (%)**	**Rwd (%)**	**Kd (%)**	Wd (%)	**Ra (%)**	**Rwd (%)**
UP	20.12 a	6.15 a	2.68 a	0.60 a	37.56 a	12.02 a	6.10 a	1.65 a
MID	3.24 b	0.71 b	2.39 a	0.46 b	5.84 b	0.62 b	4.08 b	0.45 b
BAS	0.76 c	0.94 b	0.11 b	0.26 c	8.27 b	1.05 b	1.21 c	0.15 c

B.

Corn cob portion	Year 2009				Year 2010			
	Kd (%)	Wd (%)	**Ra (%)**	**Rwd (%)**	**Kd (%)**	Wd (%)	**Ra (%)**	**Rwd (%)**
UP	33.46 a	8.26 a	4.00 a	0.68 a	33.93 a	8.20 a	5.37 a	1.06 a
MID	3.43 b	0.63 b	2.64 b	0.51 a	9.87 b	0.90 b	6.93 a	0.67 b
BAS	2.18 b	0.73 b	0.22 c	0.06 b	3.11 c	0.53 b	0.40 b	0.34 b

C.

Corn cob portion	Year 2009				Year 2010			
	Kd (%)	Wd (%)	**Ra (%)**	**Rwd (%)**	**Kd (%)**	Wd (%)	**Ra (%)**	**Rwd (%)**
UP	26.68 a	8.09 a	3.04 a	0.81 a	32.2 a	7.55 a	5.34 a	0.96 a
MID	3..93 b	1.07 b	2.90 a	0.86 a	5.04 b	0.57 a	3.49 b	0.42 b
BAS	4..25 b	0.78 b	0.44 b	0.1 b	4.71 b	1.02 b	0.69 c	0.17 b

D.

PLOT	Year 2009				Year 2010			
	Kd (%)	Wd (%)	**Ra (%)**	**Rwd (%)**	**Kd (%)**	Wd (%)	**Ra (%)**	**Rwd (%)**
Control	4. 90 a	2.14 a	4. 90 a	2.14 a	11.39 a	3..30 a	11.39 a	3.30 a
15 kg ha^{-1}	6.88 a	2.40 a	6.88 a	2.40 a	12.70 a	2.68 a	12.70 a	2.68 a
30 kg ha^{-1}	6.29 a	2.04 a	6.29 a	2.04 a	9.52 a	2.59 a	9.52 a	2.59 a

E.

Fig. 26. Corn cob injured by ECB larvae (left) and corn kernels incubated on MDRB Petri dishes (right).

After having evaluated and quantified ECB damages on the 10 corn cobs, we chose randomly three groups of ten grains from the apical, median and basal part of each corn cob to investigate the relation between ECB damages and *A. flavus* infection (Fig. 26). Conversely to the kernel damages, the analysis of variance indicated a significant difference of kernel infection due to biocontrol treatment. From the Tukey's HDS is highlighted that this variability is due to the higher infection recorded on 2010 on the plot treated with 30 kg ha^{-1} of microbiological formulation (data not shown) according with previous microbiological analysis .

In Fig. 27 and 28 are summarized data about kernel contamination with *A. flavus* in both years. As shown in the scatterplot no correlation between *A. flavus* infection and ECB damages was observed. These findings confirmed the unclear relation between ECB injuries and *A. flavus* infection (Buntin et al., 2001; Ovodi et al., 2000; Masoero et al., 1999; Pietri et al., 2000; Munkvold et al., 2000, Maupin et al., 2001; Lillehoj et al., 1976).

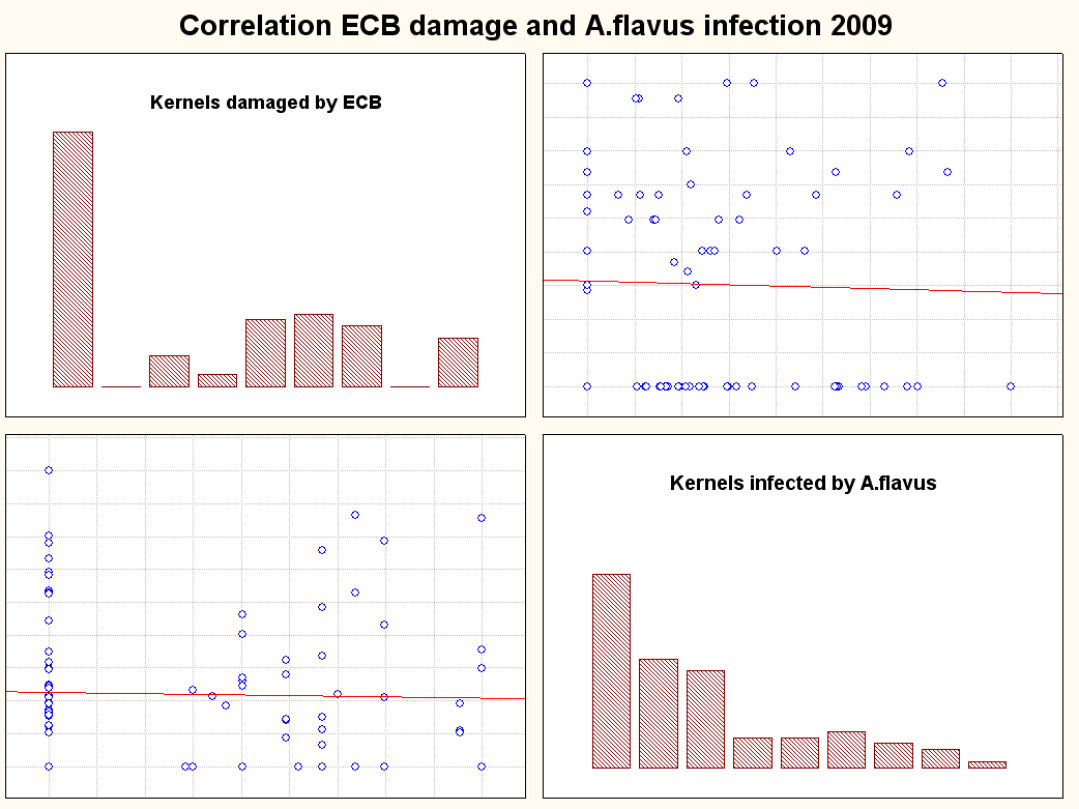

Fig. 27. Correlation between ECB damage and *A. flavus* infection during the corn growing season 2009. Data reported are the number of observation for each group of values.

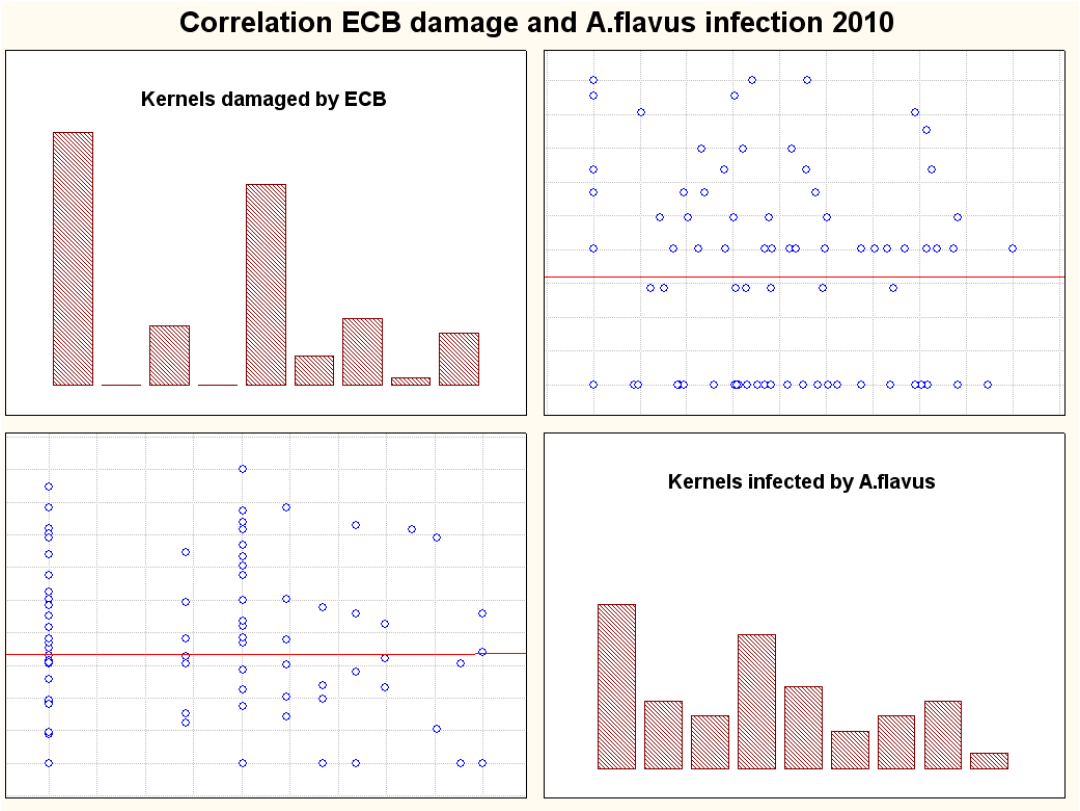

Fig. 28. Correlation between ECB damage and *A. flavus* infection during the corn growing season 2010. Data reported are the number of observation for each group of values.

3.2.3. ECB bioassay

Bioassay studies were included in this study to investigate the potential effects of AFL/A. flavus on ECB larvae. In Fig. 29 is shown the lethal effect on 4th instar ECB larvae after exposure for 14 days to high doses of AFB1. Rates of AFB1 were transformed in $\log_{10}$ and the mortality was transformed in probit. According to Jarvis et al. (1984) we found that AFB1 had a slow toxic effect to ECB larvae, needing approximately 14 days to completely kill the larvae. Our data also showed that ECB 4th instar larvae can tolerate high levels of AFB1. In fact, our experimental estimated LC50 at 14 days was 2.30± 0.3ppm. This is in accord with the literature. Mc Millian et al. 1980 feeding 2nd and 5th instar larvae with a dose of 250 ppb of AFB1 find that this rate determined significant effects on larval development on the 2nd larval instar, conversely 5th instar larvae were not affected by this concentration. Jarvis et al. (1984) find that larval mortality was delayed and 4th instar larvae development was negatively influenced if larvae was feed whit more than 1 ppm of AFB1. In another study, larvae feed with AFB1 ≥5 ppm showed reduction of weight and an increase of mortality (Accinelli et al., 2008a).

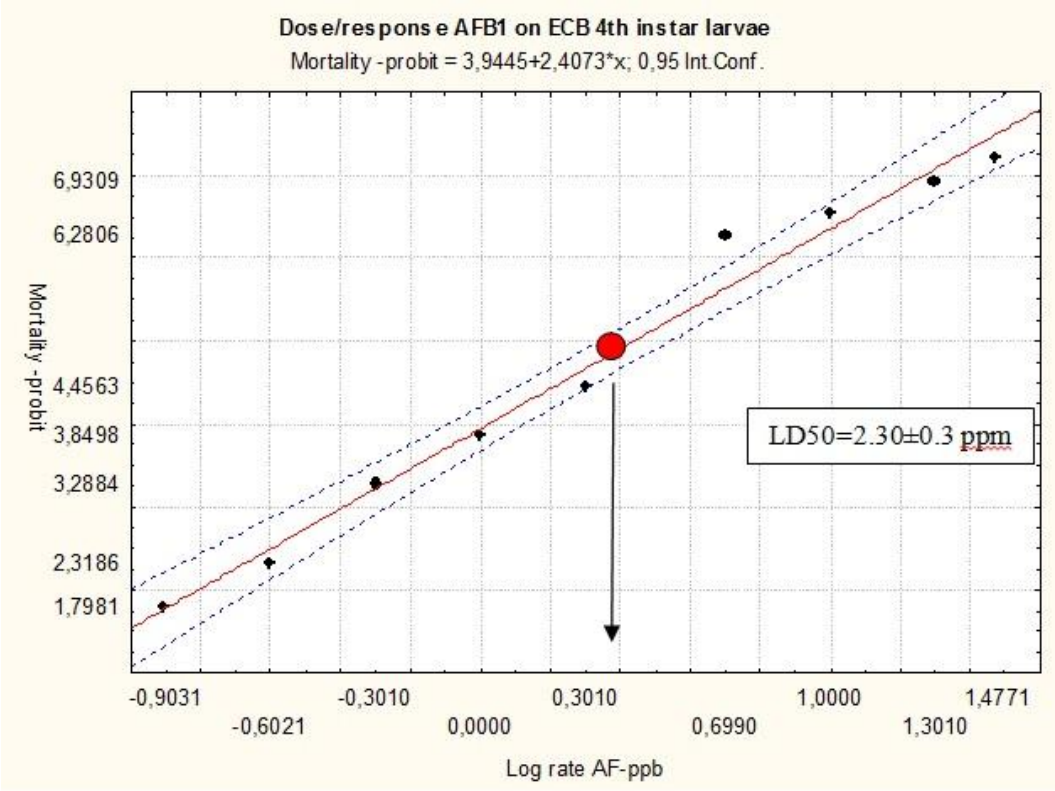

Fig. 29. Dose-response of AFB1 on ECB 4th instar larvae.

Because of AFB1 is a potent carcinogen and have also shown some insecticidal propriety, some authors have hypnotized that AFL in field can act as a natural regulator of the ECB population (Jarvis et al., 1984; Xinzhi et al., 2011). Although AFL were also found in surface soil associated with agricultural debris, in plant stalks and cob without kernels (Accinelli et al., 2008b), in general the maximum level of AFL is found in corn kernels (Accinelli et al., 2008b; Abbas et al., 2007; Jarvis et al., 1984). As reported in the literature, and according to our finding, ECB larval feeding activity is more centered in internal cob and stalk than on kernels. In addition, AFL occurrence is influenced by erratic biotic and abiotic environmental factors and AFL and the ECB distributions in field are not correlated (Xinzhi et al., 2011). According to Jarvis (1984), even though the AFL levels found in corn kernel can potentially have a negative effect on ECB, likely AFL is expected to have a little impact on ECB field population.

As shown in Fig. 30, in the bioassay whit *A. flavus* spores, 4th instar larvae after having feed or being in contact with toxigenic and non–aflatoxigenic spores of *A. flavus*, show an increase of mortality respect the control.

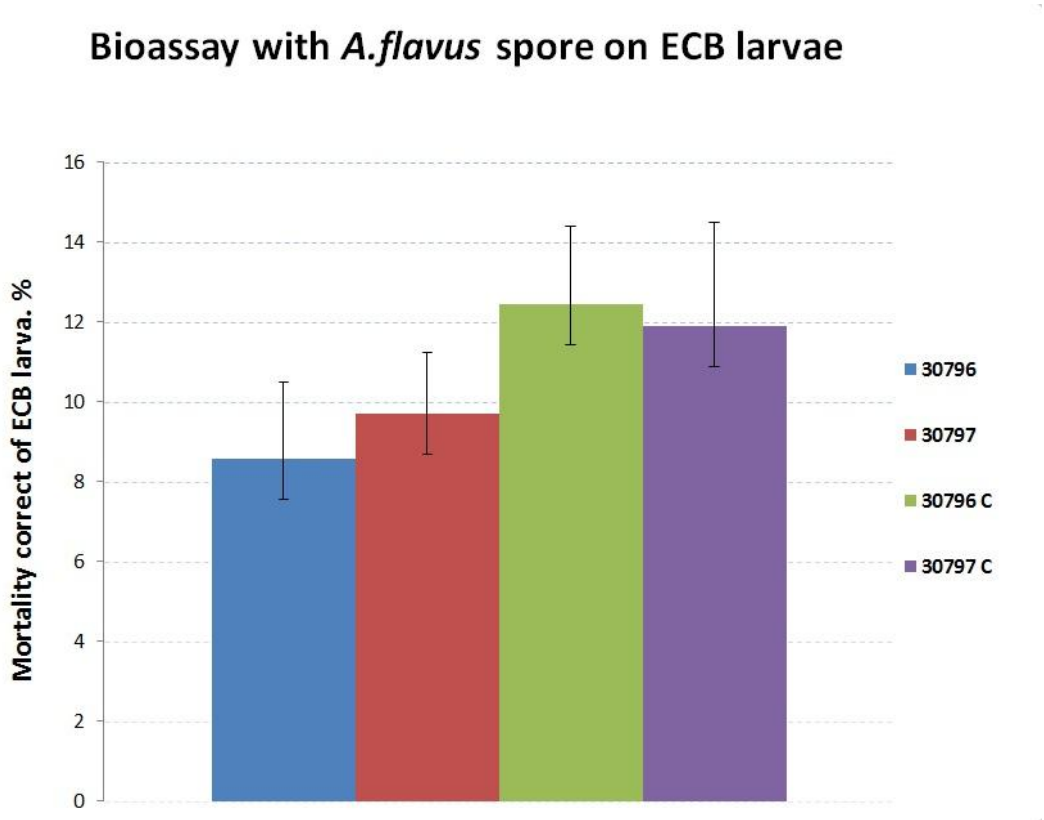

Fig. 30. Effect of aflatoxigenic and non-aflatoxigenic *A. flavus* spores of on ECB 4th instar larvae by spore suspension ingestion and indirect contact. Each point represents the Mortality correct with Schneider-Orelli formula ±STD.

In the present experiment, we recorded a greater mortality also in the untreated control (data not shown). This is likely due to the high insect manipulation. The aflatoxigenic and not-aflatoxigenic strains did not show differences on mortality. This can be linked with the low ability of the two *A. flavus* strains to colonize ECB larvae, but can be also linked with the time of contact. Although the larval mobility in field is limited to the infested corn plant, contaminated larvae in field can continue to move along and feed on corn plants. In a field where has been inundated of propagules of non-toxigenic strain of *A. flavus*, ECB larvae have a greater probability to enter in contact with Aspergillus spore. Fungal propagules can be carried asymptomatically by larvae and, after death insect cadaver can potentially serve as a food source for the fungal growth and sporulation.

CHAPTER 4. CONCLUSIONS

Two years field studies demonstrated the ability of a novel bioplastic-based formulation carrying propagules of the non-aflatoxigenic strain *A. flavus* NRRL 30797 to reduce aflatoxin (AFL) contamination in corn. The positive characteristics of this biodegradable formulation (easy preparation, handling and application, safety for operators and its eco-friendly profile) make this product an alternative solution to the formulations currently used (e.g. autoclaved grains, alginate pellets, etc…). Field studies conducted in 2009 and 2010 demonstrated the superior ecology competence of *A. flavus* NRRL 30797 against native toxigenic *A. flavus* isolates. This is in accord with previous studies conducted in Southern USA. Investigating the effect of biocontrol treatment on *O. nubilalis*, we found that the distribution of an high amount of *A. flavus* propagules did not influenced the ECB infestation and damages. In addition, it was found that *O. nubilalis* injuries and infestations and *A. flavus* infections in field were not correlated. Insect bioassays demonstrated a certain tolerance of *O. nubilalis* larvae to AFL and an high tolerance to *A. flavus* conidia. Although AFL concentration can vary, data from this study indicated that AFL contamination in field corn kernels would not affect the ECB local population. Based on the results from this two- year trial, inoculated bioplastic granules should be included in the list of available technologies for reducing AFL contamination in corn. In addition, preliminary results demonstrated that the designed bioplastic-based bait was effective for isolation of Aspergilli from soil and corn kernel samples. This simple and cost-effective bait system would be helpful for monitoring Aspergilli from soil and corn kernels. In addition it can also be used to improve the actual AFL forecasting models which are actually based on weather data (no biological measurements).

REFERENCES

Abbas H.K., Shier W.T., Cartwright R.D. (2007). Effect of temperature, rainfall and planting date on aflatoxin and fumonisin contamination in commercial Bt and non-Bt corn hybrids in Arkansas. Phytoprotection, 88: 41-50.

Abbas H.K., Wilkinson, J.R., Zablotowicz R.M., Accinelli C., Abel C.A., Bruns H.A., Weaver M.A. (2009). Ecology of *Aspergillus flavus*, regulation of aflatoxin production and management strategies to reduce aflatoxin contamination of corn. Toxin Rev, 28: 142-153.

Abbas H.K., Zablotowicz R.M., Bruns H.A., Abel C.A. (2006). Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigeenic *Aspergillus flavus* isolates. Biocontrol Science and Technology, 16: 437-44.

Abbas H.K., Zablotowicz R.M., Bruns H.A., Abel C.A. (2008). Development of non–toxigenic strains of *Aspergillus flavus* for control of aflatoxin in maize. Crop Protection Research Advanced. Isbn.78-1-60456-040-4. Editors. E.N. Burton and P.V. Williams, Pp. 181-199. Nova Science Publisher Inc.

Abbas H.K., Zablotowicz R.M., Locke M.A. (2004). Spatial Variability of *Aspergillus flavus* soil populations under different crops and corn grain colonization and aflatoxins. Can. J. Bot., 82: 1768-1775.

Accinelli C. and Abbas H.K. (2011). New perspectives for the application of bioplastic materials in the biocontrol of *Aspergillus flavus*. Corn. Toxin Reviews, 30(2–3): 71–78

Accinelli C., Abbas H., Zablotowicz R., Maini S., Vicari A. (2008). Role of *Ostrinia nubilalis* in vectoring *Aspergillus flavus* in a corn field in northern Italy. Proceedings of American Chemical Society National Meeting. March 5, 2008

Accinelli C., Abbas H.K., Zablotowicz R.M. (2008). *Aspergillus flavus* aflatoxin occurrence and expression of aflatoxin genes biosynthesis in soil. Canadian Journal of Microbiology, 54: 371-379.

Accinelli C., Mencarelli M., Saccà M.L., Vicari A., Abbas H.K. (2012). Managing and monitoring of *Aspergillus flavus* in corn using bioplastic-based formulations. Crop Protection, 32: 30-35.

Accinelli C., Saccà M.L., Abbas H.K., Zablotowicz R.M., Wilkinson J.R. (2009) Use of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of *Aspergillus flavus*. Bioresour Technol., 100: 3997-4004.

Accinelli C., Vicari A., Rossi Pisa P., Catizone P. (2002). Losses of atrazine, motolachlor, prosulfuron and trisulfuron in subrface drain water. I. Field results. Agronomie, 22: 399-44.

Anderson H.W., Nehring E.W. and Wichser W.R. (1975). Aflatoxin contamination of corn in the field. J. Agric. Food Chem., 23 (4): 775-782.

Asao T., Buchi G., Abdel-Kader M., Chang S.B., Wick E. L. and Wogan G. N.

(1965). The structures of aflatoxins B and G. Journal of the American Chemical Society, 87: 882– 886.

Atehnkeng J., Ojiambo P.S., Ikotun T., Sikora R.A., Cotty P.J. and Bandyopadhyay R. (2008). Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Additives and Contaminants 25 (10):1264–1271.

Barry D., Zuber M.S., Lillehoj E.B., Mcmillian W.W., Adams N.J., Kwolek W.F. and Widstrom N.W. (1985). Evaluation of two arthropod vectors as inoculators of developing maize ears with *Aspergillus flavus*. Environmental Entomology, 14(5): 634-636.

Blandino M., Reyneri A., Vanara F., Pascale M., Haidukowski M., Saporiti M. (2008). Effect of sowing date and insecticide application against European Corn Borer (Lepidoptera: Crambidae) on fumonisin contamination in maize kernels. Crop Protection, 27: 1432–1436.

Blownt W.P. (1961). Turkey X Disease. Turkeys, 52(9): 55–58.

Bock C., Cotty P.J. (1999). Wheat seed colonized with atoxigenic *Aspergillus flavus*: characterization and production of a biopesticide for aflatoxin control. Biocontrol Science and Technology, 9: 529-543.

Brown R. L., Cotty P. J., Cleveland T. E. (1991). Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus*. J. Food Prot., 54: 623–626.

Bruns H.A. (2003). Controlling aflatoxin and fumonisin in maize by crop management. Toxin Reviews 22, (2-3): 153–173.

Bruns H.A., Abbas H.K (2005). Ultra-high plant populations and N-fertility effects on corn in the Mississippi Valley. Agron J., 97: 1136–1140.

Bryden W.L. (2007). Mycotoxins in the food chain: human health implications. Asia Pac J Clin Nutr; 16 (Suppl 1):95-101.

Bryden W.L., Suksupath S., Taylor D.J. and Prasongsidh B.C. (2004). Cyclopiazonic acid: food chain contaminant? In: Acamovic T., Stewart C.S. and Pennycott T.W. Eds. Poisonous Plants and Related Toxins Cab International, Wallingford, U.K; 134-139.

Buntin D., Lee D., Wilson D.M. and Mcpherson R.M. (2001). Evaluation of yieldgard transgenic resistance for control of Fall Armyworm and Corn Earworm (Lepidoptera: Noctuidae) on corn. Florida Entomologist, 84(1) 37-42.

Canadian grain commission (2011). Official Grain grading Guide ISSN 1704-5118 http://www.grainscanada.gc.ca/oggg-gocg/2011/17-corn-2011-eng.pdf.

Chang P.-K., Abbas H. K., Weaver M.A., Ehrlich K.C., Scharfenstein L. L., Cotty P.J. (2012). Identification of genetic defects in the atoxigenic biocontrol Strain *Aspergillus flavus* K49 reveals the presence of a competitive recombinant group in field populations. International Journal of Food Microbiology. 154 (3): 192–

196.

Cleveland T.E., Yu J., Fedorova N., Bhatnagar D., Payne G.A., Nierman W.C. and Bennet J.W. (2009). Potential of *Aspergillus flavus* genomics for applications in biotechnology. Trends in Biotechnology,27 (3): 151-157

Codex Alimentarius Commission (1997). Code Of Practice For The Reduction Of Aflatoxin B1 In Raw Materials And Supplemental Feedingstuffs For Milk Producing Animals Cac/Rcp 45-1997 1. *Cac/Rcp (45) 1- 4*

Codex Alimentarius Commission (2004). Code Of Practice For The Prevention And Reduction Of Aflatoxin Contamination In Peanuts *Cac/Rcp (55): 1-7*

Commission Regulation (Ec) No 1881/2006 of 19 December 2006 Setting Maximum Levels For Certain Contaminants in Foodstuffs. Official Journal of the European Union L 364/ 5-24 20.12.2006.

Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union 9.3.2006. L 70/12-34.

Commission Regulation (EU) No 165/2010 of 26 February 2010 Amending Regulation (EC) No 1881/2006 Setting Maximum Levels For Certain Contaminants in Foodstuffs As Regards Aflatoxins.

Commission Regulation (EU) No 178/2010 of 2 March 2010 amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. Official Journal of the European Union 3.3.2010, L 52/32-43.

Cotty P.J. (1990). Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis., 74: 233–235.

Cotty, P.J. (1994). Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. Flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. Phytopathology, 84: 1270– 1277.

Cotty, P.J. and Jaime-Garcia R. (2007) influences of climate on aflatoxin producing fungi and aflatoxin contamination. International Journal of Food Microbiology, 119: 109-115.

Covarelli L., Beccari G., Salvi S. (2011). Infection by mycotoxigenic fungal species and mycotoxin contamination of maize grain in Umbria, central Italy. Food and Chemical Toxicology, 49: 2365–2369.

CYMMIT (2011). Maize a global alliance for improve food security and the livelihoods of the resource of poor the developing world. On Line Document. http://www.cimmyt.org/en/component/docman/doc_view/500-maize-global-alliance-for-improving-food-security-and-the-livelihoods-of-the-resource-poor-in-the-

Daigle D.J., Connick W.J. Jr., Boyette C. D., Lovisa M. P., Williams K.S.,

Watson M. (1997). Twin-Screw extrusion of 'Pesta'-encapsulated biocontrol agents. World J. Microbiol. Biotechnol. 13: 671–676.

Daigle, D.J., Connick, W.J., Boyette, C.D., Jackson, M.A., Dorner, J.W. (1998). Solid-state fermentation plus extrusion to make biopesticide granules. Biotechnol. Tech., 12: 715–719.

Daigle, D.J., Cotty, P.J. (1995). Formulating atoxigenic *Aspergillus flavus* for field release. Biocontrol Sci. Technol., 5: 175–184.

De Curtis F., De Cicco V., Haidukowski M., Pascale M., Somma S., Moretti A. (2011). Effects of agrochemical treatments on the occurrence of Fusarium ear rot and fumonisin contamination of maize in southern Italy. Field Crops Research, 123: 161–169.

Denning D.W (1998). Invasive Aspergilliose. Clinical and Infective Disease 26: 781-783.

Dietzgen, R.G. Ed. (1999). Elimination of Aflatoxin Contamination in Peanut. Isbn 1 86320 270 6 Canberra, Aciar Proceedings No. 89. 98 P. Australian Centre For International Agricultural Research, Gpo Box 1571, Canberra, Act 2601.

Dohlman E. (2003). Mycotoxin hazards and regulations impacts on food and animal feed crop trade in International Trade and Food Safety: Economic Theory and Case Studies. Agricultural Economic Report No. (Aer828) 145 Pp, November 2003. Jean C. Buzby, Editor.

Dorner J.W. (2004). Biological control of aflatoxin contamination of crops. Toxin Review, 23(2-3): 425–450.

Dorner J.W., Cole R. J., Connick W. J., Daigle D. J., Mcguire M. R., Shasha B. S. (2003). Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. Biol. Control., 26: 318–324.

Dorner J.W., Cole R.J., Blankenship P.D. (1992). Use of a biocompetitive agent to control pre-harvest aflatoxin in drought stressed peanuts. J. Food Prot. 55:888–892.

Dorner J.W., Cole R.J., Wicklow D.T. (1999). Aflatoxin reduction in corn through field application of competitive fungi. J. Food Prot., 62: 650–656.

Dorner, J.W. (2008). Management and prevention of mycotoxin in peanuts. Food Additive Contaminants, 25: 203-208.

Dorner, J.W., Cole, R.J. (2002). Effect of Application of non-toxigenic strains of *Aspergillus flavus* and A. parasiticus on subsequent aflatoxin contamination of peanuts in storage. J. Stored Prod. Res., 38:3 29–339.

Dowd P.F. (1998). Involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions. In: Sinha K. K., Bhatnagar D., Eds. Mycotoxins in Agriculture and Food Safety. Newyork: Marcel Dekker, Pp. 307–350.

Dowd P.F. (2003). Insect management to facilitate pre-harvest mycotoxin management. Toxin Reviews, 22(2-3): 327–350.

Dowd P.F., Bennett G.A., Mcguire, M.R., Nelsen, T. C., Shasha B. S., Simmons, F.W.(1999). Adherent malathion flour granules as an environmentally selective control for chewing insect pests of dent corn ears: indirect reduction of mycotoxigenic ear molds. Journal of Economic Entomology, 92 (1): 68-75(8).

Dowd P.F., Mehta A.D. and Boston R.S. (1998). Relative toxicity of the maize endosperm ribosome-inactivating protein to insects. J Agric Food Chem., 46: 3775–3779.

Drummond J. and Pinnock D.E. (1990). Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus* Journal of Invertebrate Pathology, 55: 332-336.

Eguchi N., Kondo K., Yamagishi N. (2009). Bait twig method for soil detection of *Rosellinia necatrix*, causal agent of white root rot of Japanese pear and apple, at an early stage of tree infection. J. Gen. Plant Pathol., 75: 325-330.

Ehrlich K. (1987). Effect on aflatoxin production of competition between wild-type and mutant strains of *Aspergillus parasiticus*. Mycopathologia, 97: 93-96.

Emilia Romagna Region Integrate Pest Management strategy in corn, http://www.ermesagricoltura.it/Sportello

Eurostat (2010). Pocket Book Agricultural Statistics Main Results 2008–09. Issn 1830-463x, Luxembourg: Publications office of the European Union, 2010, Isbn 978-92-79-15246-7, Doi: 10.2785/44845, Cat. No Ks-Ed-10-001-En-C.

FAO (2004). Worldwide regulations for mycotoxins 2003: a compendium, food and nutrition paper 81, Food and Agriculture Organization, United Nations, Rome, Italy.

FAO Statistics. http://faostat.fao.org/site/339/default.aspx

Fennell D. I., Kwolek W.F., Lillehoj E.B., Adams G., Bothast R.J., Zuber M.S., Calvert O.H., Guthri W.D., Bockholt A.J., Manwiller A., and Jellum M.D.(1977) *Aspergillus flavus* presence in silks and insects from developing and mature corn ears. Cereal Chemistry, 54(4): 770-778.

Fennell D.I., Lillehoj E.B. and Kwolek W.F. (1975) *Aspergillus flavus* and other fungi associated with insect-damaged field corn. Cereal Chemistry, 52(3): 314-321

Finney D.J. (1971). Probit analysis. Cambridge University Press, London.

Folcher L., Jarry M., Weissenberger A., Gerault F., Eychenne N. Delos M, Regnault-Roger C. (2009). Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (Zea Mays L.). Fields. Crop Protection, 28: 302–308.

Giorni P., Magan N., Piertri A., Bertuzzi T., Battilani P., (2007). Studies on *Aspergillus* section Flavi isolated from maize in northern Italy. Int. J. Food Microbiol, 113: 330-338.

Guo B.Z., Zhang Z.J., Butron A., Widstrom N.W., Snook M.E., Lynch R.E. and Plaisted D. (2001). Quantitative effects of loci P1 and A1 on the concentrations of maysin, apimaysin, methoxymaysin and chlorogenic acidinmaize silk tissue. Maize Genetics Newsletter, 75: 64-66.

Gupta A. and Gopal M. (2002). Aflatoxin Production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. World Journal of Microbiology & Biotechnology, 18: 325–331.

Hedayati M.T., Pasqualotto A.C., Warn P.A., Bowyer P., Denning D.W. (2007). *Aspergillus flavus*: human pathogen, allergen and micotoxin producer. Microbiology 153:1677-1692.

Horn B.V., Dorner J. W. (1998). Soil populations of Aspergillus species from section Flavi along a transect through peanut-growing regions of the United States. Mycologia, 90: 767-776.

Hua, S.-S.T. (2002). Potential use of saprophytic yeast to reduce populations of Aspergillus flavus in almond and pistachio orchards. – In: Proceedings of the Third International Symposium of Pistachio and Almond (eds, I. Battle, I. Hormaza, and M. T. Espiau). International Society for Horticultural Science, Leuven, Belgium. Acta Horticulture, 591: 527-530.

Hussein H.S., Brasel J.M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology, 167: 101–134.

International Agency For Research On Cancer IARC (2002). Monographs on the evaluation of carcinogenic risks to humans (82): Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. Aflatoxins: 171-300.

Istat Istituto Statistico Italiano/Italian Statistical Center Statistiche Agrarie 2010 Http://En.Istat.It/Agricoltura/

Jaime-Garcia R., Cotty P. J. (2010). Crop rotation and soil temperature influence on the community structure of *Aspergillus flavus* in soil. Biology & Biochemistry, 42: 1842-1847.

Jarvis L., Guthrie W.D. and Lillehoj E.B. (1984a). Aflatoxin and selected biosynthetic precursors: effects on the European Corn Borer in the laboratory. Agric. Entomol., 1(4): 354-359.

Jarvis L., Guthrie W.D. and Lillehoj E.B. (1984b). Effect of Aflatoxin B1 on the European Corn Borer survival and development in the laboratory. J. Agric. Entomol. 1(1): 17-22.

Jones R.K., Duncan H.E., Payne G.A. (1980). Factors influencing infection by *Aspergillus flavus* in silk inoculated corn. Plant Disease, 64(9): 859-863.

Jones R.K., Duncan H.E., Payne G.A., Hamilton P.B. (1981). Planting date, harvest date, and irrigation effects on infection and aflatoxin production by *Aspergillus flavus* in field corn. Phytopathology, 66: 675– 677.

Kellerhals M.D. (2000). EU food safety rule may cost Africa $700 Million in trade. World Bank Says Rule Does Little to Protect Human Health. [Available On Line] Http://Usinfo.State/Gov.

Kensler T.W., Roebuck B.D., Wogan G.N. and Groopman J.D. (2011). Aflatoxin: A 50-Year Odyssey of Mechanistic and Translational Toxicology. Toxicological Sciences, 120(S1): S28–S48.

Klun J.A. and Cooperators (1975). Insect sex pheromones: intraspecific pheromonal variability of *Ostrinia nubilalis* in Northern America and Europe. Environ. Entom., 4 (6): 891-894.

Krumm J.T., Hunt T.E., Skoda S.R., Hein G.L., Lee D.J., Clark P.L. and Foster J.E. (2008). Genetic variability of the European Corn Borer, *Ostrinia nubilalis*, suggests gene flow between populations in the Midwestern United States Journal of Insect Science: Vol. 8 |Article 72Www.Insectscience.Org

Lee L.S., Lillehoj E.B., Kwolek W.F. (1980). Aflatoxin distribution in individual corn kernels from intact ears. Cereal Chemistry, 57: 340-343.

Leger R.J. St., Screen S.E. and Shams-Pirzadeh B. (2000). Lack of host specialization in *Aspergillus flavus*. Applied and Environmental Microbiology, 66 (1): 320-324.

Lewis L., Onsongo M., Njapau H., Schurz Rogers H., Luber, G., Kieszak S., Nyamongo J., Backer L., Dahiye A. M., Misore A., Decock K., Rubin C. and the Kenya Aflatoxicosis Investigation Group.(2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. Environ. Health Perspect., 113:1763–1767.

Lillehoj E.B., Fennell D.I., Kwolek W.F. (1976). *Aspergillus flavus* and Aflatoxin in Iowa Corn Before Harvest. Science, New Series, 193 (4252): 495-496.

Lillehoj E.B., Fennell D.I., Kwolek W.F., Adams G.L., Zuber M.S., Horner E.S. Widstrom N.W, Warren H., Guthrie W.D., Sauer D.B., Findley W.R., Manwiller A., Josephson L. M. and Bockholt A.J. (1978). Aflatoxin Contamination of corn before harvest: *Aspergillus flavus* association with insects collected from developing ears. Crop Science 18: 921-924.

Liu Y., and Wu F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ. Health Perspect., 118(6): 818-24. Epub 2010 Feb 19.

Lubulwa A.S.G., and Davis J.S. (1994). Estimating the social costs of the impacts of fungi and aflatoxins in maize and peanuts. In: Highley E., Wright E. J., Banks H. J. and Champ B. R.. Eds. Stored Product Protection: Proceedings of the 6th International Working Conference On Stored-Product Protection. Wallingford,

Uk: Cab International, Pp. 1017–1042.

Lyn M.E., Abbas H.K., Zablotowicz R.M., Johnson B.J., (2009). Delivery Systems For Biological Control Agents to Manage Aflatoxin Contamination of Pre-Harvest Maize. Food Addit. Contam. A 26 381-387.

Magg T., Melchinger A. E., Klein D. and Bohn M. (2001). Comparison of Bt maize hybrids with their non-transgenic counterparts and commercial varieties for resistance to European Corn Borer and for agronomic traits. Plant Breeding, 120: 397-403.

Maini S., Burgio G. (1990). Influence of trap design and phenilacetaldehyde upon field capture of male and female *Ostrinia nubilalis* Hr Lepidoptera, Pyralidae and other moths. Bull. Ist. Ent. G.Grandi. Uni. Bo., 45: 157-165.

Maini S., Pallotti G., Platia G. (1978). Ricerche sull'identificazione del feromone sessuale in popolazioni bolognesi di *Ostrinia nubilalis* Hr (Lepidoptera: Pyralidae) e relative prove di campo. Boll. Ist.Entom. Uni. Bologna, 34: 15-25.

Mannon J. and Johnson E. (1985). Fungi down on the farm. New Scientist, 105:12-16

Marsh S.F. and Payne G.A. (1984). Preharvest infection of corn silk and kernels by *Aspergillus flavus*. Phytopathology 74 (11): 1284-1289.

Masoero F., Moschini M., Rossi F., Prandini A., Pietri A.L. (1999). Nutritive value, mycotoxin contamination and in vitro rumen fermentation of normal and genetically modified corn (*Cry1Ab*) grown in northern Italy. Maydica, 44: 205–209.

Mason C. E., Rice M. E., Calvin D. D., Van Duyn J. W., Showers W. B., Hutchinson W.D., Witkowski J. F., Higgins R. A., Onstad D. W. and Dively G. P. (1996). European Corn Borer ecology and management. North Central Regional Extension Publication 327. Iowa State University Press, Ames.

Matsumoto M.,(2003). A qualitative baiting technique for selective isolation and DNA diagnosis of *Rhizoctonia Spp*., causal agent of rice sheath disease, from soil. J. Fac. Agric. Kyushu Univ 48, 13-20.

Maupin L.M., Clements M.J., Walker S.L., White D.G. (2001). Effects of Cryiab on Aspergillus Ear Rot and Aflatoxin in commercial corn hybrids. Phytopatology, 91 (6): 6.

Mcmillian W.W. (1987). Relation of insects to aflatoxin contamination in maize grown in the Southeastern USA. Aflatoxin in Maize, Zuber, M.S.Lillehoj, E.B.Renfro, B.L. (Eds.). - Mexico, Df (Mexico): Cimmyt, 1987. - Isbn 968-6127-12:194-200

Misaghi I.J., Cotty P.J., Decianne D.M. (1995). Bacterial Antagonists of *Aspergillus flavus*. Biocontrol Sci. Technol., 5:387–392.

Munkvold G.P., Hellmich R.L., Biggerstaff C.M. (2000). Interactions among *Fusarium verticillioides*, insect pests and *Aspergillus flavus* in transgenic and

conventional maize hybrids. In: Proceedings of the Aflatoxin/Fumonisin Workshop 2000, Yosemite, Ca. 25-27 P. 142.

Murakoshi S. (1977). Productivity of aflatoxins and some biological effect of *Aspergillus flavus* link isolated from cadaver of the silkworm, *Bombyx mori* L. Applied Entomology Zoology, 12 (3): 255-259.

Nechwatal J., Schlenzig A., Jung T., Cooke D.E.L., Duncan J.M., Osswald W.F. (2001). A combination of baiting and PCR techniques for the detection of *Phytophthora quercina* and *P. citricola* in soil samples from oak stands. Forest Pathol., 31: 85-97.

Niu G., Siegel J., Schuler M.A., Berenbaum M.R (2006). Comparative toxicity of mycotoxins to Navel Orangeworm (*Amyelois transitella*) and Corn Earworm (*Helicoverpa zea*) J Chem Ecol., 35:951–957

Otsuki T., Wilson J. and Sewadeh M. (2001) Saving two in a billion: quantifying the trade effect of European Food Safety Standards on African Exports. Food Policy, 26 (5): 495-514.

Ovoid G.N., Chilcutt C.F., Parker R.D., Benedict J.H. (2000). Aflatoxin and insect response of near-isogenic Bt and Non-Bt Commercial Corn Hybrids in South Texas. In: Proceedings of the Aflatoxin/Fumonisin Workshop 2000, Yosemite, Ca, 25-27 October, Pp. 121-122

Parsons M.W, Munkvold G.P (2010). Associations of planting date, drought stress, and insects with Fusarium ear rot and fumonisin B1 contamination in California maize. Food Addit Contam Part A Chem Anal Control Expo Risck.27 (5):591-607.

Paulitz T.C., Schroeder K.L., (2005). A new method for the quantification of *Rhizoctonia solani* and *R. oryzae* from. Soil. Plant Dis., 89: 767-772.

Payne G.A. (1992). Aflatoxin in Maize. Crc Crit Rev Plant Sci. 10: 423–440.

Pena A., Arn H., Buser H.-R., Rauscher S., Bigler F., Brunetti R., Maini S. and Toth M. (1988). Sex pheromone of European Corn Borer *Ostrinia nubilalis* polymorphism in various laboratory and field strains. Journal Of Chemical Ecology, 14 (5): 1359-1366, DOI: 10.1007/BF01020140.

Penchan T, Ye L, Chang YM, Mitra A, Lin L, Davis FM, Williams WP and Luthe DS (2000). A unique 33-kd cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to Fall Armyworm and other Lepidoptera. Plant Cell., 12: 1031–1040.

Pietri A., Bertuzzi T., Pallaroni L., Piva G. (2004). Occurrence of micotoxyns and ergosterol in maize harvested over 5 years in Northern Italy. Food Addit. Contam. A 21, 479-487.

Pietri A., Piva G. (2000). Occurrence and control of mycotoxins in maize grown in Italy. In: Proceedings of the 6th International Feed Production Conference, Food Safety: Current Situation and Perspectives in the European Community.

Piacenza (Italy) 27-28 November, pp. 226-236.

Pitt J.I. (2000). Toxigenic fungi and mycotoxins British Medical Bulletin, 56 (1): 184-192.

Pitt J.I., Hocking A.D. (2006). Mycotoxins in Australia: biocontol of aflatoxin in peanuts. Mycopathologia 162(3): 233-243.

Ritchie S.W., Hanway J.J. (1982). How a corn plant develops. Iowa State Coop. Ext. Serv. Spec. Rep. 48 Ames, Ia.

Robens J., and Cardwell K. (2003). The costs of mycotoxin management to the USA: management of aflatoxins in The United States. J. Toxicol. Toxin. Rev. 22(2–3): 143–156.

Rodriguez Del Bosque (1996). Impact of Agronomic factors on Aflatoxin Contamination in Pre harvest Field Corn in Northeastern Mexico. Plant Disease, 80 (9): 988-993.

Rodriguez-Del-Bosque L. A., Leos-Martinez J., Dowd P. F. (1998). Effect of ear wounding and cultural practices on abundance of *Carpophilus Freemani* (Coleoptera: Nitidulidae) and other microcoleopterans in maize in Northeastern Mexico. Journal of Economic Entomology, 91(4):796-801.

Sargent K. and Carnaghan R.B.A. (1963) Britannic Veterinary Journal. 119: 178

Scheffer R.P. (1991). Role of Toxins in Evolution and Ecology of Plant Pathogenic Fungi. Experientia, 47: 804–811.

Scheidegger K.A. and Payne G.A. (2003). Unlocking the Secrets Behind Secondary Metabolism: A Review of *Aspergillus flavus* from pathogenicity to functional genomics. Journal of Toxicology-Toxin Reviews. , 22 (2-3): 423-459.

Scheidegger K.A. and Payne G.A. (2003).Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. Toxin Reviews, 22(2-3)423-459.

Schneider-Orelli A. (1947). *Entomologisches praktikum*. H.R. Sauerlander, Aarau, Switzerland

Scully L.R. and Bidochka M. J. (2006). A Cysteine/Methionine auxotroph of the opportunistic fungus *Aspergillus flavus* is associated with host-range restriction: A model for emerging diseases. Microbiology 152: 223–232

Shier, W.T., Abbas, H.K., Weaver, M.A., Horn, B.W. (2006). The case for monitoring *Aspergillus flavus* aflatoxigenicity for food safety assessment in Developing Country. In: Abbas, H.K. (Ed.), Aflatoxin and Food Safety. Crc Press, Pp. 291e311.

Siegel D., Babuscio T. (2011). Mycotoxin Management in The European Cereal Trading Sector. Food Control 22: 1145-1153

Smith J. S. Jr., Dorner J. W., Cole R. J. (1990). Testing *Bacillus subtilis* as a possible aflatoxin inhibitor in stored farmers' stock peanuts. Proc. Am. Peanut Res. Educ. Soc. 22:35.

Smith M. S., Riley T. J. (1992).Direct and interactive effects of planting date, irrigation, and Corn Earworm (Lepidoptera: Noctuidae) damage on aflatoxin production in pre-harvest. Field Corn. Journal of Economic Entomology, 85(3): 998-1006.

Sobolev V.S., Dorner J.W. (2002). Cleanup procedure of aflatoxins in major agricultural commodities by Liquid Chromatography. J. Aoac Int., 83: 642-645.

Taubenhaus J.J. (1920). A study of black and yellow molds of ear corn. Texas Agricultural Experimental Station Bulletin, 270: 3–38.

The Aspergillus Website Http://Www.Aspergillus.Org.Uk/

U.S. Department of Agriculture (2009). Aflatoxin Handbook Us Department of Agriculture (USDA), Grain Inspection, Packers and Stockyard Administration (Gipsa)http://www.gipsa.usda.gov/publications/fgis/handbooks/aflatoxin/aflatoxin-hb.pdf

USDA Foreign Agricultural Services Statistic Http://Www.Fas.Usda.Gov/Data.Asp

Vardon P., Mclaughlin C., and Nardinelli C. (2003). Potential economic costs of mycotoxins in The United States. In Council For Agricultural Science and Technology (Cast): Mycotoxins: Risks in Plant, Animal, and Human Systems. Task Force Report No. 139. Ames, Ia, Cast.

Vasileiadis V.P., Sattin M., Otto S., Veres A., Pálinkás Z., Ban R., Pons X., Kudsk P.,Van Der Weide R., Czembor E., Moonen A.C., Kiss J. (2011). Crop Protection in European Maize-Based Cropping Systems: Current Practices and Recommendations For Innovative Integrated Pest Management. Agricultural Systems 104: 533–540

Vujanovic V., Smoragiewicz W., Krzysztyniak K. (2001). Airbrone fungal ecological niche determination as one of possibilities for indirect mycotoxin risk assessment in indoor air. Environmental Toxicology, 16: 1-6

Wicklow D. T., Mcalpin C. E. and Platis C. E. (1998). Characterization of the Aspergillus flavus Population Within An Illinois Maize Field. Mycological Research (1998), 102 : 263-268

Wicklow D.T, Wilson D.M., Nelsent C. (1993).Survival of *Asperfillus flavus* sclerotia and conidia buried in soil in Illinois or Georgia. Phytopathology 83 (11): 1141-1147

Wicklow D.T. (1988). Metabolites in the coevolution of fungal chemical defense systems. In: Pirozynski K. A., Hawksworth D. L., Editors. Coevolution of Fungi With Plants and Animals. London, England: Academic Press;: 173–202

Wicklow, D. T., Dowd P. F., and Gloer J. B. (1994). Antiinsectan effects of

Aspergillus metabolites, pp 93–114. In K. A. Powell, J. Perberdy, and A. Renwick (Ed.), The Biology of Aspergillus. Plenum Press, New York.

Widstrom N.V., Lillehoj E.B., Sparks A.N. and Kwolek W.F. (1976).Corn Earworm damage and Aflatoxin B1 On corn ears protected with insecticide. Journal of Economic Entomology, 69(5): 677-679.

Widstrom N.W, Sparks A.N., Lillehoj E.B., Kwolek W.F. (1975). Aflatoxin Production and Lepidopteran insect injury on corn in Georgia. Journal of Economic Entomology, 68: 855-856

Williams W.P., Buckley P.M, and Windham G.L. (2002) Southwestern Corn Borer (Lepidoptera: Crambidae) damage and Aflatoxin accumulation in maize. Journal of Economic Entomology, 95(5): 1049-1053

Williams W.P., Davis F.M, Buckley PM, Hedin P.A., Baker G.T. and Luthe D.S.. (1998). Factors associated with resistance to Fall Armyworm (Lepidoptera: Noctuidae) and Southwestern Corn Borer (Lepidoptera: Crambidae) in corn at different vegetative stages. J Econ Entomol., 91: 1471–1480.

Williams W.P., Windham G.L., Buckley P.M., Perkins J.M. (2005) Southwestern Corn Borer damage and Aflatoxin Accumulation in conventional and transgenic corn hybrids. Field Crops Research 9:329–336

Windham G.L. (1999). Aflatoxin Accumulation in commercial corn hybrids in 1998. Miss. Agric. For. Exp. Stn. Res. Rep,

Windham G.L., Williamsw.P.And.Davis F. M (1999). Effects of the Southwestern Corn Borer on *Aspergillus flavus* kernel infection and aflatoxin accumulation in maize hybrids. Plant Disease 83 (6): 535-540.

World Bank (2005). Food Safety and Agricultural Health Standards. Challenges and Opportunities For Developing Country Exports. Report No. 31207, Washington, D.C., USA.

World Health Organization (WHO). (2008). The Global Burden of Disease: 2004 Update.
Geneva(Switzerland).http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf

Wright V.F., R.F. Vesonder, and A. Ciegler. (1982) Mycotoxins and Other Fungal Metabolites as Insecticides, 559–583. In E. Kurstak (Ed.), Microbial and Viral Pesticides. Marcel Dekker, New York.

Wu F. (2007). Bt Corn and impact on mycotoxins. Cab Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 2 (060) Doi: 10.1079/Pavsnnr20072060. http://www.cababstractsplus.org/cabreviews.

Wu F. (2008). A tale of two commodities: how EU mycotoxin regulations have affected U.S. tree nut industries. World Mycotoxin Journal, 1(1): 95-102.

Wu F. and Khlangwiseta P. (2010). Health Economic Impacts and Cost-Effectiveness of Aflatoxin Reduction Strategies in Africa: Case Studies in Biocontrol and Postharvest interventions. Food Addit Contam Part A Chem Anal Control Expo Risk Assess; 27(4): 496–509.

Wu F., (2004) Mycotoxin Risk Assessment For the Purpose of Setting International Regulatory Standards. Environmental Science & Technology. 38, (15): 4049-4054.

Wu F., Bhatnagar D., Bui-Klimke T,. Carbone I., Hellmich R., Munkvold G., Paul P., Payne G., Takle E. (2011). Climate change impacts on mycotoxin risks in US maize. World Mycotoxin Journal, 4: 79-93.

Xinzhi NI.., Wilson J.P., Buntin G.D., Guo B., Krakowsky M.D., Dewey L.R., Cottrell T.E., Scully B.T., Huffaker A. and Schmelz E.A (2011). Spatial patterns of aflatoxin levels in relation to ear-feeding insect damage in pre-harvest corn. Toxins 3:920-931; Doi: 10.3390/Toxins3070920.

Yu J. and Keller N. (2005). Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol., 43: 437–58.

Yu J., Cleveland T.E., Nierman W.C., Bennett J.W. (2004). *Aspergillus flavus* genomics: gateway to human and animal health: food safety, and crop resistance to diseases. Rev. Iberoam. Micol., 22: 194-202.

Yu J., Payne G.A., Campbell B.C, Guo B., Cleveland T.E., Roberts F., Keller N.P, Bennett J.W., and Nierman W.C. (2008). Mycotoxin Production and Prevention of Aflatoxin contamination in Food and Feed. The Aspergilli 457-472.

Zablotowicz R.M., Abbas H.K., Locke M.A. (2007). Population ecology of *Aspergillus flavus* associated with Mississippi Delta soils. Food Addit Contam 24:1102-1108.

Zeng R.S., Niu G., Zhimou W., Schuler M.A., Berenbaum M.R. (2006). Toxicity of Aflatoxin B1 to *Helicoverpa Zea* and Bioactivation by Cytochrome P450 Monooxygenases. J Chem Ecol., 32: 1459–1471.

TABLE OF FIGURES

TABLE OF TABLES