TOXIGENIC FUNGI: ECOLOGY AND PREVENTION OF THEIR MYCOTOXIN PRODUCTION (A REVIEW)

(Hongos toxicogénicos: Ecología y prevención de la producción de sus micotoxinas (Revisión))

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Palabras Clave: ***Aspergillus, Fusarium, Penicillium, Alternaria***, micotoxinas, hongos toxicogénicos, ecología, prevención

Key words: ***Aspergillus, Fusarium, Penicillium, Alternaria,*** mycotoxins, toxigenic fungi, ecology, prevention.

RESUMEN

Las actividades humanas, entre ellas la agricultura, interfieren con la dinámica de las poblaciones fúngicas, pudiendo afectar en mayor o menor medida los tamaños y la estructura de la comunidad. Si bien muchas especies de Hyphomycetes son beneficiosas y aún esenciales para el desarrollo de ciertos cultivos, otras son fitopatógenas y/o toxicogénicas, pudiendo estas últimas producir micotoxinas, que por su acción tienen relevancia en salud pública.

Desde un punto de vista agrícola, se ha establecido que las especies toxicogénicas más significativas pertenecen principalmente a los géneros ***Aspergillus, Fusarium, Penicillium*** *y* ***Alternaria*** *.*

En la presente revisión, se discuten los principales determinantes ecológicos así como la capacidad para producir micotoxinas de ciertas especies integrantes de estos taxa, contribuyendo de esta manera a la comprensión del aspecto ecotoxicológico del problema.

Las micotoxinas son contaminantes naturales de los alimentos y materias primas alimentarias, por lo tanto, las estrategias ideales para su control tienden a prevenir el crecimiento fúngico y la producción de sus metabolitos secundarios tóxicos. Para ello se ha diseñado una serie de medidas temporales con el propósito de reducir esta contaminación. Entre ellas pueden destacarse el desarrollo de programas de manejo integrado de cultivos y el control de condiciones durante la cosecha y almacenamiento del producto final. Las soluciones definitivas al problema de contaminación con micotoxinas, requieren un tiempo más prolongado para su desarrollo e implementación. Dichas estrategias, incluyen el tradicional desarrollo de resistencia a la invasión fúngica en la planta hospedera . Otros métodos alternativos, incluyen el control químico o biológico del crecimiento fúngico y la manipulación genética de especies toxicogénicas, con el propósito de interrumpir su metabolismo de formación.

En la presente revisión, se discuten los procedimientos provisorios y a largo plazo tendientes a disminuir o evitar la contaminación de los alimentos con micotoxinas

SUMMARY

Human activities, such as agriculture, interfere with the dinamics of fungal populations by affecting in high or low degree the size and structure of the community. Even though, many fungal species are beneficial and essential for the development of certain crops, there are other groups having a phytopathogenic and /or atoxigenic nature which makes them to produce mycotoxins that, because of their action, become relevant in public health.

From the agricultural point of view, it may be stated that the most significant toxigenic fungi belong to the ***Aspergillus, Fusarium, Penicillium*** *and* ***Alternaria*** *genera.*

The main ecological characteristics as well as the ability of certain species belonging to these taxa to produce mycotoxins are discussed in this review so as to help in the understanding of the ecotoxicologic feature of the problems.

Mycotoxins are considered natural contaminants of foods and alimentary raw materials, therefore the ideal strategies designed for their control aim to prevent the fungal growth as well as the production of their

secondary toxic metabolites. A series of temporary measures has been designed to this effect in order to minimize this contaminations, such as the development of programs to get an integrated handling of cultures and the control of conditions during the crop and storage of the final product. Definitive solutions to the problem of contamination by toxins, require a longer period of time for their evolution and implementation. These strategies include the usual resistance to the fungal invasion in the host plant. Another alternative methods include chemical or biological control of the fungal growth and the genetic handling of toxigenic species with the purpose of interrupting their metabolism of production.

In this review, both the temporary and long-term procedures tending to minimize or else avoid the contamination of food by mycotoxins are also discussed.

I) Fungi in nature

Fungi are ubiquitous both in nature and domestic environments, and any kind of organic matter provides an adequate substrate for their growth (De Nijs *et al.*, 1996).

Human activities affect dimensions as well as structures of fungal populations. Construction, wars, recreation, and agriculture disrupt great areas of soil and vegetation; disruption causes redistribution of fungal propagules and provides nutrients to fungi. Many fungi take advantage of these resources supplied by man. This results in the association of fungal populations with different human activities, especially agriculture. Nevertheless, human activity, determines in a partial way, quality and quantity of the fungi, while this influences man's activities, domestic animals and even man himself (Cotty *et al.* 1994).

One fungal characteristic is the ability to produce secondary metabolites. In comparison with primary metabolites which are essential for the functioning of metabolic tracts and which are synthesized during the active growing phase, secondary metabolites have no apparent biochemical role, and their biosynthesis occurs mainly in the stationary phase of growth (Smith & Moss, 1985; Basílico, 1995).

Thus, through their metabolites, fungi may be beneficial for humanity, in many ways, for instance, playing important roles in the production of cheese, antibiotics, vitamins, enzymes, and glycerol (Davies & Diener, 1987; Ellis *et al.*, 1991). However, many fungi are also harmful and may cause damage to foods or produce toxic substances such as secondary metabolites known as mycotoxins. They can be produced on a wide range of agricultural commodities and in a diverse range of situations. Due to their various toxic effects and their generally good thermal stability, the presence of mycotoxins in foods and feeds is potentially hazardous for the health of both humans and animals and causes very important economic losses (Smith & Henderson, 1991; Shotwell, 1991; Pier, 1992; Laciaková *et al.*, 1995; Pittet, 1998). This topic has a special relevance for rural populations in developing countries, for whom the hazards posed by natural compounds can exceed the effects of manmade chemicals (Thiel, 1998).

None of the mycotoxins has an acute toxicity approaching those of the macromolecular toxins of bacteria. However they may be quite widespread in foods, or in the raw materials used in their production, and some of them may pass through the food chain to commodities which have not been contaminated by moulds (Moss, 1998).

Among terrestrial fungi, filamentous fungi may produce mycotoxins, while yeasts do not produce them (Miller, 1995). The production of these toxic metabolites is not limited to one group of fungi. Although we may find toxigenic fungi within **Zygomycetes** and **Ascomycetes** classes, the principal genera of fungi known to be producers of mycotoxins may be found in the **Hyphomycetes** (Smith & Moss, 1985).

II) Toxigenic fungi and their ecology

II.a) Toxigenic fungi in the fields and in storage

Although all plants may suffer fungal infection, most of the researches have been carried out with cereal grain, since human diet as well as that of domestic animals throughout the world is based on these grains (De Nijs *et al.*, 1996).

Toxigenic fungi associated with crops have been, throughout history, included in two main categories: field fungi and storage fungi (Miller, 1995). However, both concepts are not always representative because of the presence of species which are able to grow in both habitat.

Fungal attack on grains of cereals may already occur when they are reaching maturity in the spikes. The damaging organisms are, among others, species of the genera ***Alternaria***, ***Cladosporium*** and ***Fusarium***. Because they occur in the fields they are called field fungi. They are adapted to changing field conditions related with the gradual maturation of the grains. Humidity contents in the seeds higher than 25 % are necessary for the activity of these fungi (Agarwal & Sinclair, 1987; Moss, 1991). The activity of this mycota reduces the values of seeds and the ability of some species to produce toxic metabolites.

The organisms that produce deterioration and loss of quality in barns, farms, and silos are known as storage fungi. Fungi present in stored seeds have long been known, as saprophytic opportunistic invaders of naturally dried plant tissue or dead organic matter (Hudson, 1986; Chelkowsky 1991). The group comprises a number of xerotolerant species of the *Aspergillus* and *Penicillium*

genera, some of which may be metabolically active in seeds with humidity contents as low as 13 % (Wicklow, 1994). Where their activity is not stopped, by keeping low temperature and storage humidity, they are capable of damaging the seeds in a very short time period (Agarwal & Sinclair, 1987). In developing countries it has been reported that these fungi destroy more than 30 % of the stored grains. Besides quantitative losses, the quality of the stored grains may also be affected, especially in terms of vigour, germinability and nutritious value. A certain number of the species involved produce mycotoxins. Conidia and sclerotia of the most aggressive of these storage fungi are frequently isolated from soil (Cotty, 1989; Kozakiewicz, 1989; Wicklow, 1994). Therefore, the original source of the fungi in both cases is the field (Miller, 1995). Primarily the fungus-host plant relationship and other biological interactions (relations with other microorganisms, insects, etc.) govern fungal invasion before harvest. The growth of fungi after harvest is governed by crop factors (nutrients, genotype and seed resistance), physical factors (temperature, humidity) and biotic factors (interactions with other microorganisms, presence of insects and inoculum ratio). Seeds developed in a plant may therefore be exposed to an infection by field fungi and storage fungi. However, as the seed loses humidity in the maturation process, the intraseminal conditions become more favorable for storage fungi. So, at harvest time, a mixture of both groups is associated with grain tissue, although field fungi are still more frequently isolated. There are not definite limits with respect to the deterioration effects produced by field fungi and those produced by storage fungi (Chelkowski, 1991; Moss, 1991).

From the agricultural point of view, it may be stated, up to this moment, that the most significant toxigenic fungi belong to the *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* genera (Wyatt, 1991; Pittet, 1998; Sweeney & Dobson, 1999;).

II.b) The genus *Fusarium* and its mycotoxins

The genus exhibits a remarkable degree of variability in morphological, physiological and ecological attributes (Logrieco *et al.*, 1999). Some are specifically pathogenic for vegetables, causing putrefaction in roots, death of plantules and cancer in mature plant tissues and other species are saprophytic on senescent plant matter, while there are even some those that cause degradation of industrial products (Smith & Moss, 1985).

Most *Fusarium* spp. are distributed throughout the world and may be isolated from a wide variety of samples, especially in temperate climate zones, where they are considered the most important plant pathogens (Lacey, 1990; Langseth *et al.*, 1993). However, they are found in tropical regions too (Samson *et al.*, 1995; Placinta *et al.*, 1999).

A large number of species belonging to the genus ***Fusarium*** are capable of producing secondary toxic metabolites (De Nijs *et al.*, 1996) and differ from the genus ***Aspergillus*** in that only a few species of the latter produce mycotoxins. De Nijs *et al.* (1996) reported that, out of more than 61 ***Fusarium*** species, 35 were capable to produce some mycotoxins. The main groups of ***Fusarium*** toxins commonly recognized in grains are trichothecenes, zearalenones, and fumonisins (Placinta *et al.*, 1999). In addition, moniliformin, beauvericin, and fusaproliferin were also found in ***Fusarium*** infected cereal ears (Bottalico, 1998).

The ability to produce a particular mycotoxin may vary among isolates of the same species (Miller *et al.*, 1991). Production of mycotoxins may therefore be used chemiotaxonomically to identify ***Fusarium*** isolates as a strain or a variety (Miller *et al.*, 1991; Lori *et al.*, 1992).

The trichothecenes are subdivided into four basic groups, with types A and B representing the most important members. The synthesis of the two types of trichothecenes appears to be characteristic for a particular ***Fusarium*** species (Placinta *et al.*, 1999). ***F. sporotrichioides***, ***F. poae*** and ***F. equiseti*** are considered to be the most important species producers of trichothecenes of Group A (fundamentally T-2 and HT-2 toxins, diacetoxyscirpenol DAS, neosolaniol NS). Production of Group B trichothecenes (deoxynivalenol DON, 3-AcDON, nivalenol NIV and fusarenon-X FUS-X) is generally associated with ***F. graminearum***, ***F. culmorum*** and ***F. croookwellense*** (also zearalenone producers) (WHO 1990, Lori *et al.*, 1992; Miller, 1995). ***F. graminearum***, ***F. culmorum*** and ***F. croookwellense*** (= ***F. cerealis*** (Cooke) Sacc.)also vary in pathogenicity. ***F. graminearum*** is regarded as the most virulent, although all three species can cause pathogenicity. Wheat, corn and barley seem to be the grains that are mostly affected by these pathogens and these crops represent two thirds of the world production of cereals. Contamination with these ***Fusarium*** toxins on rye, oats and triticale have also been reported (Chelkowski, 1989; Miller, 1995). Contamination Tricho-thecene-producing ***Fusarium*** spp. are destructive pathogens and attack a wide range of plant species. Trichothecenes appear to be examples of fungal toxins that can function as virulence factors without strong host selectivity (Desjardins *et al.*, 1996*b*; Desjardins & Hohn, 1997; Hohn *et al.*, 1998).

F. vericillioides (=F. moniliforme) is a serious pathogen of cereals such as maize and rice and its secondary metabolism includes the production of at least three mycotoxins, namely fumonisins, moniliformin and fusarin C (Abbas *et al.*, 1993; Wicklow, 1994; Miller, 1995; Marasas, 1996; Moss, 1998). The production of fumonisins is quite

widespread and a number of other species of *Fusarium*, including *F. proliferatum*, *F. anthophilum*, *F. dlamini*, *F. napiforme* and *F. nygamai*, were able to produce it (Nelson *et al.*, 1992; Moss, 1998). The fumonisins (FB_1, FB_2 and FB_3) are also structural congeners of certain toxins produced by *Alternaria alternata* (Chen *et al.*, 1992; Placinta *et al.*, 1999). It is also believed that fumonisins might have an important role in the pathology of *F. verticillioides* on corn (Nelson *et al.*, 1993; Desjardins & Hohn, 1997). Other studies indicate a probable association between fumonisins production and high levels of virulence on corn, tomato and other plant buds (Desjardins *et al.*, 1995).

II.c) The genus *Aspergillus* and its mycotoxins

The genus *Aspergillus* has a high metabolic versatility, a great ability to disperse its conidia, and many of its species are capable of developing at low values of water activity (a_w). This fact allows them to grow in a wide range of natural substrata and climatic conditions, and to be associated with deterioration of materials that are too dry to be attacked by other microorganisms (Gourama & Bullerman, 1995*b*). Thus, the *Aspergillus* species commonly affect food products and other materials such as wood, leather, textile fibres, kerosene, paints, plastics, rubber, cement and pharmaceutical products (Smith & Moss, 1985; Gourama & Bullerman, 1995*b*). Some *Aspergillus* species are used in the manufacture of foods and compounds used in the food industry. Thus *Aspergillus oryzae* has been used in the manufacture of "koji", an intermediate product in the manufacture of oriental fermented foods. *Aspergillus niger* has been used for the production of citric acid.

Though most of aspergilli are essentially saprophytic, other species may be allergenic, toxigenic and pathogenic for men and animals (Gourama & Bullerman, 1995*b*).

Aspergillus ochraceous (= A. alutaceus) is present in soil, grains and vegetables and in the process of decomposition. This species and other related ones, as well as *Penicillium verrucosum* and *Penicillium cyclopium* (belong to *P.aurantiogriseum/verrucosum* complex) produce a group of structurally related metabolites known as ochratoxins (Prelusky *et al.*, 1994). The most important toxin in the group, ochratoxin A, has been isolated from a wide variety of plant products, cheese and tissues of animals that had eaten contaminated feed, though its phytotoxicity is not known (Fink-Gremmels *et al.*, 1995; Desjardins & Hohn 1997). *A. ochraceous* is also capable of producing penicillic acid.

Aspergillus versicolor may be isolated from soil, mature cheese, cured meat or decomposing vegetables and is capable of producing sterigmatocystin (ST), an intermediary in aflatoxin biosynthesis, as well as cyclopiazonic acid (CPA). The latter, originally isolated from a *Penicillium cyclopium* culture during a routine screening of toxigenic fungi, may be also produced by different fungi found in agricultural products (*A. versicolor*, *A. flavus*, *A. tamarii*) or by fungi used in the production of fermented foods (*Penicillium camembertii* and *A. oryzae*). CPA has been found to occur naturally in corn, peanuts and cheese.

Aspergillus clavatus may be found in animal excrements, soil and decomposing organic matter. Among other toxic metabolites, this species may synthesize patulin, also produced by species of *Penicillium*.

The species of Aspergillus belonging to the Section Flavi W. Gams *et al.*,1985(=*A.flavus* group.) are present in soil and contaminate a wide variety of agricultural products in the fields, storage areas, processing plants and during distribution of such products. *A. flavus*, *A. parasiticus* and *A. nomius* may produce aflatoxins (Gourama & Bullerman, 1995*b*; Moss, 1998). The four main naturally produced aflatoxins are B_1, B_2, G_1 and G_2, with B_1 (AFB_1) usually being the aflatoxin found at the highest concentration in contaminated food and feed. The *A. flavus* strains vary from non-toxic to those which produce aflatoxins B_1 and B_2, while *A. parasiticus* may produce aflatoxins B_1, B_2, G_1 and G_2. *A. parasiticus* tends to be more stable in its production of aflatoxins than *A. flavus* (Gourama & Bullerman, 1995*b*).

All aflatoxin producer fungi are soilborne microorganisms, but there are some differences in the occurrence pattern (Diener *et al.*, 1987; Domer *et al.*, 1989). *A. flavus* conidia are more common in air than in soil, and are generally found in tropical and mild regions. *A. parasiticus* is adapted to warm environments such as tropical and sub-tropical regions and has been often found to be associated with soil. So, *A. parasiticus* is a more frequent contaminant for peanuts while *A. flavus* contaminates corn. Wicklow, has reported (1994) hat sclerotia constitute the primary inoculum in cornfields. Nevertheless, high concentrations of aflatoxin are produced as a result of postharvest spoilage of commodities stored under warm moist conditions, and significant concentrations may also be produced in the field before harvest (Payne, 1992; Moss, 1998; Placinta *et al.*, 1999). Although aflatoxins have been reported to be phytotoxic, it is still necessary to determine the true role of aflatoxins in fungus-plant interaction (Desjardins & Hohn 1997).

Biotransformation of aflatoxins B in some animal species, including humans that have consumed AFB_1 or AFB_2 contaminated materials, results in the production of aflatoxins M_1 and M_2, which are excreted in milk and urine. AFM_1 has been widely found in a number of food products including infant formula, dried milk, cheese and yoghurt (Galvano *et al.*, 1996).

II.d) The genus *Penicillium* and its mycotoxins

Man in the production of foods and antibiotics has used some species of *Penicillium*. *P. roqueforti* and *P. camembertii* are employed in the manufacture of cheese matured by fungi while *P. chrysogenum* has been the main source in the penicillin industry (Smith & Moss, 1985).

However, in moderate climate regions these are the dominant fungi associated with food decay. Although they are essentially saprophytic, some species show their ability to colonize certain fruits and vegetables. Thus *P. digitatum* and *P. italicum* are the green and blue fungi respectively found in citrus fruit while *P. expansum* attacks apples and *P. gladioli* attacks bulbs, including onions.

The genus *Penicillium* contains many toxigenic species (approximately 100) and the range of mycotoxin classes produced is much broader than that of any other genus (Sweeney & Dobson, 1998). Pitt (1991) and Pitt & Leistner (1991) list 27 mycotoxins produced by 32 species, which possess demonstrated toxicity.

As previously mentioned ochratoxin A is produced not only by *A. ochraceous* but also by *P. verrucosum* and related species *(P.aurantiogriseum/ verrucosum* complex*)*. *Penicillium* species primarily produce ochratoxin in temperate climates while *A. ochraceous* strains are more commonly associated with warmer climates (Sweeney & Dobson, 1998).

P. citrinum is widely distributed in the world in foods, decomposing plant matter and textile fibers and may produce citrinin. This mycotoxin may be also synthesized by *P. verrucosum* strains present in cereal and by-products, and by *P. expansum* (Smith & Moss, 1985; Sweeney & Dobson ,1998).

P. expansum, a fruit pathogen, may be isolated mainly from apples and pears. It can be a producer of citrinin as well as patulin (McKinley & Carlton, 1991). The major source of patulin in the food supply is juice from apples infected with *P. expansum* (Pittet, 1998). *P.aurantiogriseum/verrucosum* complex (including *P. cyclopium* and others (Lund & Frisvad, 1994; Sam-son *et al.*, 1995) is also frequent in cereal and is capable of producing CPA and penicillic acid, and even penitrem A. Some strains of *P. purpurogenum*, a soil fungus primarily associated with the deterioration of many substrates, produce rubratoxins. *P. roqueforti* isolated from blue cheese and from other products stored in cold, may produce PR toxin, isofumigaclavines and roquefortine (Smith & Moss, 1985).

IIe) The genus *Alternaria* and its mycotoxins

The genus *Alternaria* is ubiquitous in the atmosphere as well as in soil, seeds, and in agricultural commodities. It includes both plant pathogenic and saprophytic species that may damage crops in the field or cause postharvest decay of plant products in storage (Bottalico & Logrieco, 1998). Some species may often grow at low temperatures and may be associated with important damage to fruit and vegetables during transportation and storage in refrigerated conditions (Magan & Lacey, 1985).

Species of *Alternaria* are known to produce many metabolites, mostly phytotoxins, which play an important role in the pathogenesis of plants. Conidia of certain species, in particular *A. alternata* aggr. are abundant in the air, especially during cereal maturation and harvest. They are capable of producing several toxic metabolites in infected plants and/or in agricultural commodities, which can contaminate foods and feeds and elicit adverse effects in animals (Bottalico & Logrieco, 1998). Besides at least 70 secondary metabolites can be produced by species of *Alternaria*, only seven major toxins are known as possible food contaminants with a potential toxicological risk. These are tenuazonic acid (TA), alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), and altertoxin I, II, and III (ATX-I, ATX-II, ATX-III). The effects of environmental factors on the production of these mycotoxins have rarely been studied in detail (Bottalico & Logrieco ,1998).

It has been demonstrated that AAL toxins (structurally similar to fumonisins) play an important role in the pathogenesis of *A. alternata* f.sp. *lycopersici* on certain tomato genotypes (Desjardins & Hohn, 1997).

III) Control of food contamination with mycotoxins

In the last thirty years a tremendous expansion of research on mycotoxins has taken place. The knowledge gathered during these three decades has introduced us to a new era of multiple discoveries (Stoloff, 1979). They include a better understanding of the biology of the involved fungi, the mechanisms that initiate the production of mycotoxins, their biosynthetic production paths, factors and conditions that determine fungal infection and colonization of a substrate, and the type and quantity of mycotoxin produced.

Perhaps the most important challenge today is to develop a package of control measures which, combined with the achieved knowledge might help in the development of cultural behaviours that would help to prevent or reduce contamination to manageable levels (Widstrom, 1996). With this aim in mind, provisional strategies aiming to solve the present problem have been proposed while control measures intended to be definitive are being developed.

IIIa) Provisional strategies

III.a.1) Handling of conditions during the sowing and development of the plant

Fungal growth and mycotoxin biosynthesis are

a consequence of an interaction between the fungi, the host (the plant) and the environment. In natural conditions there exists an interaction – more complex than laboratory conditions - between the toxigenic fungus and the substrate, no matter whether this association occurs in a field crop, in stored grain or in any other type of food. Variability of environmental conditions is added to the interaction of other biological factors, different from the fungal and plant genotypes, which may have influence on their growth and metabolism.

Assuming that the farmer is restricted to cultivate in a limited area, climatic conditions and the kind of soil are almost firmly fixed. The importance of the soil as a source of inoculum has been demonstrated (Widstrom, 1996). It was determined that some plowing techniques such as continuous crops favour the formation of sclerotia on the residues that remain in the fields after harvest, turning them into an important source of inoculum for the following years (Wicklow, 1994). The use of rotations - routinely recommended to obtain a good production - is also effective to reduce mycotoxin contamination of the crops (Wilson *et al*,. 1989; Widstrom, 1992). Elimination of weeds reduces water consumption and removes an additional reservoir of fungal propagules. Processes of fertilization to obtain a good production are also adequate to diminish contamination with mycotoxins.

The selection of the appropriate seed is of vital importance. The suggestions include use of seeds without fungi, use of hybrids that combine both adaptation to the medium and greater resistance to insects though the thickness of the cover (Widstrom *et al.*, 1994). It has also been detected that certain cotton and peanut tissues, under certain conditions, react to the fungal attack producing considerable levels of certain antifungal compounds called phytoalexins. These substances are believed to have different effects on *A. flavus* physiology such as to prevent fungal growth or to inhibit the first stages of aflatoxin synthesis (Santamarina *et al.*, 1995).

The stress produced by excess water, drought, high temperatures, malnutrition and damage produced by rodents, birds or insects on the host plant may contribute significantly to increase the infection with toxigenic fungi in the spikes, damaging grain, transporting conidia and notoriously increasing the mycotoxin concentration (Moss , 1991).

In warm climates, conidia and sclerotia of the *A. flavus* group are usually present in the soil or on cereal plants providing an important inoculum source (Wicklow , 1994; Widstrom, 1996). It is generally affirmed that the process of infection occurs in the field where *Aspergillus* propagules may attack developing flower stigma (spikes in anthesis), or infect spikes from one to two weeks after their fertilization, increasing aflatoxin accumulation through grain maturation (Widstrom, 1996). Conidia may germinate and the germinative tubes penetrate the tissues of developing seeds, or the fungus may grow throughout the spike and eventually penetrate through lesions or ruptures in the pericarp and move through areas of the pedicel (Zummo,1991). The main place of grain invasion is apparently the same for grain matured in the field as for storage grain (Widstrom, 1996).

Detailed studies of associated climatic conditions have concluded that high temperatures and low humidity content during the development of some crops (corn, peanuts, cotton, etc.) are significantly correlated to an important contamination with aflatoxins (Widstrom *et al.*, 1990; Gourama & Bullerman, 1995*b*). Using an irrigation system, especially during the reproductive period of the plant (Jones, 1987), or adapting the sowing date for this critical period to coincide with the moment of minimum stress for lack of water could reduce the stress produced by drought (Widstrom *et al.*, 1990).

In mild climates, after a series of rains, both ascospores and macroconidia of *F. graminearum* and related species may infect wheat and corn with high humidity. They are more susceptible to infection during the first stages of anthesis (Snijders,1994). If the temperature later becomes moderate, an important contamination with DON may be produced, but if the temperature drops *F.graminearum* may produce zearalenone (Martin, 1993). In these cases there are not provisional solutions available and it is necessary to carry out strict control prior to storage.

Other fungi found in the soil or on decomposing plant material, for instance *P. verrucosum* and *A. ochraceous*, may also invade grain during development in the fields and later proliferate in storage if conditions are favourable, producing penicillic acid or ochratoxin A (Miller, 1995).

In nature a fungal strain generally interacts with other microorganisms and the simultaneous growth of different species usually occurs on a certain food. That may alter the metabolism of toxigenic fungi, compete for the necessary substrate, produce unfavourable conditions for the formation of mycotoxins, metabolise the toxins produced or even stimulate the production of one or more mycotoxins (Ellis *et al.*, 1991). (These interactions will be discussed in the section on biological control).

Some biocides used in agriculture inhibit the formation of mycotoxins, while others may favour their biosynthesis. Up to now, only laboratory tests have been carried out (Zaika & Buchanan, 1987, Gourama & Bullerman, 1995*b*). Therefore, the application of herbicides and/or insecticides on growing plants must be carefully evaluated.

Summarizing, serious programs for crop handling must include conditions that lead to the decrease of stress

throughout the whole life of the plant. Also, frequent field inspections could help in early detection of drought stress, nutritional deficiencies or damage caused by insects, birds and rodents (Widstrom, 1996). This procedure may be critical to minimize the risk of an eventual contamination with mycotoxins.

III.a.2) Handling of the product during harvest and storage

It is important to harvest as soon as possible once physiological maturity has been reached to maintain the grain quality and minimize losses. Intact grains must be separated from foreign materials and broken grains, and dried until a moisture content is attained which prevents a later fungal growth (12 to 14 %) (Gourama & Bullerman, 1995*b*). With favourable climatic conditions (especially temperature and humidity) grains may be dried in the fields, since artificial drying represents the most important expense of harvest.

Storage conditions play an important role in the physicochemical and microbiological quality of products. Humidity levels and temperature are the most important factors to be taken into account in the protection of stored grains from fungal growth and mycotoxin production (Chatterjee 1990; Ellis *et al.*, 1991; Widstrom, 1996).

Humidity is essential for fungal growth. It has been demonstrated that *A. flavus*, for example, will not invade stored cereal grains and oleaginous seeds with a relative humidity of less than 70 %. At this relative humidity, wheat humidity content is approximately 13 % and 7-10 % for products rich in oil, such as peanuts and cotton (Ellis *et al.*, 1991).

To control fungal growth, various studies have verified that temperature should be reduced to 5 °C as soon as possible, especially in perishable products. However, the use of low temperatures for the storage of agricultural products on a large scale is generally economically unfeasible.

The presence of insects in stored products indicates that the temperature and / or humidity have increased in some "hot spots" since most insects are not capable of living and reproducing at low levels of humidity and temperature. Good aeration is indispensable if one aims at keeping the grains cold and uniformly dry (Smityh, 1990).

Conservation of products in plastic hermetic containers or sealed plastic folders under refrigerated conditions may maintain the humidity, especially on the surface, providing favourable conditions for fungal growth, thus precautions must be taken so as not to create a propitious environment for mycotoxin synthesis.

The gaseous atmosphere is another critical environmental factor that has its influence on fungal growth. For the conservation of some foods either storage in controlled atmosphere (CAS) or package in modified atmosphere (MAP) may be used (Ellis *et al.*, 1991). In CAS the gaseous atmosphere is modified to a desired level and kept at that level throughout the whole storage. Thus CAS has been applied to control the growth of *A. flavus* and the production of aflatoxins in peanuts stored in bulk (Pitt *et al.*, 1993) and to prevent contamination by insects in stored grains (Ellis *et al.*, 1991). MAP consists of packing food products in materials with a gaseous barrier, where the gaseous environment has been changed (generally increasing the CO_2 content) to slow down breathing, reduce the microbiological growth and delay enzymatic damage. Not many data with respect to the production of mycotoxins in foods packed in MAP are available yet. However, both CAS and MAP seem to be natural and economical methods to control fungal growth as well as the production of mycotoxins in stored products.

III.b) Measures of definitive control

Permanent solutions to the problem of contamination of grain and oleaginous seed with mycotoxins require time for their development and implementation. A definitive method for the control of pests and diseases is based on the creation of resistance in the host plant. A second alternative to achieve a solution in the long run has been the imposition of chemicals and / or conditions on the fungus, that inhibit mycotoxin production. Another means of control is the use of other microorganisms that might limit the growth of toxigenic fungi by different mechanisms. A last alternative might be genetic manipulation of the fungus to cause an interruption in its ability to produce mycotoxins (Widstrom, 1996).

III.b.1) Development of resistant hybrids

From the beginning most of the research has been focused on obtaining plants resistant to aflatoxin contamination (Zuber, 1977). A reliable identification of the type of plants resistant to fungal invasion was not possible until a uniform inoculation could be achieved under field conditions. It was also necessary to determine the most appropriate moment and method for inoculation and the time and way in which the samples had to be taken in developed grains (Widstrom, 1996). The subsequent screening revealed that even if evaluation and selection were difficult, the processes of infection and contamination would be under genetic control (Zuber & Lillehoj, 1979). Various important sources of genetic resistance have recently been identified (Scott & Zummo 1988; Campbell *et al.*, 1993) resulting in obtaining resistant germplasm (Scott & Zummo,1992; McMillian *et al.*, 1993). Therefore, the chances of finding and developing other sources of resistance are good and could, in the future, provide the cereal with defense against invasion of toxigenic fungi and subsequent production of mycotoxins (Sweeney & Dobson, 1999).

III.b.2) Interruption of the toxin production

Interruption of the mycotoxin production processes may be also achieved by controlling the microenvironment where these fungi develop or by establishing methods of chemical or biological control.

III.b.2.a) Chemical interference in the production of toxins

A number of compounds that inhibit or suppress fungal growth and / or the formation of toxins have been tested. Most of them have been evaluated in stored products and consequently they have not been suggested as control agents for developing plants. Among these compounds are potassium metabisulfite, sodium bicarbonate, different phosphate compounds and several alkenals (Lebron *et al.*, 1989; Montville & Goldstein, 1989; Sharma *et al.*, 1988; Zeringue, 1991). However, none of them has been recommended as an adequate control agent from a commercial point of view.

Compounds from different biological sources, as o-vanillin, volatile extracts and compounds of *Azadirachta indica* leaves, spices and their oils, have also been tested as inhibitors of mycotoxin production (Bhatnager & McCormick ,1988; Chatterjee, 1990; Ranjan *et al.*, 1992; Zeringue & Bhatnager, 1994).

Finally, compounds isolated from corn itself have also been reported as having limiting activity against *A. flavus* and aflatoxin production (Neurece & Godshall, 1991; Neurece, 1992). Experiments by Brown *et al.* (1993), provide evidence that resistance to aflatoxin contamination is related to metabolic activities in the living corn embryo.

III.b.2.b) Interference or biological control

In Nature, fungi not only interact with the host but also with many microorganisms present in the infection zone. In order to succeed, the fungus must attack the host and repel or compete with other organisms at the same time (Cook & Baker, 1983). Therefore, the control of plant diseases using biological strategies may be achieved by regulating the pathogen population or excluding them through beneficial microorganisms (Gabriel & Cook, 1990; Scott, 1995). It is desiderable, in general, that the introduced organisms are already part of the ecosystem and not part of exotic environment. The biological control offers less exposure to potentially toxic pesticides, fewer residues on the surface of marketed products and in consequence a lower risk of environmental pollution than chemical control as well as a more profitable cost-benefit relation (van Lenteren, 1992; Gloer, 1995).

Interest in new biological methods of preservation has increased in recent years, supported by investigations that point out that those antagonistic organisms or their antimicrobial metabolites might act as natural preservatives to control toxigenic fungal growth (Schillinger *et al.*, 1996).

Thus, tests of intraspecies or interspecie competition have been made. The first type of biocon petitive control involves the use of non-toxigenic strai isolated from the same habitat where the toxigenic strai are found, either as agents of growth limitation and / or a inhibitors of mycotoxin production. The strain stability is fundamental criterium in the selection of this type biocontrol agent (Cotty, 1990). In these tests, carried out i greenhouses or in the fields, atoxigenic strains (highl competitive) were inoculated together with th corresponding toxigenic strains in corn ear (Brown & Cott 1991), peanuts or cotton bolls (Cotty, 1990), or roots i developing plants (Chourasia & Sinha, 1994), or added t the soil where cereals or oleaginous plants were growin (Dorner *et al.*, 1992; Cotty,1994; Dorner *et al.*, 1998; Dorn *et al.*, 1999). In most cases the atoxigenic strain diminishe mycotoxin contamination of the seeds when it was applie simultaneously with, or previously to the toxigenic strai (Cotty, 1990; Brown & Cotty, 1991; Dorner *et al.*, 199 Chourasia & Sinha, 1994; Cotty,1994). However, differenc were found in the ability of atoxigenic strains to preve mycotoxin production, especially of aflatoxins (Cotty Bhatnager, 1994; Dorner *et al.*, 1998; Dorner *et al.*, 1999 Data also indicated that a higher degree of control migl be achieved when plots or fields were retreated wit biocontrol agents in subsequent years (Dorner *et al.*, 1998

Interspecies competition tests, in which toxigen strains of ***Aspergillus***, ***Fusarium*** or ***Penicillium*** we confronted in the laboratory, in greenhouses or in the fiel with different biocompetitive agents (also isolated fro the same ecological niche as the organism to be controlled presented more variable results (Misaghi *et al.*, 199 Among them different species of filamentous fun (***Aspergillus niger***, ***Aspergillus auriconus***, ***Penicillium*** sp., ***Fusarium*** sp., ***Trichoderma*** sp., ***Rhizopus*** sp., etc (Ehrlich *et al.*, 1985; Devi & Polasa, 1987; Wicklow *et a* 1987; Cuero *et al.*, 1988; Choudhary,1992; Widstrom *et a* 1995) and even a yeast (***Pichia guillermondii***) (Schilling *et al.*, 1996) could inhibit the development of toxigenic fun and/or the production of mycotoxins or they could influen the fungal patterns of colonization *in vitro* (Marin *et a* 1998*a*, Marin *et al.*, 1998*b*; Marin *et al.*, 1998*c*).

In greenhouse and field tests a significa decrease in infection of corn spikes with ***A. flavus***, and the subsequent production of aflatoxins, due to inoculati with ***F. moniliforme*** was reported (Wicklow *et al.*, 198 Widstrom *et al.*, 1995). The reduction in the mycotox production by competition between ***Aspergillus*** a ***Fusarium*** sp. was smaller in the field than in the laborato (Ehrlich *et al.*, 1985; Widstrom *et al.*, 1995). Besides, it h also been suggested that the prospective use of other fung strains as competitors of toxigenic fungi is not promisin It may probably be due to the fact that, in Nature, the mc

appropriate time and means of infection are not the same for both fungi (Widstrom *et al.*, 1995).

In the last few years the objective of different investigations was to study the influence exerted by exometabolites of various microorganisms (other fungi and bacteria) on the growth and production of mycotoxins by certain fungal strains. Inhibition of growth of *A. flavus* and *A. parasiticus* was produced by metabolites of *Rhizopus microsporus*, *R. arrhizus* and *Neurospora sitophila* (Nout, 1989; Lanciotti & Guerzoni ,1993; Nout ,1995). Similar effects were produced on *Penicillium roqueforti* by volatile antagonistic compounds of *Pichia anomala* (Bjornberg & Schnurer, 1993). Substances produced by other aspergilli (*A. niger*, *A. oryzae* and *A. tamarii*) also inhibited the growth of *A. flavus*, *A. ochraceous* and their mycotoxin production (Shanta & Rati ,1990; Shanta *et al.*,1990; Paster *et al.*, 1992, Sardjono *et al.*, 1992).

Similarly, there have been attempts at biocontrolling toxigenic strains with several bacteria. Some lactic acid bacteria (LAB) have been tested. In the majority of reports it was postulated that the cause of this inhibition was the action of a metabolite present in the culture supernatants of lactic bacteria (Gourama & Bullerman , 1995*a*). It was reported that the effect of two strains of lactic bacteria (*Lactococcus lactis* and *Streptococcus termophilus*), with antifungal activity against *A. parasiticus*, *A. fumigatus* and *Rhizopus* sp. The activity was produced by a compound probably of polypeptide nature since its activity was destroyed by the action of proteolytic enzymes (pronase E and trypsin) (Batish *et al.*, 1990; Batish *et al.*, 1991). The culture supernatant of a mixture of *Lactobacillus* species inhibited the production of aflatoxins by *A. parasiticus* also reducing the fungal growth (Gourama & Bullerman, 1995*c*). This inhibition was probably due to a metabolite of molecular mass > 6000 D. Gourama & Bullerman, (1995*a*). Karutnaratne *et al.*, (1990) have reported that several *Lactobacillus* spp. strains totally inhibited the conidial germination of aflatoxigenic fungi. Other compounds produced by *Lactobacillus* spp. only inhibited the biosynthesis of aflatoxins B_1 and G_1 by *A. parasiticus* (Karutnaratne *et al.*, 1990) and were to be sensitive to the action of proteolytic enzymes (trypsin and α-chymotrypsin) and of heat (Gourama & Bullerman ,1997).

The antimicrobial activity of other bacteria among toxigenic species of *Aspergillus*, *Fusarium* and *Penicillium* was studied (Motomura & Hirooka, 1996). Among them strains of *Bacillus subtilis* produced substances which suppressed the growth of toxigenic *A. parasiticus* and *A. flavus* and blocked the synthesis of aflatoxins (Kimura & Hirano, 1988; Ono & Kimura, 1991). Some of these metabolites had peptide characteristics (Ono and Kimura, 1991). In other cases, certain strains of *B. subtilis* were active against the development of *Fusarium verticillioides*, *A. parasiticus* and *Penicillium expansum* (Motomura & Hirooka, 1996). Another bacterial strain, identified as *Pseudomonas cepacia*, completely inhibited *A. flavus* growth in synthetic media and reduced the damage produced in cotton bolls by this fungus. This was the first report of an antagonistic bacterium capable of reducing the damage caused by a toxigenic fungus on a plant in the fields (Misaghi *et al.*, 1995).

Bacteria of the *Streptomyces* genus are very abundant in soil and they are included in the literature within the main producers of bioactive compounds and extracellular enzymes. Their effectiveness has been demonstrated against bacteria, fungi, some protozoa and nematodes (Dicklow *et al.*, 1993; Coelho *et al.*, 1995;Trejo-Estrada *et al.*, 1998). Substances produced by *Streptomyces* spp. strains that could inhibit growth of toxigenic fungi or their mycotoxin production are very few. A *Streptomyces* sp. strain produced a new compound that inhibits the synthesis of aflatoxin B_1 by *A. parasiticus* (Ono *et al.*, 1997). This compound, a polyalcohol of 63 carbon atoms called aflastatin A, did not present any effect on the fungal development.

As part of a screening of *Streptomyces* strains isolated from soil of cereal crop, a strain with activity inhibiting conidia germination of *A. parasiticus* (aflatoxin producer), *Fusarium graminearum* (deoxynivalenol and nivalenol producer) and *Fusarium tricinctum* (T-2 and HT-2 toxins producer) was selected. This effect was due to the presence of antifungal substances (Borghi *et al.*, 1988; Fulgueira *et al.*, 1989; Borghi *et al.*, 1992). In experiments carried out on developing wheat plants (in greenhouse) it was determined that the *Streptomyces* sp. strain stopped fungal colonization and its effects: decrease in weight of the wheat grains caused by *F. graminearum* and its mycotoxin production (Fulgueira *et al.*, 1992, Fulgueira *et al.*, 1996; Fulgueira, 1998). Subsequent studies demonstrated that the inhibiting activity was sensitive to proteolytic enzymes (pronase and elastase) and to heat and the substance could have a molecular mass between 8 and 20 kDa. Up to now the majority of experiments on the control of toxigenic fungi have been developed for corn, peanuts and cotton plants, and different *Streptomyces* spp. have been successfully used to decrease damage produced by pathogenic fungi soilborne in some cereals and cruciferous plants (Tahvonen *et al.* ,1994). These reports represent the first finding corresponding to the reduction of the effect produced by toxigenic fungi in wheat plants achieved by the application of an antagonist strain of *Streptomyces* sp.

III.b.3) Genetic manipulation of the toxigenic fungi

Information about mycotoxin synthesis slowly began to appear in the literature during the 1970s (Papa,

1979). Work on the biosynthetic pathway of aflatoxin B_1 began using *A. parasiticus* as fungal model, developing new strategies for identifying genes and pathways for different aflatoxins (Bhatnager *et al.*, 1989; Bhatnager *et al.*, 1991).

A wide range of *Aspergillus* spp. can synthesize a precursor of aflatoxins, sterigmatocystin. The first step in the biosynthesis of sterigmatocystin/aflatoxins is catalyzed by a type I polyketide synthase (Feng & Leonard, 1995). In contrast with most polyketide synthases that use acetate as a precursor, the precursor for the aflatoxin/sterigmatocystin enzyme is hexanoate (Brobst & Townsend 1994). The synthase reaction product and first stable intermediate in the pathway is norsolorinic acid, which undergoes a complex series of modifications to yield sterigmatocystin and, finally aflatoxin. Up to now, many of the genes involved in the sterigmatocystin biosynthetic pathway have been cloned and their functions identified. Studies in *Aspergillus nidulans* have shown that the gene encoding the polyketide synthase (*pksST*) is part of a gene cluster containing at least 25 pathway-related genes (Brown *et al.*, 1996). This gene cluster occupies a 60-kb region and contains genes for regulatory factors in addition to all of the required pathway enzymes. The genetics of the aflatoxin biosynthetic pathway have also been elucidated. Mapping studies indicate that all the cloned genes involved in the aflatoxin biosynthetic pathway are contained within a 75-kb cluster located on a single chromosome in *A. flavus* and *A. parasiticus* (Trail *et al.*,1995; Yu *et al.*, 1995; Brown *et al.*, 1996; Silva *et al.*, 1996; Desjardins *et al.*,1997). Genetic studies on aflatoxin biosynthesis in *A. nidulans*, led to the cloning of 17 genes responsible for 12 enzymatic conversions in the AF/ST pathways. Pathway-specific regulation is by a Zn (II) 2 Cys 6 DNA - binding protein that regulates aflatoxin biosynthesis but there is a clear link between development and aflatoxin biosynthesis (Payne & Brown 1998).

The biosynthesis of trichothecenes by *Fusarium* spp. proceeds from the hydrocarbon trichodiene through a complex series of steps to trichothecenes such as DAS, DON and T-2 toxin. The details of trichothecene biosynthesis have been established through experiments with a number of *Fusarium* sp. (Desjardins *et al.*, 1993). In common with the biosynthetic genes for aflatoxins and many other microbial antibiotics, trichothecene pathway genes in *Fusarium* are closely linked and constitute a gene cluster (Hohn *et al.*, 1995). At least 10 pathway genes involved in trichothecene biosynthesis have been identified within a 23-kb region of chromosomal DNA in *F. sporotrichioides* (Hohn *et al.*, 1998). The cluster contains *Tri5*, the gene encoding trichodiene synthase, which catalyzes the first step in trichothecene biosynthesis (Desjardins & Hohn 1997). Recent investigations of the trichothecene pathway gene cluster have provided new information concerning the transcriptional regulation of pathway gene expression (*Tri6*) and the transport of pathway products (*Tri2*). A trichothecene resistance gene (*Trir*) has also been identified in *F. sporotrichioides*. Expression of microbial trichothecene resistance gene in wheat map provides a means for further investigating the importance of trichothecenes in *Fusarium* wheat head scab (Hohn *et al.*, 1998).

In the case of fumonisins, their structural similarity to the long chain sphingolipid bases suggests that their biosynthesis may be similar to sphingolipid biosynthesis. Genetic crosses using naturally occurring fumonisin production variants have identified the fumonisin biosynthetic genes in Gibberella fujikuroi (*Fusarium verticillioides*). Three genes have been identified, *fum1*, *fum2* and *fum3*. These genes are linked and appear to form a fumonisin biosynthetic gene cluster on chromosome 1 of *Gibberella fujikuroi* (Desjardins *et al.*, 1996*a*, Desjardins & Hohn 1997).

Molecular genetic analysis of the biosynthetic pathways of other mycotoxins of *Fusarium*, *Aspergillus* and *Penicillium* is not very far advanced.

Much progress has been made on the molecular characterization of the genes involved in the biosynthesis of various mycotoxins. This knowledge is useful in order to understand the organisation, regulation and expression of these genes, the physiological factors controlling these processes and the role of each mycotoxin in the plant pathogenesis. In addition it aids improvement of molecular-based detection methods for mycotoxins and mycotoxigenic fungi in food systems. Also, these findings may allow the development of measures for the biological control of toxigenic fungi and the development of genetically engineered resistant crop plants (Sweeney & Dobson 1999).

The availability of a variety of molecular techniques has made possible precise studies on genetics affinity and phylogeny of populations of toxigenic fungi. Correct identification by morphological, genetical, and molecular approaches as well as toxicological characterization of toxigenic species that colonize crop plants and food is a major task in order to assess the potential risks of mycotoxin accumulation and prevent animal and human mycotoxicoses (Logrieco *et al.*, 1999).

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