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Doctoral School on the Agro-Food System

Cycle XXII

S.S.D. AGR/12

Fusarium verticillioides in maize: how abiotic and biotic factors can influence growth and fumonisins production in field and during storage

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Reviewers: Prof Rossi Vittorio
Prof Meriggi Pierluigi

Academic Year 2008/2009

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CHAPTER 1

INTRODUCTION

1.1 The origin of maize

Maize (*Zea mays L*. ssp. *mays*; also known as corn) is one of the most widely distributed food plants in the world and is a cereal grain that was domesticated in Mesoamerica and then spread throughout the American continent. During the 1st millennium, maize cultivation spread from Mexico into the Southwest and a millennium later into Northeast and south-eastern Canada transforming the landscape as Native Americans cleared large forest and grassland areas for the new crop (Sprague, 1977).
Maize was spread to the rest of the world after European contact with the America in the late 15th and early 16th century.
The term *maíze* derives from the Spanish form of the Arawak Native American term for the plant. This word literally means “that sustain life”.
There are several theories about the specific origin of maize in Mesoamerica. First it is a direct domestication of a Mexican annual teosinte and this model was proposed by Nobel Prize winner George Beadle in 1939.
Second it derives from hybridisation between small domesticated maize and teosinte of section *Luxuriantes.* The second explains many conundrums but is dauntingly complex.
For the third model it underwent two or more domestications either of wild maize or of a teosinte.
In the late 1930s, Paul Mangelsdorf suggested that domesticated maize was the result of a hybridisation event between an unknown wild maize and a species of *Tripsacum*, a related genus.
However, the proposed role of *Tripsacum* (gama grass) in the origins of maize has been refused by modern genetic analysis, negating Mangelsdorf’s model.
The third model (actually a group of hypotheses) is unsupported.
The domestication of maize is of particular interest to researchers.
The process is thought by some to have started 7,500 to 12,000 years ago (corrected for solar variations). Recent genetic evidence suggests that maize

domestication occurred 9000 years ago in central Mexico. The wild teosinte most similar to modern maize grows in the area of the Balsas River. Archaeological remains of early maize cobs, found at Guila Naquitz Cave in the Oaxaca Valley, date back roughly 6,250 years.
Perhaps as early maize began to spread widely and rapidly as it was introduced to new cultures, new uses were developed and new varieties selected to better serve in those preparations. Maize was the staple food, or a major staple, of most the pre-Columbian North American, Mesoamerican South America, and Caribbean cultures.
The Mesoamerican civilization was strengthened upon the field crop of maize; through harvesting, its religious and spiritual importance and how it impacted their diet.
Maize formed the Mesoamerican people identity.

1.2 The maize plant

1.2.1 Cultivation

Maize is widely cultivated throughout the world, and a greater weight of maize is produced each year than any other grain. While the United States produces almost half of the world's production (about 42.5%), other top producing countries are as widespread as China, Brazil, France, Indonesia, India and Africa. Worldwide production was around 800 million tonnes in 2007 with over 150 million hectares and a yield of 4971 kg/hectare (FAO, 2007).

1.2.2 Genetic

The maize is an annual graminaceous plant belonging to the tribe of Maydeae.
Many types of maize are used for food, sometimes classified as various subspecies:

- Fluor corn− *Zea mays var. amylacea*
- Pop corn− *Zea mays var. everta*
- Dent corn− *Zea mays var. indentata*

- Flint corn− *Zea mays var. indurata*
- Sweet corn− *Zea mays var. saccharata* and *Zea mays var. rugosa*
- Waxy corn− *Zea mays var. ceratina*
- Amylomaize− *Zea mays*
- Pod corn− *Zea mays var. tunicate* Larrañaga ex A. St. Hil
- Striped maize− *Zea mays var. japonica*

This system has been replaced over the last 60 years by multi variable classifications based on ever more data. Agronomic data was supplemented by botanical traits for an initial classification, than genetic, cytological protein and DNA evidence was added.
Maize has 10 chromosomes. In 2005, the U.S. National Science Foundation (NSF), Department of Agriculture (USDA) and the Department of Energy (DOE) formed a consortium to sequence the maize genome. The resulting DNA sequence data will be deposited immediately into Gen Bank, a public repository for genome-sequence data. Sequencing the corn genome has been considered difficult because of its large size and complex genetic arrangements. The genome has 50,000–60,000 genes scattered among the 2.5 billion bases that make up its 10 chromosomes.
On February 2006, researchers announced that they had sequenced the entire genome of maize (www.maizegdb.org).

1.2.3 Physiology

Maize has a very distinct growth form; the stalk is erect, conventionally 2–3 meters in height, with many nodes, casting off flag-leaves at every node. The leaves number usually are within 12 and 18. The leaves are long between 30 and 150 centimeters and wide between 5 and 15 centimeters. Under these leaves and close to the stalk grow the ears. The ears are female inflorescences, tightly covered over by several layers of leaves (and so closed in by them to the stem that they do not show themselves easily until the emergence of the pale yellow silks from the leaf whorl at the end of the ear) (Figure 1.1).

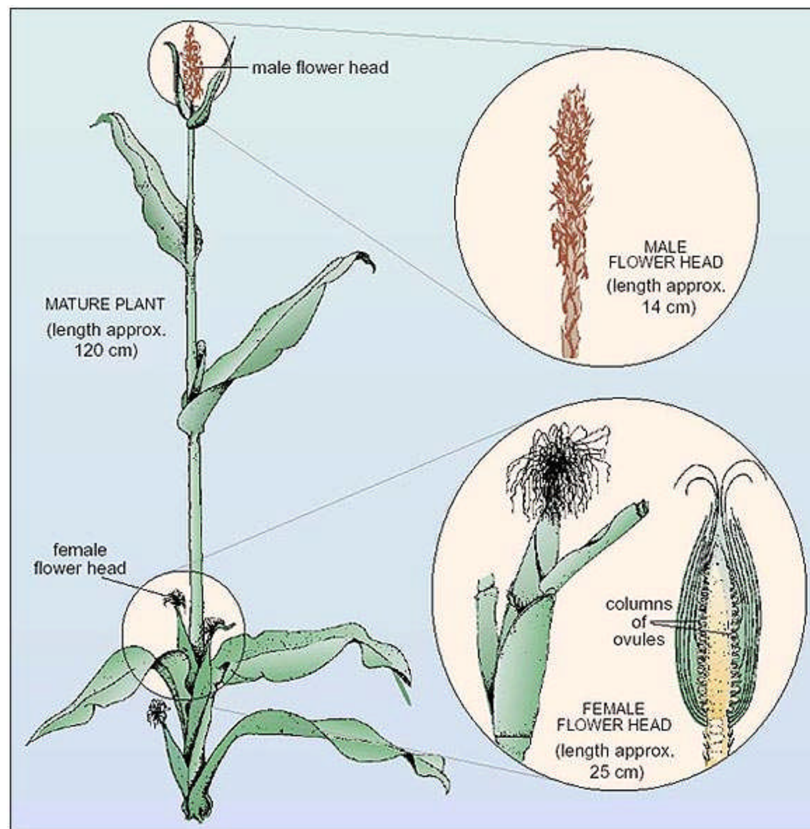

Fig. 1.1 - Structure of male and female flowers on a maize plant (openlearn.open.ac.uk).

The silk are elongated stigmas. Plantings for silage are denser and achieve an even lower percentage of ears and more plant matter. Certain varieties of maize have been bred to produce many additional developed ears, and these are the source of the “baby corn” that is used as a vegetable in Asian cuisine (Sprague, 1977). The apex of the stalk ends in the tassel, the male inflorescence. Young ears can be consumed raw, with the cob and silk, but as the plant matures (usually during summer) the cob becomes tougher and the silk dries to inedibility. By the end of the growing season, the kernels dry out and become difficult to chew without cooking them tender first in boiling water (Sprague, 1977). The kernel of corn has a pericarp, hull or bran, fused with the seed coat, typical of the grasses; the germ; the endosperm and the tip cap: dead tissue found where the kernel joins the cob (Figure 1.2).

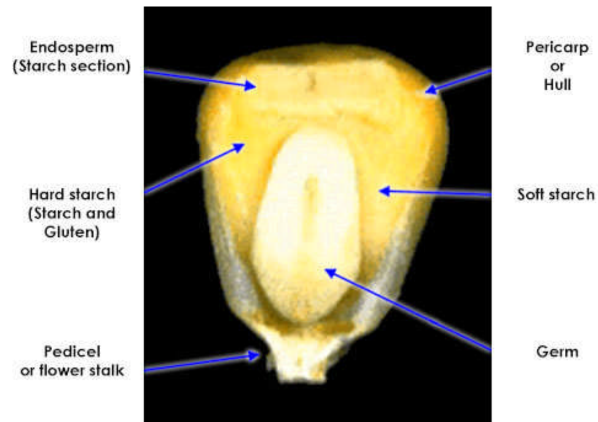

Fig. 1.2 – The maize kernel and their parts (maize.agron.iastate.edu)

The endosperm provides about 83% of the kernel weight, while the germ averages 11% and the pericarp 5%.The grains are about the size of peas, and adhere in regular rows round a white pithy substance, which forms the ear. An ear may contain from 300 to 1000 kernels, and is from 10–25 centimeters in length. They are of various colors: blackish, bluish-gray, red, white and yellow. When ground into flour, maize yields more flour, with much less bran, than wheat does. However, it lacks the protein gluten of wheat and therefore makes baked goods with poor rising capability (Bonsembiante, 1983).

Maize is a facultative long-night plant and flowers in a certain number of growing degree days >10°C in the environment to which it is adapted. The magnitude of the influence that long nights have on the number of days that must pass before maize flowering is genetically prescribed and regulated by the phytochrome system. Photoperiodicity can be eccentric in tropical cultivars, where in the long days at higher latitudes the plants will grow so tall that they will not have enough time to produce seed before they are killed by frost. These characteristics, however, may prove useful in using tropical maize for bio-fuel. Because maize is cold-intolerant, in the temperate zones it must be planted in the spring. Its root system is generally shallow, so the plant is dependent on soil moisture. As a C4 plant (a plant that uses C4 photosynthesis), maize is a considerably more water-efficient crop than C3 plants like the small grains and soybeans. Maize is more sensitive to drought at the time of silk emergence,

when the flowers are ready for pollination. Maize used for silage is harvested at early dough stage (Bonsembiante, 1983).

The staging system employed divides plant development into vegetative (V) and reproductive (R) stages (table 1). Subdivisions of the V stages represents the last leaf stage before tasseling for the specific hybrid under consideration. The first and last V stages are designated as VE (emergence) and VT (tasseling). The six subdivisions of the reproductive stages are designated numerically with their common names in table 1.1. Each leaf stage is defined according to the uppermost leaf whose leaf collar is visible. The first part of the collar that is visible is the back which appears as a discoloured line between the leaf blade and leaf sheath. The characteristically oval-shaped first leaf is a reference point for counting upward to the top visible leaf collar. Beginning at about V6, however. increasing stalk and nodal root growth combine to tear the small lowest leaves from the plant. Degeneration and eventual loss of the leaves results. To determine the leaf stage after lower leaf loss, split the lower stalk lengthwise and inspect for internode elongation. The first node above the first elongated stalk internode generally is the fifth leaf node. This internode usually is about one centimeter in length. This fifth leaf node may be used as a replacement reference point for counting to the top leaf collar.

Tab. 1.1 – Vegetative and reproductive stages of a corn plant.

Vegetative Stages	**Reproductive Stages**
VE emergence	R1 - silking
V1 first leaf	R2 - blister
V2 second leaf	R3 - milk
V3 - third leaf	R4 - dough
V6 - sixth leaf	R5 - dent
V9 ninth leaf	R6 - physiological
V12 - twelfth leaf	
V15 - fifteenth leaf	
V18 -eighteenth leaf	
VT - tasseling	

Corn plants increase in weight slowly early in the growing season. But as more leaves are exposed to sunlight. the rate of dry matter accumulation gradually increases.

The leaves of the plant are produced first, followed by the leaf sheaths, stalk, husks, ear shank, silks, cob and finally the grain. By stage V10, enough leaves are exposed to sunlight so the rate of dry matter accumulation is rapid. Under favorable conditions, this rapid rate of dry matter accumulation in the above-ground plant parts will continue at a nearly constant daily rate until near maturity.

Cell division in the leaves occurs at the growing tip of the stem. Leaves enlarge, become green and increase in dry weight as they emerge from the whorl and are exposed to light, but no cell division or enlargement occurs in the leaves after they are exposed. All leaves are full size by V12, but only about half of the leaves are exposed to sunlight. If a corn plant is grown under low plant density, prolificness may result. Increasing the number of plants in a given area reduces the number of ears per plant and the number of kernels per ear. This reduction is greater for some hybrids than for others. Grain production per hectare will increase with an increase in number of plants per hectare until the advantage of more plants per hectare is offset by the reduction in number of kernels per plant. The optimum plant population is different for different hybrids and in different environments. Highest yields will be obtained only where environmental conditions are favourable at all stages of growth. Unfavourable conditions in early growth stages may limit the size of the leaves (the photosynthetic factory). In later stages, unfavourable conditions may reduce the number of silks produced, result in poor pollination of the ovules and restrict the number of kernels that develop; or growth may stop prematurely and restrict the size of the kernels produced.

1.2.4 Kernels Composition

The main constituents are glucides, proteins, lipids and minerals.

In the endosperm, small concentration (1.0-3.0%) of saccharose, glucose, maltose and other water-soluble oligomers are to be found, the concentration whereof decreases during the kernel ripening, in proportion to the increase of the starch content.

Starch. Maize is the most important source of starch for industrial processing, both for the production of native starch and its hydrolysed derivates, as well as the production of rubber and adhesives.

Starch can be fractionated into two main constituents (amylose and amylopectine), having respectively a linear and branched structure.

The amylose/amylopectine ratio in common maize (dent) is 26/74; genetic research to obtain maize varieties with different ratios has been developed since the 40s.

Cellulose and emicellulose. In maize integument the cell walls constitute the bran; they are made of cellulose by 15-16% and of emicellulose by 44%. Maize is therefore richer in “diet” fibre than wheat.

Maize protein constituents, which represent approx. 10% of the kernel, are divided as follows:

- prolamine 47.2%
- glutenine 35.1%
- albumin 3.2%
- globulin 1.5%

Lipid concentration in the different morphological parts of the kernel varies from 4 to 7.5% depending on the variety.

The above composition changes during storage due to the oil migration toward the endosperm, where it can reach a content over 1%. Such migration can cause some problems in the production of flours destined to brewing and glucose syrups, the fat content whereof must be very low.

From the dry or humid separated germ the oil is extracted, whose percentage composition in fatty acids constitutes an alimentary lipid having excellent features.

Other oil constituents are:

- sterols 1%;
- waxes;
- phosphatides 1.5%;
- tocopherols 0.13%
- pigments;
- terpenes;
- hydrocarbons.

Among these minor constituents, pigments, waxes and phosphatides are particularly important; their separation is necessary for the production of refined alimentary oil.

About 80% of minerals present in maize are located in the germ. Potassium and phosphorus are present in higher quantity, in concentration of 0.28 and 0.27% respectively; calcium, on the contrary, is present in very limited quantity (0.03%), which represents the limiting element in maize-based diets.

Maize has a good content of vitamin E, biotin, vitamin B_6, thiamine and riboflavin. Only yellow maize is a good source of pro-vitamin A.

The lack of vitamin PP or niacin in maize has been considered responsible for cases of pellagra, a typical manifestation of hypovitaminosis when people ate almost exclusively polenta. Nicotinic acid has tryptophan as its precursor, of which it is necessary to evaluate the quantity as usable equivalent niacin (Bonsembiante, 1983).

1.2.5 Economic importance and maize widespread

In world cereal lists, maize figures in the third place after wheat and rice for spreading and total production and for unit production increase rate more than doubled over the past fifty years. Furthermore it figures in the second place after wheat in terms of import-export volume and it is the most used cereal in livestock feeding.

The current maize production could be considered sufficient to satisfy the caloric needs of almost 2 billions peoples. However the use of maize in direct human nutrition is progressively decreased whereas the usage in animal

nutrition and the industrial processing for the production of starch, oil, proteins and their by-products is increasing.

Maize currently spread over a surface of 142 million hectares with a production of 784 million tons of dry kernels.

More then 40% of the total production is located in the United States and 12 % in Europe.

Inside Europe, France occupies the first place for the crop form kernels with almost 1.7 million hectares and a production of 13 million tons, followed by Italy with about 1 million hectares and about 10 million of tons of kernels (Table 1.2). Together these 2 countries represent over 90% of the European maize growing area and production.

Maize crop in Italy concerns mostly the Northern regions where 77% of the surface and slightly more than 80% of production are located (Figure 1.3).

Veneto, Friuli, Lombardy, Piedmont provide almost 65% of the national production.

About 12-13% of the national production is currently produced in Central Italy and slightly more than 6% in Southern Italy and on the Islands. Tuscany and Campania are the central and Southern regions with the highest production (FAO, 2007). The amount of kernels involved in worldwide import-export is equal to 70 million tons. The USA are the main exporter whereas in Europe (a strong importer in the past) imports and exports are almost balanced (FAO, 2007).

Tab. 1.2 – Countries maize producer in 2007

Country	Production (Tons)
United States	332,092,180
China	151,970,000
Brazil	51,589,721
Mexico	22,500,000
Argentina	21,755,364
India	16,780,000
France	13,107,000
Indonesia	12,381,561
Canada	10,554,500
Italy	9,891,362
World	**784,786,580**

Source: Food And Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division

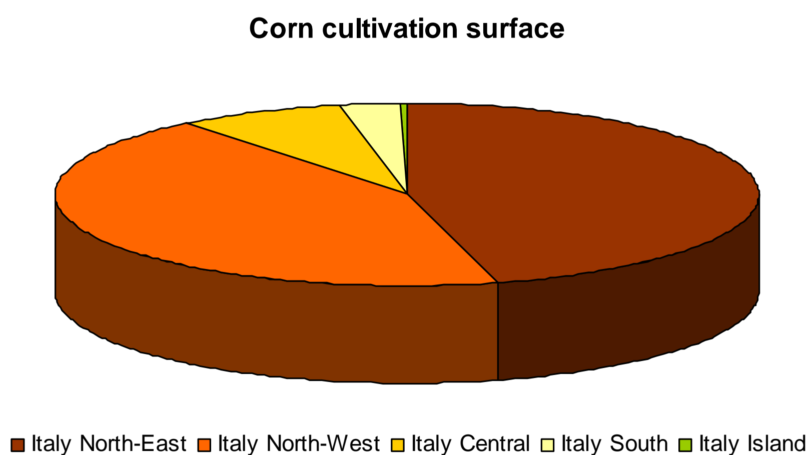

Fig. 1.3 – Corn cultivation surface in Italy (FAO, 2007)

1.2.6 Use of maize

Maize is able to provide nutrients for humans and animals, also serving as a base raw material for the production of starch, oil and protein and alcoholic beverages and food sweeteners (FAO, 1992). Hybrid maize is preferred by farmers over conventional varieties for its high grain yield.

Corn is used for three main purposes: as feed for livestock, particularly in temperate and advanced countries, providing over two-thirds of the total trade in feed grains as a staple human food, particularly in the tropics, as a raw material for many industrial products.

Corn produces a number of important industrial products, which are usually obtained by the wet-milling process, in which the grain is steeped, after which the germ and bran are separated from the endosperm. The main product is starch, which, when dried, is also used converted into dextrins; if not dried, it can be processed into syrup or sugars. Oil, obtained from the germ, is made into soap or glycerine, but can be refined to produce cooking or salad oil. The starch may be used as human food or made into sizing, laundry starch, urethane plastics, and other products.

Corn can be fermented and distilled to provide industrial products such as ethyl, butyl or propyl alcohol, acetaldehyde, acetone, glycerol and acetic, citric and lactic acids. Zein, the protein in corn, is used to produce synthetic fibers of good tensile strength, and as a substitute for shellac. The fiber in the stems has been used for making paper, and the pith for explosives and light packing material. The cobs are used for fuel, smoked pork products and pipes. Over 500 important products may be obtained from corn.

In the USA and Canada, the primary use for maize is as feed for livestock, forage, silage or grain. Silage is made by fermentation of chopped green cornstalks. The grain also has many industrial uses, including transformation into plastics and fabrics. Some is hydrolysed and enzymatically treated to produce syrups, particularly high fructose corn syrup, a sweetener, and some is fermented and distilled to produce grain alcohol. Grain alcohol from maize is traditionally the source of bourbon whiskey (Bonsembiante, 1983).

Maize is increasingly used as a biomass fuel, such as ethanol, which as researchers search for innovative ways to reduce fuel costs, has unintentionally caused a rapid rise in food costs. This has led to the 2007 harvest being one of the most profitable in modern history for farmers. A biomass gasification power plant in Strem near Güssing, Burgenland, Austria, was begun in 2005.
In Italy 82% of maize is destined to feed, approximately 4% is for human consumption (food and drugs) and 12% is used for starch industry; 2% for other uses (ISTAT, 2005).

1.3 Toxigenic fungi

Contamination by toxigenic fungi and their mycotoxins of agricultural products grown world wide is a problem, and differences in environmental conditions in various countries significantly influences the distribution of toxigenic fungi and related mycotoxicological risks. Emerging problems due to climate change and new mycotoxin and commodity combinations increase these concerns. Global transposition and trade exchanges of plant products also contribute significantly to the spread of toxigenic fungi worldwide and represent an important source of inoculum for new plant diseases in Europe (Logrieco and Moretti, 2008).
Toxigenic fungi are able to produce mycotoxins (secondary metabolites) that cause toxic response when ingested by animals and humans. Consumption of a mycotoxin-contaminated diet may induce acute and chronic effects resulting in teratogenic, carcinogenic and oestrogenic or immune-suppressive effects (Binder et al., 2007).
The main toxigenic fungi belong to the genera: *Fusarium*, *Aspergillus* and *Penicillium*. Aflatoxins (AFs) are produced by *A.* section *Flavi*, especially *A. flavus* and *A. parasiticus*. They are largely associated with commodities produced in the tropics and subtropics, such as groundnuts, other edible nuts, figs, spices and maize. Aflatoxin B_1 (AFB_1), the most toxic, is a potent carcinogen and has been associated with liver cancer.
Ochratoxin A is produced by *P. verrucosum*, which is generally associated with temperate climates, and *Aspergillus* species which grow in warm humid

conditions. *A. ochraceus* is found as a contaminant of a wide range of commodities including cereals and their derived products, fruit and a wide range of beverages and spices. *A carbonarius* is the other main species associated in warm humid conditions found mainly on grapes and wine products particularly in the Mediterranean basin. It causes kidney damage in humans and is a potential carcinogen.

The species *Fusarium* can attack both corn and wheat with different effects. In wheat, they cause *Fusarium* head blight and produce mainly deoxynivalenol (DON). In corn, *Gibberella* Ear Rot and *Fusarium* Ear Rot are signalled and produce respectively DON, zearalenone, T-2 toxins and fumonisins (FUM).

1.4 *Fusarium*

The *Fusarium* genus belongs to the kingdom of Eumycota, phylum Dyckariomicota, subphylum Ascomycotina and order Hypocreales.

Fusarium species cause a vast range of diseases on a range of host plants. The most important are the crown and root rots, stalk rots, head and grain blights, and vascular wilt diseases.

The fungus can be soil-borne, airborne or carried in plant residue and can be recovered from any part of a plant from the root to the flower. *Fusarium* species recovered from both natural and agricultural ecosystems have distinct climatic preferences. The climate, and even local variation in weather, can limit the series of species observed. There are species that prefer tropical climates, hot arid climates, or temperate climates (Summerell et al., 2003). The distribution and the prevalence of different *Fusarium* species, causing two kinds of ear rot disease, are largely governed by environmental conditions, primarily temperature, as well as by many other factors including agro-technical practice (Arino and Bullerman, 1994). *Fusarium* infection of maize ears and kernels comprises two distinct diseases that differ, but also overlap, in their epidemiological characteristics. *Gibberella* ear rot or “red ear rot” usually initiates from tip of the ear and develops a red or pink mould covering a large proportion of the ear. Usually, it is caused by *F. graminearum* (Bechtel et al.,

1985), although in Europe several other *Fusarium* species may be associated with this disease, especially *F. culmorum* (Logrieco at al., 2002). *Gibberella* ear rot predominates in cooler areas or those with higher precipitation during the growing season (Bechtel et al., 1985; Logrieco et al., 1993). *Fusarium* ear rot typically occurs on random groups of kernels or on physically injured kernels (Miller, 1995) and consists of a white or light pink mould. Light pink fusariosis prevails in drier and warmer climates of Southern Europe areas (Bottalico and Logrieco, 1988). Identical symptoms are caused by *F. verticillioides, F. proliferatum* or *F. subglutinans*, but occasionally other *Fusarium* species are associated with these symptoms. Historically *F. moniliforme* has been reported as the most common pathogen causing *Fusarium* ear rot (Bottalico, 1998).

Fusarium taxonomy has been plagued by changing species concepts, with as few as nine or well over 1000 species being recognized by various taxonomists during the past 100 years, depending on the species concept employed. The literature stabilized significantly in the early 1980s with the publications of Gerlach and Nirenberg (1982) and Nelson (1983), who defined morphological species concepts that were widely accepted and successfully used. In *Fusarium,* there currently are three different basic species concepts: morphological, biological and phylogenetic. Morphological species concepts are based on the idea that an individual can represent the variation within an entire species and observe the similarity of morphological characters (spore size and shape). Both physical and physiological characters have been used as morphological characters to discriminate *Fusarium* species. The shape of the macroconidia often is given the greatest weighting when defining species, but other spore like microconidia and chlamydospores, are also important in morphological species definition.

Biological species concepts require that members of the same species are sexually cross-fertile and that the progeny of the crosses are both viable and fertile. These species concepts shift the focus from individuals to populations and define species in terms of how the members of these populations interacts and relate to one another rather than by comparisons with a static standard. Biological species concepts treat species as categories defined by an actually

or potentially shared gene pool, rather than as a taxon or type as is always done with a morphological species concept. There are practical difficulties with applying a biological species concept to *Fusarium*, including high levels of asexual reproduction and limited strains are fertile as females.

Finally, DNA sequences have been used to explain to phylogenetic concept and generate characters that usually are treated to form phylogenies and those that are part of the same monophyletic group that have a common genetic origin. Although any group of sufficiently numerous characters can form the basis of a phylogenetic lineage, in practice DNA sequences of one to several conserved genes are used for this purpose (Summerell et al., 2003). Application of the phylogenetic species concept to *Fusarium* is a relatively new development. Although DNA sequence are now the most commonly used characters for delineating phylogenetic species, in practice any marker that is sufficiently informative, including morphological characters, can be used in the phylogenetic process (Leslie and Summerell, 2006).

For many species of *Fusarium*, morphological characters are the only ones that are well described and widely available. For a limited number of *Fusarium* species, there is biological species information and publicly available tester strains can be used to make the crosses required for identification. For still other *Fusarium* species, DNA sequence information is available to support phylogenetic species concepts.

The distribution and the prevalence of different *Fusarium* species causing two different kinds of ear rot disease are largely governed by environmental conditions: temperature, water activity (a_w), relative humidity, agro-technical practices. In general Red fusariosis is severe in years and locations characterized by frequent rainfall ad low temperatures during the summer and early fall, while Pink fusariosis prevails in drier and warmer climates of southern areas (Logrieco et al., 2002).

Red ear rot is mainly caused by species of the *Discolour* section and Pink ear rot mainly caused by representatives of the *Liseola* section. The predominant species causing maize red ear rot are *F. graminearum*, *F. culmorum*, *F.*

avenaceum. The species frequently isolated from maize pink ear rot are *F. verticillioides, F. proliferatum* and *F. subglutinans.*

Many reports from Europe on maize contamination by *Fusarium* show that the colonization of this important crop plant could be determined by organisms with a broad range of mycotoxin production and different environmental niches (Logrieco et al., 2002).

Fusarium species on maize can produce many mycotoxin some of which are of remarkable importance. The naturally occurring *Fusarium* mycotoxins belong to the trichotecenes, zearalenones, and FUM.

Fusarium ear rot is characterized by cottony mycelium growth that typically occurs on a few kernels or is limited to certain parts of the ear, unlike *Gibberella* ear rot. Mycelium is generally white, pale pink or pale lavender. Infected kernels typically display white streaking on the pericarp and often germinate on the cob. Infection through silks is a significant source of *Fusarium* ear rot and symptomless infection by *F. verticillioides* (Desjardins et al., 2000, Munkvold et al., 1997; Nelson et al., 1992). In the absences of, or in addition to, insect injury, infection through silks appears to be the most important infection pathway for *F. verticillioides* (and probably *F. proliferatum* and F*. subglutinans*). Most methods for screening maize hybrids for resistance to *Fusarium* ear rot have employed silk inoculation or a method that wounds the kernels (Clements et al., 2003). Resistance factors have been identified both in silks (Headrick and Pataky, 1989) and in the pericarp of the kernels (Scott and King, 1984), which may reflect relative ability to avoid kernel injury. In fact, typically, infection occurs close to ear tips and is commonly associated with damage and injury caused by ear borers. Under severe infestation, the entire ear appears withered and is characterized by mycelium growth between kernels. The species of *Fusarium* causing maize ear rot are worldwide in distribution and are characterized by co-occurrence or succession of different species (Logrieco et al., 2002).

Maize pink ear rot is commonly observed from southern to central European areas and in Italy where environmental conditions are often conducive to a high incidence of maize pink ear rot and *F. verticillioieds* predominates.

Investigations carried out in Italy revealed that *F. verticillioieds* was the most frequently isolated fungus from infected maize plants and from commercial maize kernels associated with fumonisin B_1 (FB_1, Battilani et al., 2005).

1.5 *Fusarium verticillioides* and fumonisins

Fusarium verticillioides (Sacc.) Nirenberg was first described and associated with animal diseases in 1904 and *F. verticillioides* is likely to be the most common species isolated worldwide from diseased maize (Munkvold and Desjardins, 1997). Doko et al. (1996) reported *F. verticillioides* as the most frequently isolated fungus from maize and maize-based commodities in France, Spain and Italy, and it was confirmed by Orsi et al. (2000) in Brazil.

F. verticillioides is associated with disease at all stages of corn plant development infecting the roots, stalk and kernels and symptomless infection can exist throughout the plant in leaves, stems, roots, grains, and the presence of the fungus is in many cases ignored because it does not cause visible damage to the plant (Battilani et al., 2003). Under ordinary plant growth condition, *F. verticillioides* grows within the maize plant as an endophyte. The current impact of relationship between maize plant and *F. verticillioides* is exemplified by the increase in the number of literature citations in the past 10 years (Yates and Sparks, 2008).

There has been significant disagreement regarding the name of this fungus with some taxonomists calling it *F. moniliforme* and other *F. verticillioides*. The name *F. verticillioides* has priority and it is now generally accepted that this is the name that should be used for this species (Seifert et al., 2003). *F. moniliforme* encompassed stains in species other than *F. verticillioides*. The name *F. verticillioides* should be used only for strains that have the *G. moniliformis* teleomorph and not simply as a substitute for *F. moniliforme*. *F. verticillioides* is morphologically identical to strains of *F. thapsinum* that do not produce the diagnostic yellow pigment and is similar to *F. proliferatum*, but the latter species is distinguished by its ability to form chains of microconidia from polyphialides. The microconidial chains produced by *F. proliferatum* usually are shorter than

those of *F. verticillioides* and *F. thapsinum*. *F. verticillioides* and *F. thapsinum* can be reliably differentiated only using mating tests or molecular markers, although many isolates of *F. thapsinum* produce yellow pigments that are unique to *F. thapsinum*. *F. verticillioides* also is very similar to *F. andiyazi*, but does not form pseudochlamydospores. *F. verticillioides* can produce swollen cells in hyphae that may be difficult to differentiate from pseudo-chlamydospores.

F. verticillioides forms microconidia in shorts chains or false heads from monophialides (Burgess et al., 1994). Strains that are morphologically similar to and phylogenetically closely related to *F. verticillioides* have been isolated from bananas, but these strains do not produce FUM (Moretti et al., 2004). Conidia are produced on the phialides through an aeroblastic process in which the inner wall of the conidium and the phialide are continuous and to which the middle and outer layers are subsequently added (Tiedt and Jooste, 1992). Disease severity is affected by the inoculation technique and by the time of both inoculation and scoring of the diseases symptoms (Gulya et al., 1980).

F. verticillioides can enter a maize plant systemically from the seed (Oren et al., 2003), through wounds in the plant or though infections of the silks. Of these different routes, kernel infection occurs most efficiently from strains that are inoculated onto the silks (Munkvold et al., 1997). Disrupting husk integrity increase ear rot severity and drought stress increases the amount of stalk rot and can be relieved by irrigation.

The fungus usually appears in the kernels as they are near physiological maturity and continues to increases until the end of the growing season (Blish et al., 2004) when it may represent the main species of the *Fusarium* isolates from the grain (Bankole and Mabekoje, 2004). Based on electronic microscope observations, the fungus is found at the tip cap of both symptomatic and asymptomatic maize kernels (Bacon et al., 1992). In the symptomatic kernels the embryo and endosperm also were extensively colonized and in some cases microconidia were produced inside the infected kernel.

F. verticillioides is more commonly recovered from the fines, bran and germ than it is from whole kernels or coarse grit fractions (Katta et al., 1997). The

effect of seedborne *F. verticillioides* on germination and yield is a subject of debate (Oren et al., 2003), but appears heavily dependent on the conditions under which germination and growth occur.

When *F. verticillioides* is present in a maize kernel, then that kernel is less likely to be infected by *Aspergillus* spp. Kernel infected whit *F. verticillioides* also may contain less of mycotoxins produced by other fungi (Marin et al., 2001). Multiple genetically distinct isolates of *F. verticillioides* may be recovered from a single maize plant (Kedera et al., 1994). For example, “BT” maize hybrids, that are less susceptible to the European corn borer (*Ostrinia nubilalis)*, has a lower level of infection by *F. verticillioides* than comparable hybrids without the transgene (Clements et al., 2003) and accumulates less FUM (Papst et al., 2005).

Fusarium verticillioides has been recovered from teosinte (Desjardins et al., 2000), suggesting that the association between this fungus and maize is of long standing and possibly of evolutionary importance. *F. verticillioides* also can degrade the antimicrobial benzoxazinoids 6 methoxy 2 benzoaxazolinone and 2 benzoaxazolinone produced by maize (Glenn et al., 2004). This ability may be one of the reasons why this fungus is a successful maize pathogen.

Fusarium verticillioides also may be recovered from finger millet, and native North American tall grass prairie (Leslie et al., 2004) and desert soils. It may be recovered from and cause disease on sorghum. This fungus has been associated with a broad range of diseases but because of the nomenclatural confusion and the lack of understanding that there were more than one species in the older species definitions, it is difficult to determine the true causal agent in many cases. *Fusarium verticillioides* has been reported to cause top rot of sugar cane, foot rot of rice and crown rot of asparagus (Stephens et al., 1989). These pathogenic associations need to be re-evaluated to confirm that they are caused by *F. verticillioides* and not by another member of the *G. fujikuroi* species complex that used to be included in “*F. moniliforme* ”.

Fusarium verticillioides also is well adapted to air and wind dispersal and can infect the grain of maize via the silk of the cob or through wounds crested by

Lepidopterum larvae. *Fusarium verticillioides* persists in host residues on the soil surface or in the soil following mechanical incorporation.

Reducing tillage increases the amount of *F. verticillioides* inoculum available for the following season.

Maximum linear growth is reported to occur at 25°C and an osmotic potential of -1.0 MPa (Nelson et al., 1990). Spore germination is sensitive to a_w, with a 30°C optimum for an a_w between 0.90 and 0.94, and a broad range of 25-37 °C as the optimum with an a_w between 0.96 and 0.98 (Marin et al., 1996). The type maturity and physical condition of the maize tissue can determine how well it can be colonized by *F. verticillioides* (Yates and Jaworiski, 2000), with the best growth occurring on wounded, relatively immature reproductive tissues.

Many strains of *F. verticillioides* produce relatively few macroconidia. The number of macroconidia produced increases if strains are grown on media with maltose or soluble starch as a carbon source. Commercially available antioxidants can be used to treat grain and retard growth by *F. verticillioides* and reduce the amount of FUM produced *in situ*. The fungus can also grow under microaerobic conditions. The most common medium for recovering *F. verticillioides* is PPA, a peptone PCNB medium that is semi-selective for *Fusarium*. Colonies can grow on this medium for more than 1 to 2 weeks. Similarly, cultures on richer media Potato Dextrose Agar (PDA) do not produce the uniform macroconidia that are necessary for an accurate identification. Colonies should be transferred from the isolation medium to a nutritionally weak medium like water agar with a bit of sterilized plant tissue.

Accurate identification of a culture requires growing it on at least two media: carnation leaf-piece agar (CLA) is a natural substrate medium (Snyder and Hansen, 1947) prepared by placing sterile carnation leaf pieces (approximately 1 piece per 2 ml agar) in a Petri plate and then adding sterile 2% Water Agar and Potato Dextrose Agar (PDA) is a carbohydrate rich medium which contains 20 g dextrose, 20 g agar and the broth from 250 g white potatoes made up to 1L with tap water. The potatoes are unpeeled but washed and diced before boiling until just soft. The boiled potatoes are filtered through cheesecloth leaving some sediment in the broth.

Microconidia are the most common spore type produced by some species, but usually they are preferred because their larger size makes them easier to handle.

Many species of *Fusarium* readily form sporodochia or robust, uniform macroconidia on the CLA that are particularly useful for identification purposes. PDA cultures are used to assess pigmentation and gross colony morphology. Incubation conditions are the temperatures of 20 to 25 °C and the presence of light for 7 to 10 days (Summerell et al., 2003).

Fusarium verticillioides is known to be allergenic to humans and to be capable of systemically infecting cancer and HIV patients. The most common human health problem associated with *F. verticillioides* is skin lesions, but it can also infect through wounds and has been associated with keratitis. Infections with *F. verticillioides* usually are not associated with hospital settings, but nosocomial outbreaks of diseases attributable to this fungus do occurs. *Fusarium verticillioides* is resistant to most clinical antifungals (itraconazole, miconazole etc) with amphotericin B and natamycin reported as the most effective. *F. verticillioides* also can cause direct diseases in some animals including alligators and freshwater fish.

A case of chronic invasive rhinosinusitis in an apparently healthy man, caused by *F. verticillioides*, has been described. The identity of the isolate as *F. verticillioides* was established by demonstrating characteristic morphological features and by amplification of rDNA using species-specific primers. Surgical debridement of the infected nasal tissue and therapy with amphotericin B resulted in a favourable outcome (Macêdo et al., 2008).

Fumonisins were first isolated from *F. verticillioieds*. Four series of FUM have been described and named A, B, C, and P. The B series includes the most active FUM, particularly B_1.

Fusarium verticillioides typically produces FB_1, FB_2, FB_3 and FB_4. The most predominant toxin produced is FB_1. FB_1 frequently occurs together with FB_2, which may comprise 15-35% of FB_1 (Visconti and Doko, 1994). These compounds share a linear 20-carbon backbone with an amine at carbon atom 2 and tricarboxilic acid moieties esterified to C-14 and C-15. The compounds

differ from one another by the presence or absence of hydroxilic functions at C-5 and C-10.

In general, FB_1 is the most abundant fumonisin in naturally contaminated maize and in cultures of most field isolates of *F. verticillioides*.

Toxin synthesis is stimulated by the presence of methionine, a fumonisin precursor, in the culture medium. The biochemistry of fumonisin biosynthesis has been examined in some detail and both the biochemistry and the molecular genetic underlying the pathway are well understood. The International Agency for Research on Cancer (IARC) evaluated in 1992 the toxins derived from *F. verticillioides* as possibly carcinogenic to humans (IARC, 1993). More recently, based on the research results obtained so far, FB_1 has been evaluated as possibly carcinogenic to humans (class 2B) (IARC, 2002).

Fumonisins are clearly the most important toxins produced by *F. verticillioides* and some strains may produce these mycotoxins at very high levels (Glenn, 2007). Fumonisin B_1 is the best known and studied of the FUM, but other derivatives are known to occur naturally as well. Member of this family of toxins disrupt sphingolipid metabolism and cause Leukoencephalomalacia in horses (ELEM) (Marasas et al., 1988). The first case of ELEM was reported in north Italy associated with the presence of FB_1 in maize based feed (Caramelli et al., 1993); pulmonary edema syndrome in pigs (Harrison et al., 1990), liver cancer and liver and kidney toxicity in rats (Gelderblom et al., 1988), neurodegeneration in mice and apoptosis in many type of cells are other diseases signalled.

Fumonisins have been implicated in humans esophageal cancer (Marasas, 2001), birth defects and in cardiovascular problems (Fincham et al., 1992) in populations consuming relatively large amounts of food made with contaminated maize (Voss et al., 2007). Corn contaminated with FB_1 has been statistically associated with high rates of human oesophageal cancer and a similar claim has been made for north-eastern Italian areas (Franceschi et al., 1990).

The chemical structure of the FUM was first reported in 1988 (Gelderblom et al., 1988). Since then more than 28 homologues have been discovered and more

are likely to be found (Hump and Voss, 2004). FB_1 is the most common and the most thoroughly studied. FB_2, FB_3 and FB_4 are in order less prevalent and different structurally from FB_1 in the number and placement of hydroxyl groups on the molecule's hydrocarbon. FB_1 has the empirical formula $C_{34}H_{59}NO_{15}$ and is the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl–3,5,10,14,15-pentahydroxyeicosane (Figure 1.4).

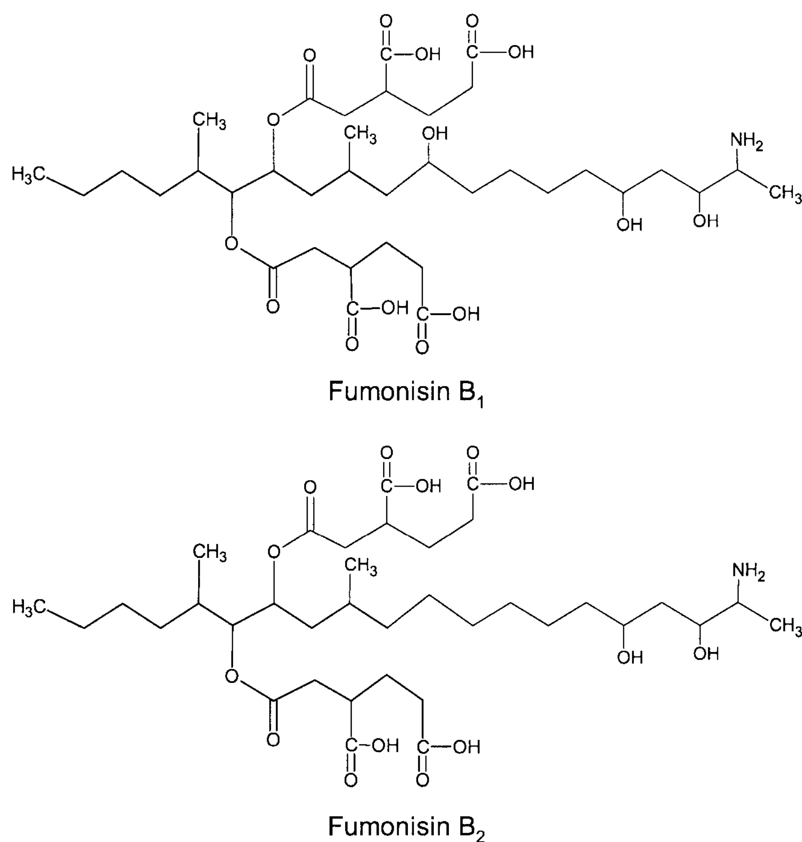

Fig. 1.4 – Chemical structure of FB_1 and FB_2.

The pure substance is a white hygroscopic powder, which is soluble in water, acetonitrile-water or methanol, is stable in acetonitrile-water (1:1), is unstable in methanol and is stable at food processing temperature and light.

The structural similarity of FUM to the sphingoid bases sphinganine is critical to their ability to disrupt sphingolipid metabolism (Riley et al., 2001).

Fumonisin's primary amine function appears necessary for its biological activity (Voss et al., 2007). Over the past decade, there have been significant advances in the understanding of the genetics and biochemistry of fumonisin biosynthesis. In general, FUM consist of a 19- or 20-carbon backbone with an amine, one to four hydroxyl, two methyl and two tricarboxylic acid constituents. Eighteen of the carbons that make up the fumonisin backbone are assembled by a polyketide synthase, an enzyme class required for the biosynthesis of numerous toxins, antibiotics, and other biologically active compounds produced by fungi. The linear polyketide precursor of FUM undergoes up to nine oxygenation, esterification, reduction, and dehydration reactions to form mature, biologically active FUM. Fumonisins are structurally similar to the sphingolipid sphinganine and disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthase. Sphingolipids play a critical role in cell membranes and a variety of cell signaling pathways. Thus, disruption of sphingolipid metabolism may account for the multiple diseases associated with FUM (Lonnie et al., 2007).

FB_1 has been detected in maize-based products worldwide at mg/Kg levels, sometimes in combination with other mycotoxins. Concentration at mg/kg levels have also been reported in food for human consumption. Dry milling of maize results in the distribution of fumonisin into the bran, germ and flour. In experimental wet milling, fumonisin was detected in steep water, gluten, fibre and germ, but not in the starch. Fumonisins may be present in beer where maize has been used as a wort additive (Scott et al., 1995). They are also stable in stored products when these are kept in airtight at very low temperatures or γ-irradiated (Visconti et al., 1996). However, instability of FUM in contaminated products over time has been shown (Scott et al., 1999; Kim et al., 2002). Fumonisins are also water soluble (IPCS, 2000).

FB_1 is stable in maize and polenta, whereas it is hydrolysed in foods processed with hot alkali solutions (Hendrich et al., 1993). FB_1 is not present in milk, meat or eggs from animals fed grain containing FB_1 at levels that would not affect the health of the animals.

Apart from maize and maize products, FUM have seldom been found in other food products, such rice (Abbas et al., 1998), asparagus (Logrieco et al., 1998) and sorghum (Shetty and Bhat, 1997).

Although the effects of FUM on humans are not yet well understood, legislation is being put in place to regulate commercial exchanges of fumonisin contaminated maize and maize-based foods. The US Food and Drug Administration (FDA) recommended that the fumonisin levels should not be higher than 4 µg/g in human foods (FDA, 2000a, FDA 2000b). In Switzerland, tolerance levels for FUM of 1 µg/g in dry maize products intended for human consumption were proposed (Marasas et al., 2001). The European Commission has established a tolerable daily intake of 2 µg/kg body weight per day for the total FB_1, FB_2, and FB_3, alone or in combination (Silva et al., 2009).

Also, the European Union recently regulated FUM (as the sum of FB_1 and FB_2) in maize-based products and unprocessed maize. The European Commission has set action limits of 4000 µg fumonisin/kg for unprocessed corn, and 200 µg fumonisin/kg for processed corn-based foods and baby foods for infants and young children (European Commission, 2007).

As the 20th century ended, *Fusarium* mycotoxicology entered the age of genomics with the discovery of the fumonisin biosynthetic gene cluster in *F. verticillioides* in 1999. The field of *Fusarium* genomics was accelerated when the United States Department of Agriculture and National Science Foundation jointly supported the sequencing and public release of the complete genomes of *F. graminearum* in 2003 and of *F. verticillioides* in 2006 (Desjardins and Proctor, 2007).

The fumonisin biosynthetic pathway in *Fusarium* species begins with formation of a linear dimethylated polyketide and condensation of the polyketide with alanine, followed by a carbonyl reduction, oxygenations, and esterification with two propane-1,2,3-tricarboxylic acids. The fumonisin biosynthetic gene cluster in *F. proliferatum* and *F. verticillioides* have both been sequenced. Molecular genetic analysis of *F. verticillioides* has identified a fumonisin biosynthetic gene (FUM) cluster that consists of 15 co-regulated genes, all of which exhibit a pattern of expression that is correlated whit fumonisin production. The roles of

some of the clustered FUM genes in FUM biosynthesis have been determined (Desjardins and Proctor, 2007). Several studies have provided indirect evidence for relationships between natural variations in fumonisin production and the clustered FUM genes. To date, fumonisin biosynthetic genes have been mapped to one locus in the *F. verticillioides* genome. Fumonisin polyketide synthase (FUM1) was the first fumonisin gene to be cloned and is the anchor of a cluster of 15 co-regulated fumonisin biosynthetic genes.

Gene-disruption studies have determined that eleven of these genes are required for fumonisin biosynthesis (Desjardins and Proctor, 2007).

Three lines of evidence suggest that the naturally occurring fumonisin non-production phenotype may result from a mutation in or near the FUM genes.

Most fields of *F. verticillioides* produce the full complement of FB_1, FB_2, FB_3 and FB_4. The scarcity of no-producer strains in natural populations of the fungus may be an indication that FB_1 production contributes to the competitiveness of the fungus. Characterization of fumonisin biosynthetic genes and elucidation of the genetic basis of naturally occurring altered fumonisin production phenotypes should contribute to understand the role of the toxins in the ecology of *F. verticillioides* (Proctor et al., 2006). The FUM genes cluster consist of FUM1 to FUM19. FUM1 is an 8163 bp gene that is responsible for the production of a polyketide synthase, an enzyme that possibly convert acetate into a polyketide and correspondes to one of the first steps in the fumonisin biosynthetic pathway (Bojja et al., 2004). Its is important in the production of these toxins has been demonstrated by Bojja et al. (2004), who observed no fumonisin production where FUM1 has been disrupted.

1.6 Other *Fusarium* Mycotoxins

Fumonisins are of notable importance, but *Fusarium* species on maize can also produce other mycotoxins that belong to trichothecenes and zearalenones. Moreover, moniliformin, beauvericin and fusaproliferin have also been found in naturally infected kernels and are considered as emerging toxicological problems (Logrieco et al., 2002).

The *Fusarium* trichothecenes have been divided into type A, characterized by a functional group other than a ketone at C-8 and type B trichothecenes with only the carbonyl at C-8. The type A include T2 and HT2 mainly produced by *F. sporotrichioides*, *F. acuminatum* and *F. poae*. The type B included deoxynivalenol (DON) and its derivates produced by strains of *F. graminearum* and *F. culmorum*. Trichothecenes cause a variety of toxic effects in laboratory and farm animals including skin inflammation, digestive disorder, haemorrhages in several internal organs, haemolytic disorder and depletion of the bone marrow, impairment of both humoral and cellular immune responses and nervous disorders. It has also been implicated in human toxicoses (Logrieco et al., 2002).

Zearalenone (ZEA) is mainly produced by *F. graminearum* and *F. culmorum*. It is among the most widely distributed *Fusarium* mycotoxin in agricultural commodities and has often been encountered even at very high concentrations in maize. ZEA is an uterotrophic and estrogenic compound responsible for recurring toxicoses in livestock, characterized by hyper-estrogenism in swine and infertility and poor performance in cattle and poultry (Kuiper-Goodman et al., 1987).

Diets containing culture material naturally contaminated with moniliformin were responsible for reduced performances, haematological disorders, myocardial hypertrophy and mortality in rodents, chicks, ducklings and pigs (Harvey et al., 1997).

Beauvericin (BEA) is a cyclic hexadepsipeptide isolated from maize and maize based feed for swine and first reported to be produced by cultures of strains of *F. semitectum*, *F. subglutinans* and *F. proliferatum* (Moretti et al., 1995). BEA

was detected for the first time in maize ear rot in Poland (Logrieco et al., 1993) and then found as a toxic contaminant of maize in Italy (Bottalico et al., 1995). It is highly toxic to insects and it is also cytotoxic to mammalian cell tissues and was reported to cause apoptosis in both murine and human cell lines.
Fusaproliferin is a novel sesterterpene first purified from a culture of *F. proliferatum* from maize ear rot in northern Italy (Ritieni et al., 1995) and in naturally infected maize.
Investigations on the toxicity indicated that it is lethal to larvae of *Artemia salina* and produced high mortality in broiler chicks.

1.7 *Aspergillus flavus* and aflatoxins

Aspergillus section *flavi* are very widely distributed in nature. They are regularly isolated from soils in tropical and subtropical areas, from forage and decaying vegetation, from stored seeds and grains and from various types of food products (Raper and Fennell, 1965).
A flavus and *A. parasiticus* are closely related fungi that can contaminate seeds and plants in the field, during harvest, in storage and during processing (Diener et al., 1987).
A flavus and *A. parasiticus* are differentiated, in part by their colour and relative conidiophore lengths, but primarily by the character of their sterigmata: *A. flavus* is typically biserate and *A. parasiticus* uniserate (Raper and Fennell, 1965).
Besides, *A. parasiticus* appears to be adapted to a soil environment, being prominent in peanuts, whereas *A. flavus* seems adapted to the aerial and foliar environment, being dominant in corn, cottonseed and tree nuts (Diener et al., 1987).
Their production of mycotoxins can also be useful to separate strains of the *A. flavus* group. It is generally accepted that *A. flavus* only produces AFB_1 and AFB_2, but it is also capable of synthesising cyclopiazonic acid, a mycotoxin confirmed as being present in the batch of contaminated groundnuts which killed turkey poults in 1960 (Smith, 1997).

On the other hand, *A. parasiticus* often produces all four of the primary AFs. This group of mycotoxins comprises AFB_1, AFB_2, AFG_1 and AFG_2 (Diener et al., 1987) (Figure 1.5).

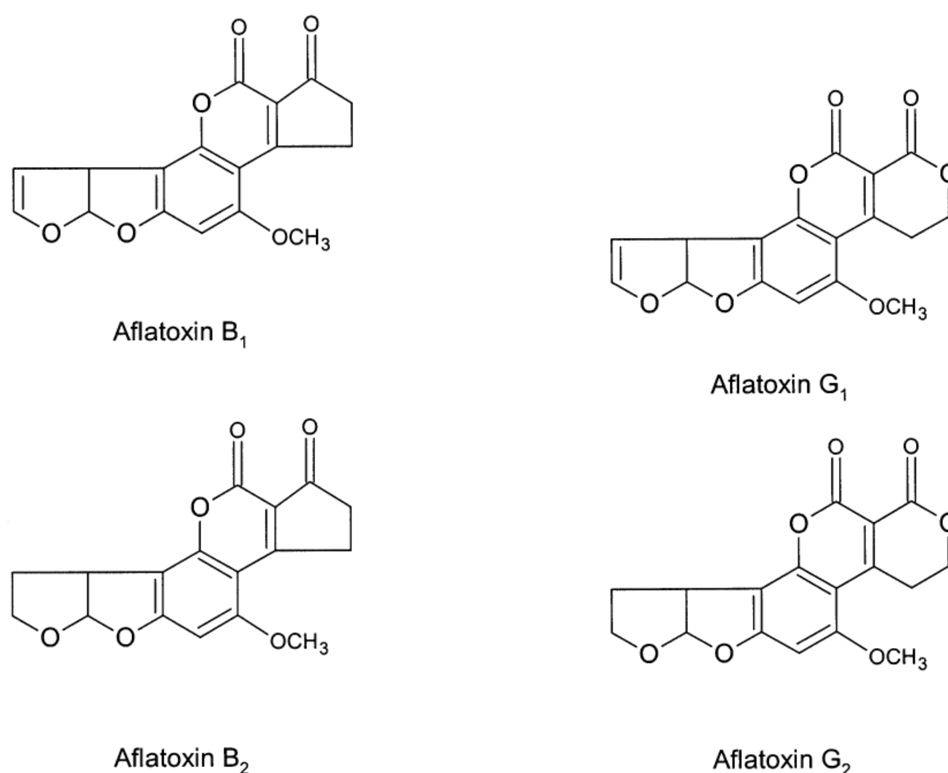

Fig. 1.5 – Chemical structure of AFB_1, AFB_2, AFG_1 and AFG_2.

In addition, AFM_1 has been identified in the milk of diary cows consuming AFB_1 from contaminated groundnut meal (van Egmond, 1989).

Aflatoxins are both acutely and chronically toxic in animals and humans. The disease primarily attacks the liver causing necrosis, cirrhosis and carcinomas. No animal has been found to be totally resistant to the effects of AFs, although susceptibility differs from species to species. Aflatoxin B_1 has been shown to be the most potent naturally occurring carcinogen in animals, with a strong link to human cancer incidence (USDA, 2004). Acute aflatoxicosis in humans is rare, however, several outbreaks have been reported. In 1967 in Taiwan people became ill because of rice from affected households containing about 200 ppb

of AFs. In India in 1974 an outbreak of hepatitis affected people and was traced to corn containing AFs (USDA, 2004). Maximum levels of AFs (AFB_1, B_2, G_1, G_2 and M_1) are laid down in Commission Regulation (EC) No 1881/2006. Special conditions governing certain foodstuffs imported from certain third countries due to contamination risks of these products by AFs are laid down in Commission Decision 2006/504/EC. This Decision was amended three times in 2007: Decision 2007/459/EC, Decision 2007/563/EC and Decision 2007/759/EC. The Scientific Committee on Food (SCF) adopted on 23 September 1994 an opinion on toxicological safety of AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1. In addition, the European Food Safety Authority (EFSA) has adopted on 3 February 2004 an opinion related to AFB_1 as undesirable substance in animal feed. EFSA adopted on 29 January 2007 an opinion related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for AFs in almonds, hazelnuts and pistachios and derived products. In order to assist the competent authorities on the official control of AFs contamination in food products which are subject to Commission Decision 2006/504/EC, a guidance document "Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins" has been elaborated.

1.8 Maize harvesting and drying

Maize harvesting is highly mechanized in developed countries of the world, while it is still done manually in developing countries. The mechanized system removes not only the ear from the plant but also the grain from the cob, while manual harvesting requires initial removal of the ear, which is shelled at a later stage. Harvesting of the seed crop may begin as soon as the corn is physiologically mature. Generally physiological maturity occurs when the kernels moisture content reaches 31%-33% and somewhat depends upon variety, environment and geographic location. Physiological maturity is regarded as that point when the grain reached its maximum dry matter accumulation. After this stage, there is no dry matter added to the corn kernel. Harvesting grain corn at moisture contents above 28% often results in significant damage

to the grain and makes it more difficult to market commercially. High quality food grade markets may require harvest moistures to be as low as 22%-25% (Giardini and Vecchiettini, 2000). Maize is usually harvested when its moisture content is in the range of 18 to 24 %. Damage to the kernel (usually during the shelling operation) is related to moisture content at harvest; the lower the moisture content, the less the damage. Changes in the physical quality of the grain are often a result of mechanical harvesting, shelling and drying. The first two processes sometimes result in external damage, such as the breaking of the pericarp and parts around the germ, facilitating attack by insects and fungi. Drying, on the other hand, does not cause marked physical damage. However, if it is carried out too rapidly and at high temperatures, it will induce the formation of stress cracks and discoloration, which will affect the efficiency of dry milling and other processes. Drying corn is widely diffused before storage, it reduce the humidity content and permit to stock grain for a long time. The aim is to reduce grain humidity at the 14% drying at a temperature of about 90°C. Stress cracking and physical kernel damages are influenced by the speed of moisture removal and also by maximum kernel temperature, combined with the rate of cooling after drying. Storage stability depends on the relative humidity of the interstitial gases, which is a function of both moisture content in the kernel and temperature. Low moisture content and low storage temperatures reduce the opportunity for deterioration and microbial growth. Aeration therefore becomes an important operation in maize storage as a means of keeping down the relative humidity of interstitial gases. A number of factors must be considered such as temperature and air velocity, rate of drying, drying efficiencies, kernel quality, air power, fuel source, fixed costs and management. Drying is an important step in ensuring good quality grain that is free of fungi and micro-organisms and that has desirable quality characteristics for marketing and final use. For a corrected storage in the conditioned silo and for prevent fermentations and spoilages, the humidity would have to be around 14% (Brooker, 1992).

1.9 Maize Storage

Corn must be stored in a manner that will preserve its quality regardless of whether it is kept for a livestock feed or processing. Corn can be sold immediately after harvest and drying, but storage of the corn for later marketing can be advantageous. Storage allows the corn grower to take advantage of price changes throughout the year. On-farm storage also offers greater flexibility in the choice of markets (Boumans, 1985). However storage adds to the cost of corn production through increased overhead or capital costs, drying and handling costs and interest charges. Damage from moulds can be prevented by proper drying and aeration of the corn. However, considerable damage can also be caused by insect infestations which can occur in dry corn. Insects are present in most grain-handling systems and it is almost impossible to eliminate them completely. The chemical components and nutritive value of maize do not lose their susceptibility to change when the grain is harvested. Subsequent links in the food chain, such as storage and processing, may also cause the nutritional quality of maize to decrease significantly or, even worse, make it unfit for either human and animal consumption or industrial use. Seed is stored at its highest quality at physiological maturity and can only deteriorate from that point onward. The best storage conditions can only maintain quality. The basic requirements for seed storage space are that it be dry, free of rodents, and grain storage insects. The efficient conservation of maize, like that of other cereal grains and food legumes, depends basically on the ecological conditions of storage; the physical, chemical and biological characteristics of the grain; the storage period; and the type and functional characteristics of the storage facility. Two important categories of factors have been identified. First are those of biotic origin, which include all elements or living agents that, under conditions favourable for their development, will use the grain as a source of nutrients and so induce its deterioration. These are mainly insects, microorganisms, rodents and birds. Second are non-biotic factors, which include relative humidity, temperature and time. The effects of both biotic and abiotic factors are influenced by the physical and biochemical characteristics of the grain.

Changes during storage are influenced by the low thermal conductivity of the grain, its water absorption capacity, its structure, its chemical composition, its rate of respiration and spontaneous heating, the texture and consistency of the pericarp and the method and conditions of drying. Nutrient losses have been reported in maize stored under unfavourable conditions. Protein quality was not affected (Bressani et al., 1982). Other changes subsequent to drying and storage included a decreased solubility of proteins; changes in nutritive value for pigs; changes in sensory properties (Abramson et al., 1980); and changes in *in vitro* digestibility resulting from heat damage (Onigbinde and Akinyele, 1989). Although damage caused by insects and birds is of importance, a great deal of attention has been paid to the problems caused by micro-organisms, not only because of the losses they induce in the grain, but more importantly, because of the toxic effects of their metabolic by-products on human and animal health.

1.10 The infection of maize with *Fusarium*

Biological interactions between maize plant and *F. verticillioides* are complex and are still under debate. In 2003, Battilani et al. proposed a theoretic and conceptual model for the dynamic simulation of the life cycle of *F. verticillioides*. *F. verticillioides* can invade maize grain via three pathways (Figure 1.6): (i) systemic growth through seed transmission or in roots, stalks, or leaves; (ii) air- or splash-borne infection by macroconidia and microconidia produced on crop residues and tassels that infect ears through silks or insect-caused wounds; and (iii) insects as vectors of conidia. Among these pathways, systemic growth from contaminated seeds seems to be less harmful while the silk and insect routes are more relevant (Munkvold et al., 1997; Oren et al., 2003; Wilke et al., 2007). Once the fungus enters the ear, FUM can be synthesized and contaminate kernels that eventually enter the food or feed chain (Maiorano et al., 2009). According to the literature, the main sources of inoculum in the field are maize residues incorporated into or covering the soil, infected seeds, and the soil itself. Inoculum can be dispersed by wind, rain, and insects even from a distance of 300–400 km (Miller, 2001). Thus, the amount of inoculum in a field

is very variable and consequently, difficult to quantify (Maiorano et al., 2009). While sporulation depends on both temperature and substrate water activity (a_w), only the relationship with the latter has been described (Cahagnier et al., 1995). The successive process of germination is controlled by air relative humidity, temperature, and a_w of the substrate. Silking and its duration are very important in relation to the meteorological conditions throughout this stage when the silks are particularly susceptible to germination by the dispersed inoculum (Stewart et al., 2002). Once the fungus enters the kernels, temperature and a_w are the main factors associated with *F. verticillioides* growth and fumonisin synthesis (Samapundo et al., 2005). In temperate maize areas, the insect most frequently associated with *F. verticillioides* and fumonisin synthesis is *Ostrinia nubilalis* (European Corn Borer – ECB) (Palaniswamy et al., 1990; Mason et al., 1996; Dowd, 2003). ECB feeding activity is crucial in maize grain fumonisin contamination; damaged ears can suffer fumonisin contamination at rates 40 times higher than healthy ones (Alma et al., 2005). The ECB facilitates the infection of *F. verticillioides* in two ways: (i) larvae directly damage kernels by breaking the pericarp and giving the fungus a direct point of entry and (ii) the same larvae can act as vectors of the inoculum (endogenous or exogenous) and carry it directly inside the kernels (Sobek and Munkvold, 1999).

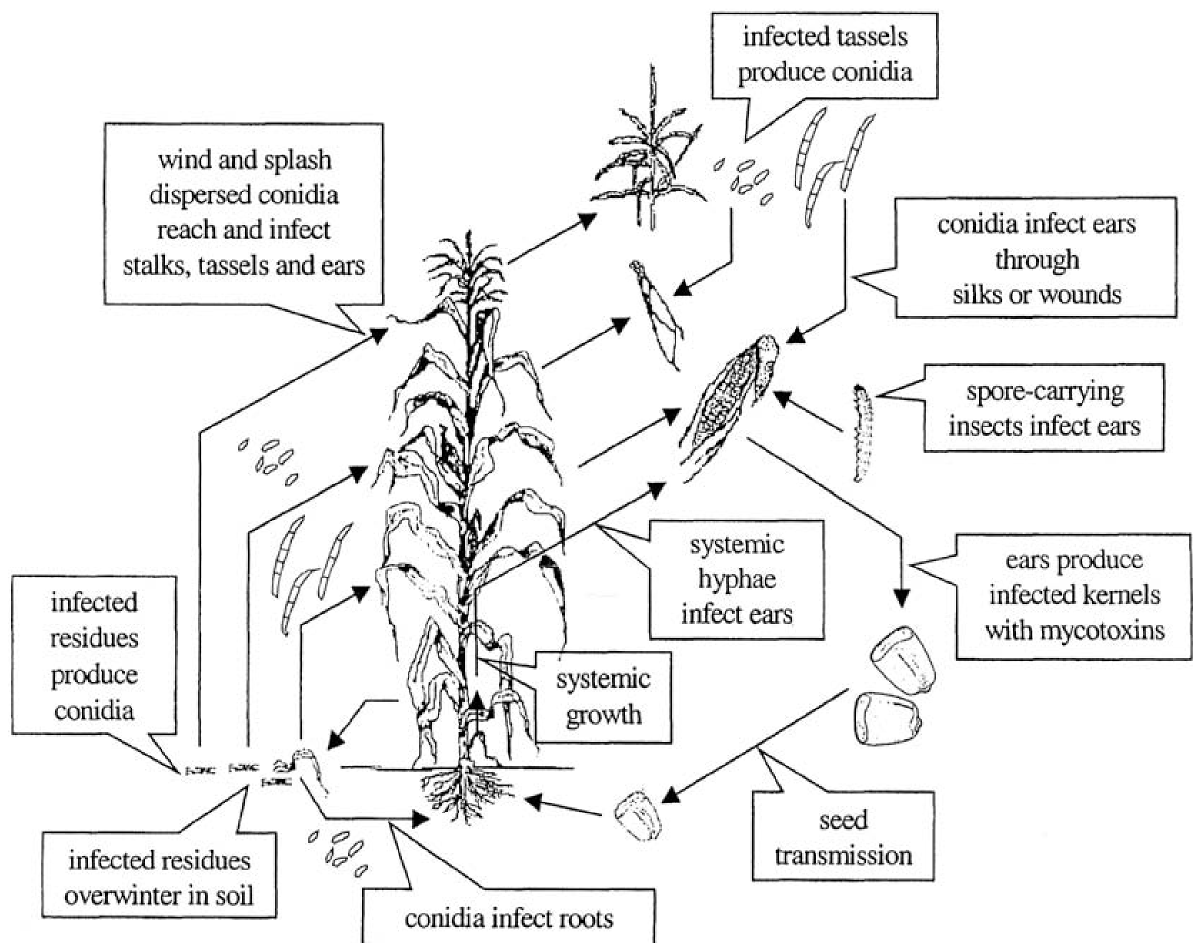

Fig. 1.6 – Life cycle of *F. verticillioides* on maize (Battilani et al., 2003).

1.10.1 pre-harvest

Worldwide surveys showed high levels of FUM associated with warmer and drier climates (Shephard et al., 1996) and when weather conditions are favorable for *Fusarium* infection (Marasas et al., 2001). At the same location, fumonisin contamination is not necessarily the same from one year to another. Hennigen et al. (2000) found in Argentina a marked difference in terms of fumonisin contamination for the same maize varieties during two consecutive growing seasons, due to the fact that environmental conditions may differ from one growing season to another.

Studying the effect of climatic conditions on fumonisin occurrence in freshly harvested maize in different regions of the State of Parana in Brazil, Ono et al. (1999) detected higher FUM levels in maize samples from the Northern and

Central-Western regions compared to that from the South. The authors suggested that it could be due to the differences in rainfall levels during the month preceding harvest. Physiological stress during the period just preceding maize harvest, due to drastic oscillations in rainfall and relative humidity, is likely to create favorable conditions for fumonisin production (Visconti et al., 1996). Shelby et al. (1994) suggested that dry weather at or just prior to pollination of maize might be an important factor for fumonisin production in maize. All this leads to the conclusion that some climatic events such as changes in rainfall patterns or stress during the last stages of maize plant development in the field are likely to have a great influence on fumonisin production in maize before harvest. Furthermore, temperature and moisture conditions during the growing season are often pointed out to affect maize infection by *Fusarium* spp. and fumonisin synthesis. Water activity, the water available for fungal growth, plays a key role. Water present in the environment can be unavailable to the fungi when bound by physical or chemical forces. The water availability in a substrate is generally described in terms of water activity a_w and water potential φ (Brown, 1990). Water activity can be defined as the ratio of the partial pressure, P, of water in the atmosphere in equilibrium with the substrate and the partial pressure, P_0 of the atmosphere in equilibrium with pure water at the same temperature. This is numerically equal to the equilibrium relative humidity (RH). Whereas a_w is a measure of the mole fraction of water in a solution, water potential is a measure of the energy of water in a system relative to that of a reference pool of pure water (Boddy and Wimpenny, 1992).

The main force most commonly included in the water-potential term are osmotic potential, matrix potential, turgor and gravimetric potential. The osmotic potential and matrix potential are the components of φ attributable a_w (Brown, 1990).

Most fungi are highly adapted to growth over a range of external water potentials. The common response to low water availability is to generate a lower internal osmotic potential by accumulating compatible solutes that do not interfere with the regulation of normal metabolic pathways. All known compatible solutes are water soluble organic compounds of relatively low

molecular mass, rarely larger than disaccharides (Brown, 1990). The ability to grow on a relatively dry substrate depends not only on the availability of water, but also on other stressful external conditions. The requirement for water is lowest when other environmental factors are most suitable for growth (Tokuoka and Ishitani, 1991). In general germination can occur at lower water activities than growth, which in turn can occur at lower a_w than both conidial production and mycotoxin production (Magan and Lacey, 1984a).

Velluti et al. (2000), working *in vitro* on fungal competition on maize found that the growth rate of *F. verticillioides* was higher at a temperature of 25°C, compared to 15°C. These researchers also found that at a constant temperature, the growth rate of *F. verticillioides* increased with a_w. Marin et al. (1999) found that the toxin was optimally produced at 30°C and 0.98 a_w. However, Alberts et al. (1990) showed that the mean FB_1 production obtained at 25°C (9.5 g/kg) was significantly higher than that at 20°C (8.7g/kg) and 30°C (0.6 g/kg). Munkvold and Desjardins (1997) reported that *F. verticillioides* generally grows in grain when moisture content is higher than 18–20%.

It has been reported that late planting of maize with harvesting in wet conditions favors disease caused by *F. verticillioides* (Bilgrami and Choudhary, 1998), and the prevalence of this fungus is considerably increased with wet weather later in the season. Moreover, repeated planting of maize and other cereal crops in the same or in nearby fields favors fungal infection by increasing the fungal inoculum and insect population that attack maize plants (Bilgrami and Choudhary, 1998). Lipps and Deep (1991) found that the rotation maize/non host crop of *Fusarium* was better than maize/maize, as the former was less favorable to *Fusarium* disease outbreak than the latter. Weed control also affects fungal infection in maize fields because it helps to eliminate non host weeds on which *Fusarium* can also be found (Bilgrami and Choudhary, 1998).

Maize hybrid and grain characteristics such as colour, endosperm type, chemical composition and stage of development may also influence fungal infection and subsequent fumonisin production. Late-maturing maize cultivars in which grain moisture content decreases slowly below 30% are most susceptible to *Fusarium* disease (Manninger, 1979). It is thought that maize cultivars with

upright cobs, tight husks (Emerson and Hunter, 1980), thin grain pericarp (Riley and Norred, 1999), and an increased propensity for grain splitting (Odvody et al., 1990) are likely to be more susceptible to *Fusarium* infection. Tight-husked varieties favour *Fusarium* problems because of slow drying (Dowd, 1998).

Fumonisins are found more concentrated in the pericarp and germ of the grain than in the endosperm, so that removal of those outer parts by mechanical processes can significantly reduce the toxin in maize (Charmley and Prelusky, 1995; Sydenham et al., 1995; FDA, 2000). However, influence of maize grain colour on fumonisin contamination does not seem to be clear. Shephard et al. (1996) reported that in some years, fumonisin levels were significantly lower in yellow than in white maize, but the reverse situation was observed in other years. Hennigen et al. (2000) compared contamination of maize varieties of flint endosperm to that of dent type and did not find significant differences. Shelby et al. (1994) tested fifteen maize hybrids and found no significant correlation between starch, lipid, fiber, and protein contents and fumonisin production in maize. Recently Pietri et al. (2009) evaluated the distribution of AFs and FUM in fractions derived from fry-milling of contaminated maize. They found that the cleaning step reduced AFB_1 and FB_1 levels and the subsequent removal of bran and germ led to a further decrease in contamination levels in the products destined from human consumption They observed also a different distribution of the two toxins in the kernels: AFB_1 contamination was more superficial and concentrated in germ, while FB_1 contamination affected the inner layers of the kernels (Pietri et al., 2009). Grain age may also influence fumonisin production in maize. Warfield and Gilchrist (1999) found higher levels of FUM in maize grains at the dent stage and significantly lower levels in grains at the immature stage, suggesting that production of the toxin may begin early in cob development and increase as the grains reach physiological maturity. Likewise, Chulze et al. (1996) reported that contamination of maize by FUM was greater after physiological maturity. For maize, the pre-harvest selection of hybrids, time of planting, plant density and insect control have all be found to have an impact on contamination of maize with these mycotoxins preharvest and during drying

and storage. A key critical control point appears to be the harvesting time. In late maturing hybrids there was an increase in FUM and zearalenone produced by different *Fusarium* species. This was found to be less significant in medium-early hybrids (Reyneri, 2006). Studies of maize of different moisture contents have also pointed out the importance of moisture content and efficiency of drying regimes required to control FUM contamination. Overall, pre-harvest factors are critical for effective post-harvest prevention of FUM from contaminated maize entering the post-harvest phase of the food chain. The key factors are:

Pre-harvest

1. proper selection of maize hybrids; prevent use of soft kernel hybrids
2. no late sowing dates
3. avoid high cropping density
4. balanced fertilization
5. avoid late harvesting
6. effective control of pests such as European corn borer

Post-harvest

1. minimize time between harvesting and drying
2. effective cleaning of maize prior to storage
3. efficient drying to 14%
4. effective hygiene and management of silos
5. absence of pests in store which can provide metabolic water and initiate heating
6. clear specifications and traceability from field to store

1.10.2 post-harvest

Contamination of cereal commodities by moulds and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain. Most grain is harvested, dried and then stored on farm or in silos for medium/long term storage. Cereal quality is influenced by a range of interacting

abiotic and biotic factors. In the so-called stored grain ecosystem, factors include grain and contaminant mould respiration, insect pests, rodents and the key environmental factors of temperature, a_w and inter-granular gas composition, and preservatives which are added to conserve moist grain for animal feed. Thus knowledge of the key critical control points during harvesting, drying and storage stages in the cereal production chain are essential in developing effective prevention strategies post-harvest (Magan and Aldred, 2007).

Post-harvest treatment of grain and the prevailing environmental factors are key determinants of the impact fungi may have on the grain quality including germinability. It is important to remember that harvested grain and contaminating microorganisms are alive under dry, safe storage conditions (Magan and Aldred, 2007). Poor post-harvest management can lead to rapid deterioration in nutritional quality of seeds. Microbial activity can cause undesirable effects in grains, contribute to heating and losses in dry matter through the utilization of carbohydrates as energy sources, degrade lipids and proteins or alter their digestibility, produce volatile metabolites giving off-odours, cause loss of germination and baking and malting quality. Filamentous fungal spoilage organisms may also produce mycotoxins that can be carcinogenic or cause feed refusal and emesis (Magan et al., 2004). Spoilage of stored grain by fungi is determined by a range of factors which can be classified into four main groups including: intrinsic nutritional factors, extrinsic factors, processing factors and implicit microbial factors (Sinha, 1995).

Wallace and Sinha (1981) in the 1970s were the first to consider stored grain as a man-made ecosystem which needed to be examined to enable a proper understanding of the processes occurring and to improve postharvest management of stored food commodities. This approach has enabled prevention strategies to be developed and implemented to avoid microbial and pest infestation from damaging stored grain-based commodities. Since most cereals are stored dry, bacteria seldom cause spoilage. At intermediate moisture content levels fungal spoilage and pests are of major concern. The development of prevention strategies today has been predominantly based on

using the HACCP approach and to identify the critical control points in the pre- and post-harvest food chain.

Grain itself and the microbial contaminants respire slowly when stored dry. However, if the a_w is increased to 15–19% moisture content spoilage fungi, particularly *Eurotium* spp., *Aspergillus* and *Penicillium* species grow, resulting in a significant increase in respiratory activity. This can result in an increase in temperature and sometimes spontaneous heating from the colonisation by a succession of fungi resulting in colonisation by thermophilic fungi (Magan et al., 2004).

The chemical process involved in heat generation is predominantly aerobic oxidation of carbohydrates such as starch. Heating occurs when this energy is released faster than it can escape from the cereal substrate. A range of studies have demonstrated that grain spoilage and dry matter loss is predominantly determined by fungal activity. Studies with maize showed that fungal invasion and mycotoxins content could be unacceptable before the grain had lost 0.5% dry matter and mould became visible (Seitz et al., 1982). There are problems with the use of visible moulding as a criterion of deterioration (Lacey et al., 1997). A number of studies have found this to be a subjective index of the safe storability of grain; Magan (1993) suggested that microscopic growth may be a more effective measurement of initial colonisation than visible moulding. Harvesting of maize is often carried out at moisture contents which are 14–15% which requires drying to reduce the available water to 0.70 a_w (=14%) which is safe for storage. Often harvested maize is left at drying facilities during this critical part of the chain if drying facilities are working at full capacity. This can create problems with an opportunity for growth and mycotoxin contamination of maize, especially by *Fusarium* section Liseola (FUM by *F. verticillioides*, *F. proliferatum*), *F. graminearum* (trichothecenes; zearalenone), and *A. flavus* (AFs).

For many years modified atmospheres or alternative gases have been examined for the medium and long term storage of cereal grain destined for food/feed. While fungi involved in bio-deterioration of grain are considered to be obligate aerobes, many are actually micro-aerophilic, being able to survive and

grow in niches where other species cannot grow and thus dominate specialised grain ecosystems. The use of integrated post-harvest systems for prevention of deterioration entails modifying O_2 and CO_2 simultaneously. The tolerance to low O_2 and high CO_2 is also influenced by interactions with grain type and water availability. The drier the grain, the more effective the treatment. Modified atmosphere storage is used to control both moulds and insects in moist stored grain. Regimes sufficient for moulds may not however be effective against some storage insects, which can survive and grow over a wider equilibrium relative humidity range.

Modified atmosphere storage has been examined for the storage of moist grain, especially for animal feed. Samapundo et al. (2007) found that fumonisin production by *Fusarium* section Liseola on maize was inhibited by 30% CO_2 over a range of a_w levels although sealed systems were used in which final CO_2 concentrations were higher. Giorni et al. (2008) made a systematic study of CO_2 and how these gases affected AFs production in maize. They showed that the treatment with 25% of CO_2 could be satisfactory to efficiently reduce *A. flavus* growth but at least 50% CO_2 was required to obtain a considerable reduction of AFs synthesis.

Moist grain specifically destined for animal feed is often treated with aliphatic acid-based preservatives. There are a number of commercial products predominantly based on salts of propionic and sorbic acids. However, these are fungistats and thus the coverage of the grain must be efficient to prevent under-treated pockets. Poor coverage can lead to growth of spoilage fungi, especially mycotoxigenic moulds which can sometime metabolise these aliphatic acids.

Studies by Marin et al. (1998c, 2000) showed that growth of *Fusarium* section Liseola species and fumonisin production was relatively unaffected by different mixtures of proprionic and sorbic acids. There is thus interest in finding alternative compounds to either enhance or to replace such compounds. Research has been carried out on both essential oils and anti-oxidants (Hope et al., 2005; Fanelli et al., 2003). These studies suggested that only few essential oils, such as cinnamon and clove leaf oil, have the capacity to control mycotoxigenic *Fusarium* species. Resveratrol has been demonstrated to have a

particularly wide spectrum of mycotoxin control, although at present this is a relatively expensive product (Fanelli et al., 2003). Pre-harvest natural contamination can only be minimized post-harvest by the application of processing techniques which will minimize subsequent entry into the food and feed chain where possible. There are however key management tools and traceability procedures which should be used to facilitate stored commodities to be effectively conserved with minimum loss in quality. These include accurate and regular moisture measurements to ensure safe thresholds are not breached.

It is essential that Good Agricultural Practice and operation approved supplier chains are in place. This also required effective diagnostic tools which can be used to monitor and quantify mycotoxins rapidly (Magan, 2006). Representative sampling remains a problem for stored commodities. While legislation exists on sampling procedures, these are not easy to achieve and the errors in actually taking samples may be significant compared to those for actually analysing for the mycotoxin contamination level. Early indication of changes in stored commodities due to insect or mould activity may be possible by monitoring of intergranular gas composition and the use of volatile fingerprints. The development of models on mycotoxigenic mould activity and the conditions which will prevent mycotoxin production and which can give an indication of tolerances relevant to the legislative limits are important (Magan and Aldred, 2007).

1.11 Post-harvest fungal ecology

Spoilage of stored grain by fungi is determined by a range of factors: intrinsic nutritional factors, extrinsic factors, processing factors and implicit microbial factors (Sinha, 1995). Factors such as grain type and quality, fungal population and community structure, mycotoxin production and pest infestation were all interlinked (Figure 1.7).

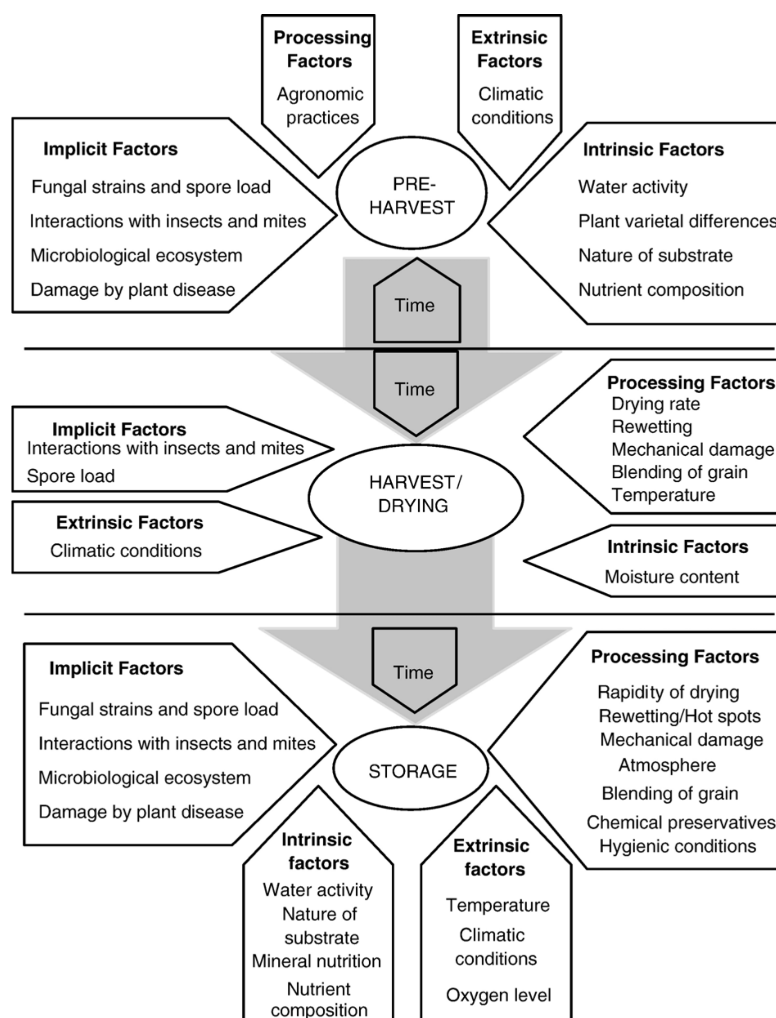

Fig. 1.7 – The interaction between intrinsic and extrinsic factors in the food chain which influence mould spoilage and mycotoxin production (Magan et al., 2004).

Fungi seldom occur on grains in isolation, but usually as a mixed consortium of bacteria, yeasts and filamentous fungi. It is thus inevitable that inter-specific and intra-specific interactions will occur depending on the nutritional status of the grain and the prevailing environmental conditions. Indeed, environmental factors may exert a selective pressure influencing community structure and dominance of individual species, especially mycotoxigenic species (Willcock and Magan, 2001).

The key environmental factors of temperature, a_w and gas composition influence both the rate of fungal growth and the production of mycotoxins.

Generally, low oxygen concentration (<1%) or increased concentrations of carbon dioxide or nitrogen can be highly effective in preventing the development

of mould on grain and inhibiting the production of mycotoxins (Paster and Bullerman, 1988).

However, the reaction of many fungi to low level of oxygen and high carbon dioxide concentrations is strongly affected by a_w and temperature (Magan and Lacey, 1984b).

The co-existence of microorganisms is also mediated by nutritional resource. Stored corn offer an excellent but finite nutritional source for spoilage fungi (Magan et al., 2003).

It has to be remembered that spoilage fungi colonising grain use different primary and secondary strategies to occupy the niche. They may have combative (C-selected), stress (S-selected) or ruderal (R-selected) strategies or merged secondary strategies (C–R, S–R, C–S, C–S–R; Cooke and Whipps, 1993).

Wilson and Lindow (1994), working with biocontrol systems, suggested that the co-existence of microorganisms particularly on plant surfaces may be mediated by nutritional resource partitioning. Thus *in vitro* carbon utilisation patterns (Niche size) could be used to determine Niche overlap indices (NOI) and thus the level of ecological similarity. Based on the ratio of the number of similar C-sources utilised and those unique to an individual isolate or species, a value between 0 and 1 was obtained. NOI of >0.9 were indicative of co-existence between species in an ecological niche, while scores of <0.9 represented occupation of separate niches. This approach was modified by Marin et al. (1998a) and Lee and Magan (2000) for a multi-factorial approach by including a_w and temperature into the system. This demonstrated that based on the utilisation of maize C-sources, the NOIs for fumonisin-producing strains of *F. verticillioides* and *F. proliferatum* were >0.90 at >0.96 a_w at 25 and 30 ◦C, indicative of co-existence with other fungi such as *Penicillium* species, *A. flavus* and *A. ochraceus*.

However, for some species, pairing with *F. verticillioides* resulted in NOI values <0.80 a_w indicating occupation of different niches. These results suggest that Niche overlap is in a state of flux and significantly influenced by both temperature and a_w.

This approach confirms that interactions and dominance are dynamic, not static, and emphasises the importance of taking into account such fluxes in any integrated approach to understanding and controlling the activity of mycotoxigenic spoilage moulds in the stored grain ecosystem.
Giorni et al. (2009) found that *A. flavus* and *F. verticillioides* occupied different ecological niches. The variability in nutritional sources utilization between A. flavus strains was not related to their ability to produce AFs. This type of data helps to explain the nutritional dominance of fungal species and strains under different environmental conditions.
In vitro and *in situ* studies have suggested that interaction between some species can result in a significant accumulation of mycotoxins, while in other cases an inhibition of mycotoxin production is observed. For example, interactions between section Liseola *Fusarium* species with *A. niger* resulted in a tenfold increase in fumonisin production especially at 0.98 a_w, although under drier conditions no increase in fumonsin occurred on maize (Marin et al., 1998b).
Finally it is important to remember that insect pests are a common problem in stored grain ecosystem. They grow and multiply at a_w much lower than those allowing fungal growth. Insects can produce metabolic heat which generates water via condensation on surfaces due to temperature differentials and develop classic hot spots which can quickly result in heating and complete spoilage.
The role of insect pests should not be neglected as they may be integrally involved in the dominance of mycotoxigenic species by helping in dispersal and acting as vectors and carriers of the toxin through grain. Overall, conditions in stored grain are not in a steady state and thus the dynamics of the system will vary over time. This needs to be taken into account in determining safe storage times for cereals without risks of spoilage and mycotoxin contamination. Any decision support system must take all these factors into account for the effective development of good management systems post-harvest.

1.12 Preventive measures

Preventive measures are generally aimed at the reduction of inoculum present in the susceptible crop and the risk of mycotoxin contamination. Preventive measures include good cultural practices, chemical control and breeding for resistance.

1.12.1 Genetic resistance

Genetic modification of corn through plant breeding or by transgenic means offers several approaches for controlling corn ear mould and mycotoxins formation. The development of corn hybrids that are resistant to environmental stresses such as heat, drought and insect damage, reduce their susceptibility to infection, prevent mycotoxins formation (Duvick, 2001). The potential for imparting resistance of corn to insect infestation through molecular genetic means is now being realized. Research by Bakan et al. (2002) and Masoero et al. (1999) has shown that corn containing genes coding for insecticidal proteins from *Bacillus thuringiensis* (Bt) had significantly less damage from the European corn borer. These Bt corn hybrids grown in United states and in Italy had reduced incidence and severity of *Fusarium* ear rot and FUM levels.

Natural differences among maize genotypes for fumonisin accumulation have been found (Clements et al., 2004). As the a_w for fungal growth plays a key role, late-maturing maize cultivars in which grain moisture content decreases slowly are more susceptible (Fandohan et al., 2006). It is thought that upright cobs and thin grain pericarp increase susceptibility to *Fusarium* infection. Tight husks have been described as an unfavourable characteristic because they slow kernel drying or as a favourable characteristic because they protect the ear from insect damage (Burton et al., 2006). Therefore, many questions remain to be answered about mechanisms for resistance of maize to fumonisin contamination. Clements et al. (2004) suggested that several dominant genes are involved, and two quantitative trait loci (QTLs) located on chromosome 5 were associated with resistance to fumonisin contamination and *Fusarium* ear rot. White corn is preferred for human consumption, and care should be taken

to avoid drought stress or damage by insects that could contribute to fungal growth on kernels. Looking for sources of resistance to mycotoxin contamination among white maize genotypes would also contribute to reducing human hazard. A efficient means of preventing fumonisin contamination in corn-based food products is planting hybrids that are highly resistant to *Fusarium* ear rot and fumonisin accumulation in grain. Relative susceptibility or resistance of food-grade dent corn hybrids to fumonisin accumulation in grain has not been reported; therefore, highly resistant (fumonisin at <2 μg/g in all environments) food-grade hybrids are commercially not available.

1.12.2 Good agricultural practices

The contamination from mycotoxins can occur along several rings of the chain (from the field to the storage) if the conditions are favourable to fungi contamination. The development of *Fusarium* is favoured in field by rainy weather and relatively low temperatures in the period between closing and harvesting of maize, while *Aspergillus* is typical of seasons with high temperatures, associated to conditions of water stress of the plant (Scudellari et al., 2008).

As neither resistant hybrids nor effective and economical techniques have been identified yet for detoxification from mycotoxins, prevention is still the best strategy for the control, provided it is applied on the complete chain, from the field to the product processing (Battilani et al., 2005).

In the maize cultivation the most effective strategy to follow includes the application of good agricultural practices, which may limit the colonization of the fungal species that have been involved in the production of mycotoxins. This aspect is important if we consider that the presence of these metabolites hardly ever increases in the operations following the harvest, provided they are carried out in a correct and careful way. Consequently, cultivation phase is the most critical, as mycotoxins accumulate in the kernels. For this reason, a particular recommendation on principles of good agricultural practices to follow to prevent contaminations from *Fusarium*-toxins has been introduced by the European Commission (2006/583Ce).

However, some agronomic techniques are useful for the control of the development only of some toxigenic fungi, while they are not effective for others.
It is certain that the plants that are not subjected to stress conditions which may affect their growth are less subject to the attack of toxigenic fungi, and are less at risk of mycotoxins presence. The crop rotation is very useful to control the spread of the fungi that are preserved in crop residue and, consequently, to reduce the inoculum sources. This could apply also to *F. verticillioides*, but the great spread of the cultivation on the territory ensures such an abundant inoculum that the importance of the alternations is greatly reduced or even lost. The field preparation before seeding must favour the growth of the plants and avoid even temporary stress conditions (Battilani et al., 2006).
Seed coating with fungicides is not a practice that can not act directly on toxigenic fungi. in fact, these are preserved in crop residue, and the inoculum reaches the ear by being transported by wind or rain. The infection of the plant caused by infected seed, although possible for *Fusarium*, is not considered important in the practice. Moreover, FUM are often correlated with some characteristics of the kernels, like specific weight and kernel composition; shot season hybrids (FAO class 300-400) are those mainly susceptible of contamination from AFs, while long season hybrids (Class FAO 600-700) are mainly predisposed to contamination from FUM (Battilani et al., 2005). The choice of the maturity class is linked to availability of water and to the harvest age. A fundamental aspect is the suitability of the hybrid for the nature of the ground and the climatic conditions of the area in which it will have to be cultivated. The late seeding (indicatively from the third decade of April in Italy) are more at risk of contaminations from fusaria-toxins, particularly for long season hybrids (Class FAO 600 - 700). It is therefore advisable to seed timely and when the agronomic and climatic conditions are good.
It is important to choose the right number of plants per hectare, because high density in fertile areas and in the first seeding time can increase the risk of water stress of plants and involve more favourable micro-climatic conditions to the development of toxigenic fungi. Some experiments carried out in Northern

Italy have shown that seeding density higher than 8,5 plants/m^2 can considerably increase contaminations from the main *Fusarium*-toxins (Battilani et al., 2005).

A correct management of the fertilization is important in order to avoid nutritional stresses to plants (deficiencies and excesses) that can favour the mycotoxin risk.

In the case of maize, the element that demands the greatest attention is nitrogen: plants with obvious symptoms of nitrogenous deficiency are more predisposed to contamination from AFs. Trials managed in Emilia-Romagna suggested that an amount of nitrogen considerably higher than balance doses can increase considerably the contamination of FUM, probably as a result of the development of more favourable micro-climatic conditions to the spread of fungi (Scandolara et al., 2008).

Irrigation is one of the most important agronomic operations for the control of the most frequent mycotoxins in maize.

A condition at high risk of infections in field from *A. flavus* is the presence of water stress after the dough maturation of the kernels. Therefore, irrigations not only must be carried out in the right way in the period immediately before the male closing, but also in the more advanced phase of the cultivation, if the humidity conditions of the field are insufficient to favour the water demand of the plant.

Weeds control is important so as to avoid water and nutritional competition with the existing crops. The development of infesting grass is an element of hard stress for the plant, therefore it must be considered as predisposing factor for the fungal infection .

In the maize areas with strong presence of *Ostrinia nubilalis*, chemical control becomes fundamental for the prevention of the contamination from FUM. In fact, the fusariosis of the ear is associated with the damage of the kernels, due to the bugs that damage the pericarp and favour the increase and the penetration of the fungus (Saladini et al., 2008).

Recent studies have shown that there is a significant correlation between the number of larvae present in the ear at dough maturation and the contamination

from FUM at harvest; particularly, the contaminations are higher than two parts per million, with more than two larvae per ear (Saladini et al., 2008).
Harvesting time is relevant for mycotoxins. Therefore a consisting reduction of the risk AFs can be obtained by harvesting the kernels with a humidity not lower than 22 - 24%.
Studies are being carried out to define models for *F. verticillioides* and *A. flavus* in order to anticipate, according to the meteorological conditions in the cultivation area, the risk of FUM and AFs contamination at harvest. The relevant search for *F. verticillioides* have been in progress since 2002, and have allowed the development of a prototype of provisional model based on the in-depth study of the infection cycle of the pathogenic agent (Scudellari et al., 2008).

1.12.3 Use of fungicides

Fungicides have been used successfully to control many diseases since their introduction in the late 1800s in small grains. Most currently registered products with activity against *Fusarium* head blight (FHB) are active against FHB pathogens involved in the complex, but are less effective or completely ineffective against the remaining pathogens. So product choice is of particular importance in the control of grain contamination by mycotoxins. Trials which demonstrate differential control of FHB pathogens have been carried out by Jennings et al. (2000). In a series of trials inoculated with a mixed conidial suspension of FHB pathogens at mid-anthesis, the demethylation inhibiting fungicide tebuconazole (as Folicur®) effectively controlled the toxigenic *Fusarium* species present on the ear, but showed little control of the non-toxigenic *M. nivale*. Conversely application of strobilurin fungicide azoxystrobin (as Amistar®) controlled *M. nivale* but not the *Fusarium* species present (Magan and Olsen, 2004). Other active ingredients which have been shown consistently good control of the toxin-producing species involved in the FHB complex come primarily from the same fungicides group as tebuconazole and include metaconazole (as Caramba®) (Jennings et al., 2000), epoxiconazole, (as Opus®) prochloraz (as Sportak®) (Matthies and Buchenauer, 2000) and propiconazole (Martin and Johnson, 1982).

The timing of fungicides application is more important than product choice when trying to control FHB and mycotoxin contamination of grain; if it is applied at the wrong time it will not control FHB. Mid-anthesis is the most susceptible time for infection of wheat by FHB pathogens (Sutton, 1982) and, as such, is the most appropriate time to apply fungicides spray aimed at controlling FHB.

Fungicides must be applied at the producer's recommended rate. Work carried out by Nicholson et al. (2003) showed that halving the rate of several fungicides led to significant reductions in control of FHB disease levels and mycotoxins production.

With any fungicides application there is always the potential for the development of resistance within a population. Results from *in vitro* investigations (D'Mello et al., 1998) indicated that more persistent patterns of toxin production may also develop in *Fusarium* populations showing resistance to fungicides.

Ellner (2000) carried out field trials whit azole fungicides (tebuconazole, metaconazole) in two seasons in Germany and found that control of head blight and reductions in the levels of deoxynivalenol (DON) did non exceed 50%. Jennings et al. (2000) and Simpson et al. (2001) demonstrated complex interactions between the type of fungicide used and effects on colonization by *Fusarium* spp. and mycotoxin production. Higher DON productions were found in plots treated with azoxystrobin with difenoconazole. They suggested that complex interactions occur between the differential impact of the fungicides, resulting different in *Fusarium* interactions which could result in this enhanced accumulation of DON.

No detailed studies of the efficacy of fungicides on maize against mycotoxigenic *Fusarium* spp. are available in literature. Only recently Folcher et al. (2009) carried out field trials to study the control of *Lepidoptera* caterpillars by agrochemical treatments and their consequences on *Fusarium* spp. mycoflora and mycotoxin levels. Although the insect populations were controlled by agrochemicals, there was no reduction in *Fusarium* spp. or mycotoxins.

CHAPTER 2

SPECIFIC AIM OF THE PROJECT

Maize quality is essential to ensure continuity in the supply of livestock feed and food reducing economic losses and minimizing potential risks to animal and human health. To obtain good quality, it is essential to manage correctly both post harvest and pre-storage of maize but poor information is available on this issue.

Surely an inadequate storage at high temperature or at high relative humidity can allow fungi present on maize to grow and to produce mycotoxins. Also an high moisture content of kernels can play a role in post harvest spoilage.

Data on mycoflora associated to maize kernels during storage could help in predicting the post harvest deterioration and help to find helpful approaches to reduce fungal action.

The use of chemical and biological control can help in reducing fungal presence and mycotoxins production in field but the study of relationship between different maize hybrids and a_w and humidity of kernels and also essential to predict potential risk of contamination with FUM and AFBs in post harvest.

The aims of this work are to:

- Monitor the main environmental parameters that regulate mycotoxigenic fungal growth and the mycotoxins contamination post harvest and during storage. In particular, it will be underlined the relationships between temperature, relative humidity and a_w of kernels with the growth of the main fungi present on maize (*F. verticillioides* and *A. flavus*) and their production of mycotoxins in storage conditions.
- Study the activity of fungicides and, above all, biological control agents in reducing fungal growth and mycotoxins production to define useful strategies to prevent or limit fungi actions in field and in post harvest.
- Understand competitiveness among fungi present on maize under stress conditions due to different levels of a_w and fungicides used.

- Study the relationship between kernels a_w, humidity and different maize hybrids on fungal and mycotoxins presence.

The detailed list of the work programme is shown in the Flow Diagram reported in Figure 2.1.

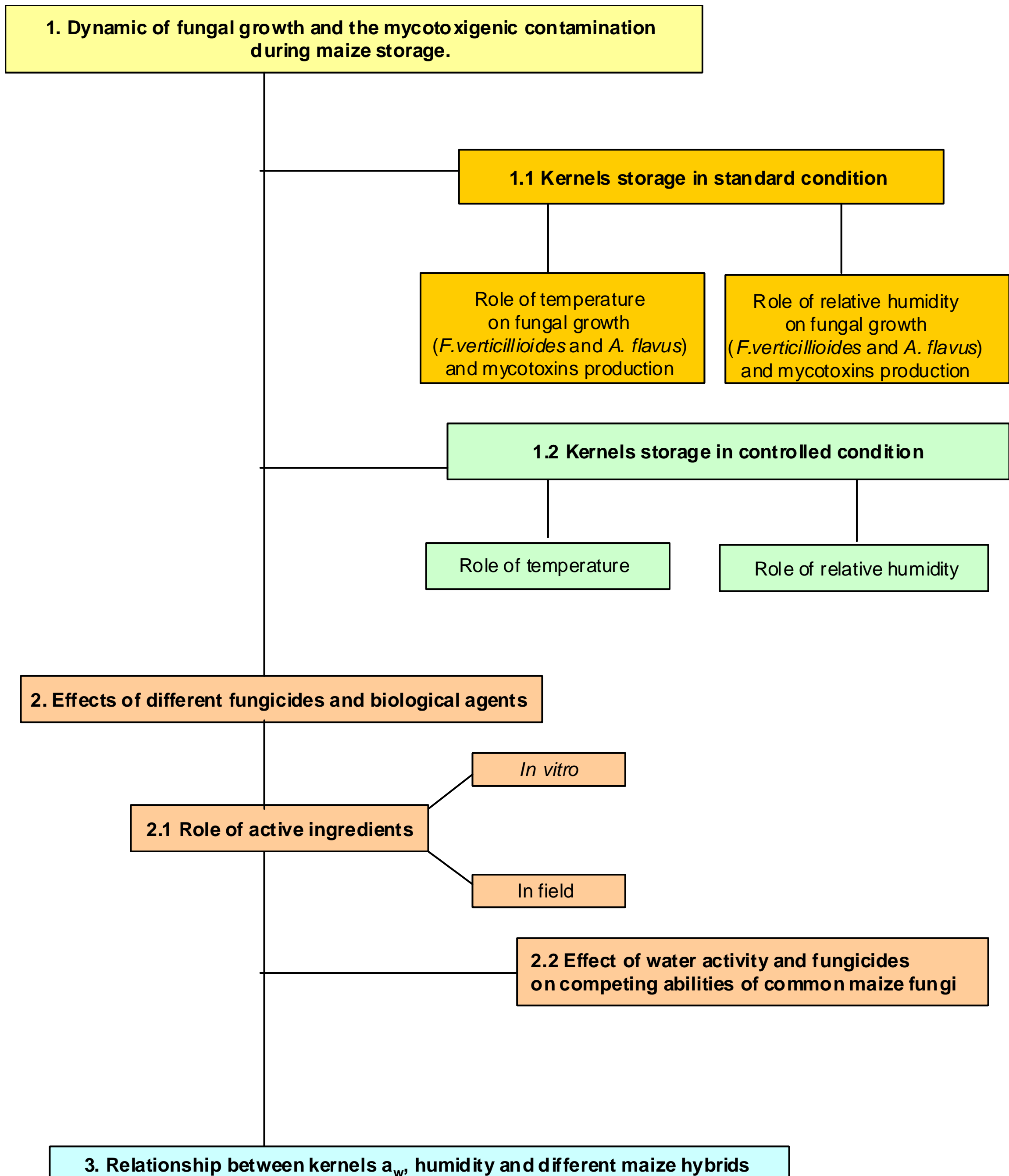

Fig. 2.1 Flow Diagram of different components of studies

CHAPTER 3

DYNAMIC OF *FUSARIUM VERTICILLIOIDES* GROWTH AND FUMONISIN PRODUCTION DURING MAIZE STORAGE

Maize is well known all over the world as host plant for several mycotoxin producing fungi. Development of species belonging to *Fusarium*, *Aspergillus* and *Penicillium* and toxin production can occur on crops in field and during storage when kernels humidity is sufficiently high (Reid et al., 1999). As effective detoxification methods are not yet available (Scott, 1991; Sinha, 1998), in field and post-harvest prevention may be considered of primary importance.

3.1 Aim

The aim of this study was to describe the dynamic of mycotoxins from harvest to the end of storage in natural conditions and to evaluate the effects of environmental factors on the growth of *F. verticillioides* and *A. flavus* and mycotoxins production in natural and controlled conditions post harvest.

3.2 Materials and Methods

3.2.1 Maize in natural conditions

3.2.1.1 Maize samples

Lots of maize kernels, DK440 hybrid (FAO class 300; 105 days as growth period length) grown in North-East Italy in 2004 and 2005, were considered in this study. In each lot, a kernels sample of 0.3 ton was randomly collected and mixed. Different steps have been considered: harvest, staying in a large square on the ground before drying and storage.

3.2.1.2 Pre-storage trial - 2004

The mass of maize kernels was collected at harvest in September 2004. Two fields were considered, 1 in Ravenna and 1 in Ferrara. The harvest moisture content of kernels collected in Ravenna was 22.4%, with the initial AFB_1 and FB_1 content respectively of 0 μg/Kg and 20395 μg/Kg. In Ferrara kernel

humidity at harvested was 16.8% with 18.1 μg/Kg and 4771 μg/Kg respectively of AFB_1 and FB_1.

Sub-samples of 7-9 Kg of kernels were taken from the two lots and put in pierced bags together with a data logger to monitor air relative humidity (RH; %) and temperature (T; °C) in the mass. They were placed inside the heap of seeds in the large square, in order to have samples in the same conditions of the whole mass.

Subsequently, sub-samples of 0.8-1 Kg were taken from the bags after 24, 48, 72, 96 hours and 18 days after harvest.

The sampled kernels were analysed for fungal presence, particularly the incidence of kernels infected by *F. verticillioides* and *A. flavus* and colony forming units (CFU) of the same fungi and for FB_1 and AFB_1 contamination (see mycological analysis and mycotoxin analysis for details).

3.2.1.3 Storage trial - 2004

Thirty kernels sub-samples of 0.8-1 Kg each were taken from maize kernels produced in Ferrara. After drying, these sub-samples were stored in three different types of stores: silos, vertical cell and horizontal store.

After 1, 3 and 6 months of storage these sub-samples were picked for the mycological and mycotoxins analysis.

3.2.1.4 Storage trial - 2005

Two lots of maize kernels, with high and low AFB_1 concentration were selected in 2005 in Ferrara for storage trials.

Samples of 7-9 Kg were stored in two different type of store: vertical cell and horizontal store. Afterwards 10 sub-samples of 0.8-1 Kg were picked at 1, 3 and 6 months of storage for the myco-toxicological analysis.

Both mycological and mycotoxins analysis were carried out as described in Annex 1 and Annex 2.

3.2.1.5 Data analysis

Data analysis were done considering the two fields together for the elaborations. Data on the CFU of *Fusarium* spp. and *Aspergillus* section *Flavi* was Ln transformed before statistical analysis. Log transformation is always required for data that cover a wide range of values from single-digit numbers to numbers in hundreds or thousands (Clewer and Scarisbrisck, 2001). Analysis of variance was performed considering all factors involved; a randomized complete block design of the statistical package SPSS 15 was applied (Statistical Package for Social Science, ver.15.0.1, 2006. SPSS Inc., Chicago, IL, USA). Means were compared using the Tuckey test.

3.2.2 Maize in controlled conditions

3.2.2.1 Trial - 2006

The research was carried out both in natural storage conditions in a horizontal store and under controlled conditions, with constant air temperature and humidity.

Kernel samples (20 kg) were collected from different lots, mixed hybrids were put in a pierced bag together with a data logger to detect RH and T.

The pierced bag was placed in a heap of seeds and in 5 different storage situations with controlled temperature and relative humidity (Table 3.1).

Tab. 3.1 – Temperature and humidity conditions considered in the study.

Temperature (°C)	**Relative Humidity (%)**
20-25 (natural conditions)	70
0-1	85
0-1	70-75
6-7	85
30	85

Maize kernels were analysed for FB_1, FB_2 and AFB_1 initial content and data are reported in Table 3.2.

Tab. 3.2 – Different mycotoxins content present in maize used for the experiment.

	FB_1 (µg/Kg)	**FB_2 (µg/Kg)**	**AFB_1 (µg/Kg)**
Maize sample	13444	4807	2.1
Maize heap	5297	1424	0

Sub-samples (1-1.5 Kg) were analysed after 10, 20, 40, 60, 90,120,180 days.

Both mycological and mycotoxins analysis were carried out on samples as described in Annex 1 and Annex 2.

3.3 Results

3.3.1 Maize in natural conditions

Mycological analysis showed that *Fusarium* and *Aspergillus* were the predominant fungal genera in maize post-harvest. The genus *Penicillium* was also detected in many samples, but with lower incidence (data not show). *Fusarium* species most commonly found in maize samples was *F. verticillioides*, detected in almost all the samples.

3.3.1.1 Pre-storage trial-2004

The analysis of variance on maize kernels collected in Ferrara and Ravenna during pre-storage period showed a temperature increase from 24.8°C to 36.6 °C in kernels respectively after harvesting and 18 days staying in the large square. The temperature remained within 24 and 27 °C until 96 days and increased at the end of the pre-storage period (Table 3.3).

The increase of temperature significantly influenced the incidence of *F. verticillioides* and the number of *Fusarium* CFU/g during the pre-storage time.

Statistically, kernels moisture content did not show significant differences during the 18 days. Relative humidity observed was between 19 and 21%. At 18 days post harvest, an important decrease in the incidence of kernels infected by *F. verticillioides* and in the number of *Fusarium* CFU/g was observed, while until 96 hours the incidence of kernels infected by *F. verticillioides* and the number of *Fusarium* CFU/g did not differ significantly. Data recorded also showed a tendency toward decreased FB_1 mean concentrations in the samples collected in both field monitored in this study (Table 3.3) with significant reduction after 18 days from harvest.

The incidence of *A. flavus* and AFB_1 content in maize were lower and did not change significantly during the pre-storage time (Table 3.3).

Tab. 3.3 – Analysis of variance of incidence of *F.* verticillioides, A .*flavus*, CFU/g of *Fusarium*, temperature (T) and relative humidity (RH) on maize kernels collected in Ferrara and Ravenna during pre-storage period of wet kernels before drying and storage (average data).

Time	T (°C)		RH (%)	*F. verticillioides* (%)		CFU_fus/ g		*A. flavus* (%)	FB_1 (ppb)		AFB_1 (ppb)
After harvest	24.8	b	19.4	61.0	a	1.5E+07	a	3.0	21244	a	0.0
24 h	26.9	b	19.2	52.5	a	1.6E+06	a	0.5	13608	a	1.2
48 h	27.2	b	20.6	54.5	a	4.0E+05	a	0.5	9494	a	4.3
96 h	24.9	b	21.6	56.0	a	3.0E+05	a	6.0	11422	a	11.7
18 days	36.6	a	20.5	10.0	b	3.06	b	2.0	1213	b	1.3

Different letters indicate significant differences according to the Tuckey test ($P \leq 0.01$)

3.3.1.2 Storage trial - 2004

After drying and before storage, the kernels T was around 21 °C, but after one month of storage a decrease was detected. From one month to six months of storage T remained almost constant (Table 3.4).

Regarding RH, ANOVA highlighted differences from dried kernels to six months of storage: RH increased in time, from 12,27% to around 14%, during the storage.

F. verticillioides incidence decreased significantly over the six months of storage from about 42% of infected kernels after drying to 0% (Table 3.4). The production of FB_1 by *F. verticillioides* increased by 30% after six months of storage.

From a statistical point of view, the number of Fusaria CFU did not show significant variations. The incidence of *A. flavus* and AFB_1 content did not change during 6 months storage. Storage types did not influence any of the factors considered (Table 3.4).

Tab. 3.4 – Analysis of variance of incidence of *F.* verticillioides, A. *flavus*, CFU/g of *Fusaria*, temperature and relative humidity on maize kernels collected in Ferrara after 4 different times of storage and considering 3 different types of storage (average data).

Storage	**T (°C)**	**RH (%)**		***F.verticillioides* (%)**		**CFU_fus/g**	***A.flavus* (%)**	**FB_1 (ppb)**		**AFB_1 (ppb)**
After drying	21.3	12.3	b	42.0	a	86.4	1.3	2667	b	17.3
1 month	16.0	14.3	a	44.7	a	303.9	0.7	5691	ab	10.9
3 months	15.7	14.0	a	42.0	a	147.4	0.7	4634	ab	1.2
6 months	16.3	13.8	a	0.0	b	8.0	0.7	9018	a	3.6
Type of storage										
silo	16.7	14.1		34.0		163.0	3.0	6798		16.9
horizontal store	16.1	13.8		–		–	2.0	4927		0.0
Vertical cell	16.7	13.7		30.0		89.0	7.0	6484		2.5

3.3.1.3 Storage trial - 2005

Taking into account initial aflatoxins contamination, the factor considered influenced significantly AFB_1 content and both *Fusarium* and *Aspergillus* CFU, except the FB_1 contamination (Table 3.5).

The population of *Fusarium* and *Aspergillus* and FB_1 contamination did not change significantly based on the store type, horizontal or vertical cells. Only AFB_1 content was significantly lower in vertical than in horizontal stores (-21%). Considering storage time, from time 0 to 6 months, both *Fusarium* and *Aspergillus* CFU did not show significant changes while an increase occurred in FB_1 content although not statistically significant.

Tab. 3.5 – Analysis of variance of CFU of *Fusarium* and *Aspergillus* and content of fumonisin B_1 and aflatoxin B_1 in maize kernels considering 2 different initial aflatoxins contamination, 2 different types of storage and 3 different storage time (average data).

Aflatoxins contamination	CFU_fus/g		CFU_asp/g		FB_1 (ppb)	AFB_1 (ppb)	
High	102.5	b	7.6E+02	a	5537	642.9	a
Low	2.4E+04	a	4.1	b	5883	4.4	b
Storage Type							
Horizontal store	1.1E+03		4.5E+01		5457	364.8	a
Vertical Cell	1.3E+04		3.5E+02		7228	76.9	b
Time Storage							
After drying	5.7E+02		1.2E+01		4392	181.1	
1 month	1.4E+03		7.6E+01		2234	296.8	
3 months	2.8E+03		6.2E+01		9711	437.6	
6 months	6.5E+03		1.1E+03		8100	435.6	

3.3.2 Maize in controlled conditions

3.3.2.1 Trial 2006 - Relative humidity of maize kernels

The relative humidity (RH) of maize kernels, during the 180 days of time incubation and under the different environmental conditions used, are given in table 3.6.

The RH of maize kernels, detected before harvesting, was 14%. This value remaind constant until the end of incubation. There were no important changes at all environmental conditions in RH of maize kernels kept at different and controlled temperature and humidity for 180 days.

Tab. 3.6 – Mean relative humidity of kernels detected at controlled environment with different levels of temperature and humidity after incubation from 0 to 180 days.

Humidity **Temperature**	**70%**	**85%**
0	14.0-15.2	14.0-15.3
6		14.0-15.7
20	14.0-15.3	
30		13.4-14.3

3.3.2.2 Maize samples at 0°C and 0.70-0.85 a_w

The population of *F. verticillioides* (lnCFU) on stored maize grain at 0°C and 0.70 a_w was small and remained below 10 with variable trend until the end of incubation (Figure 3.1).

At 0°C and 0.85 a_w, the sample showed few CFU of *F. verticillioides* and this population remained stable until 60 days of incubation. From 60 to 120 days of

storage *F. verticillioides* increased, but at 180 days the trend showed a suddenly decrease (Figure 3.1).

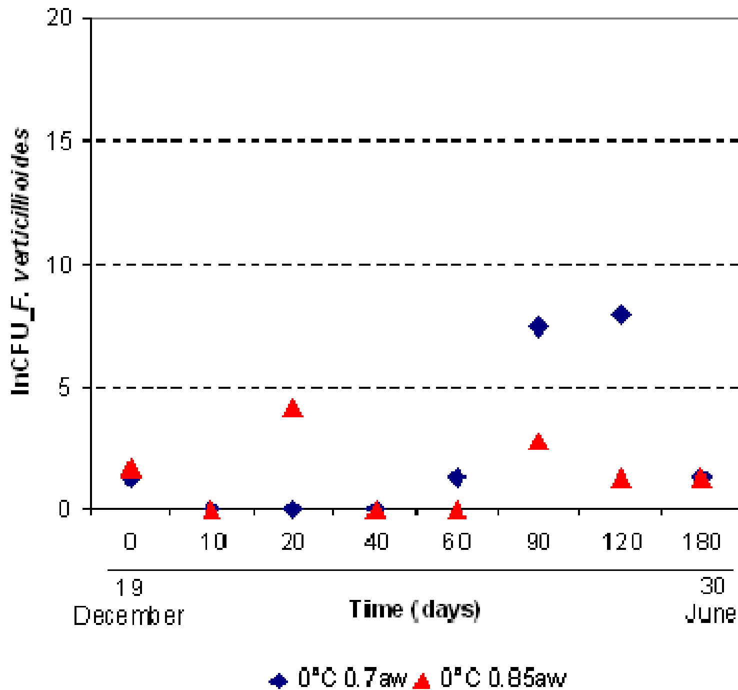

Fig. 3.1 – Colony forming units (ln) of *F. verticillioides* in maize kernels during storage (0-180 days) at 0°C and 0.70 and 0.85 a_w.

3.3.2.3 Maize samples at the other environmental conditions

Considering the environmental conditions usually present in the horizontal store and those selected in greenhouse, *F. verticillioides* populations were about ten times higher with respect to 0°C and 0.70-0.85 $a_{w,}$ mostly over 40 days of incubation.

A similar trend was observed at 6 °C and 20 °C where the *F. verticillioides* CFU remained stable and low until 20 days and the populations increased after 40 days of incubation.

On the other hand the population presence at 30 °C showed a trend extremely changeable.

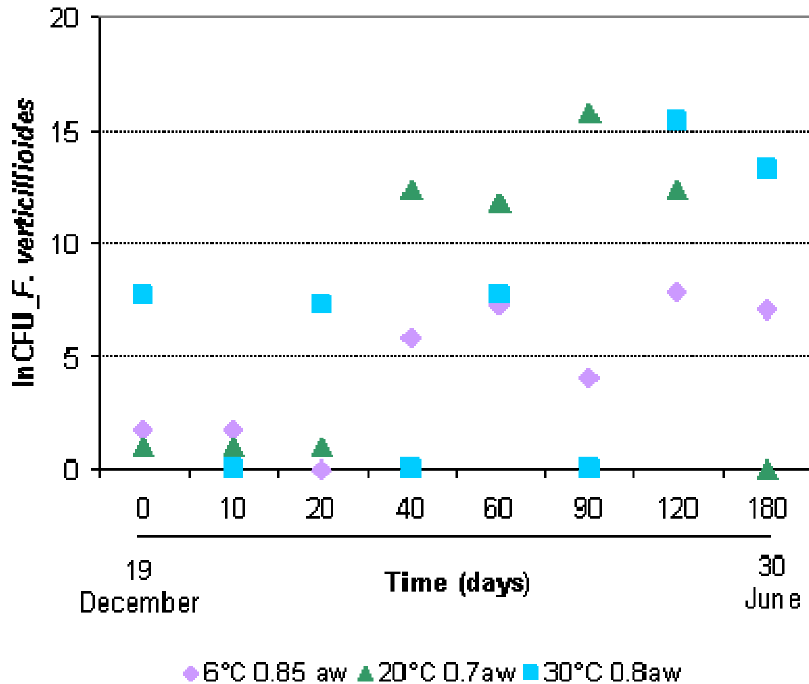

Fig. 3.2 – Colony forming units (ln) of *F. verticillioides* in maize kernels during storage (0-180 days) at different temperature and a_w conditions.

3.3.2.4 Maize mycotoxins analysis

Concentration of FUM throughout the incubation periods are shown in Figure 3.3 and are expressed as FB_1 + FB_2. In the samples at harvest, FB_1+FB_2 concentration was 18.3 µg/kg. In the sample incubated at 0°C and 0.70 a_w after 10 days from harvest a significant decrease of fumonisins (-70%) was observed, followed by an increase at 20 days. From 40 days to the end of storage, a 50% constant reduction for FUM was observed.

The FUM concentration detected at 0°C and 0.85 a_w showed a similar trend, except at 10 days where the FUM concentration showed an increase.

At all the other environmental conditions the FUM concentration trends were analogous at the trend observed at 0°C.

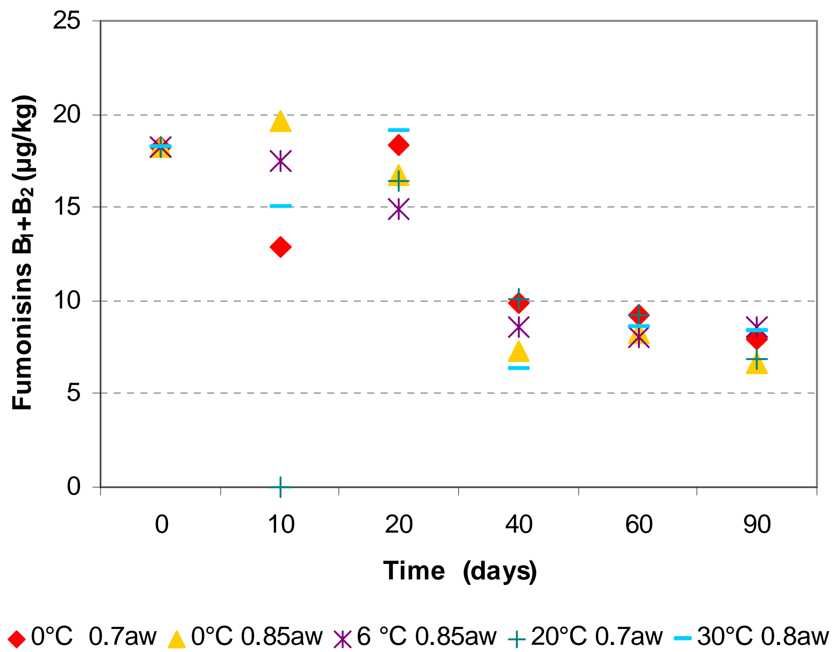

Fig. 3.3 – Concentration of FB_1+FB_2 (µg/kg) in maize stored at different incubation conditions and sampled at five subsequent times (10,20,40,60, 90 days).

Aspergillus flavus was also detected in the samples, but with limited CFU (data not showed). Considering the AFB_1 concentration, it remained under 10 µg/kg and very variable data were found in different sampling time (Fig. 3.4).

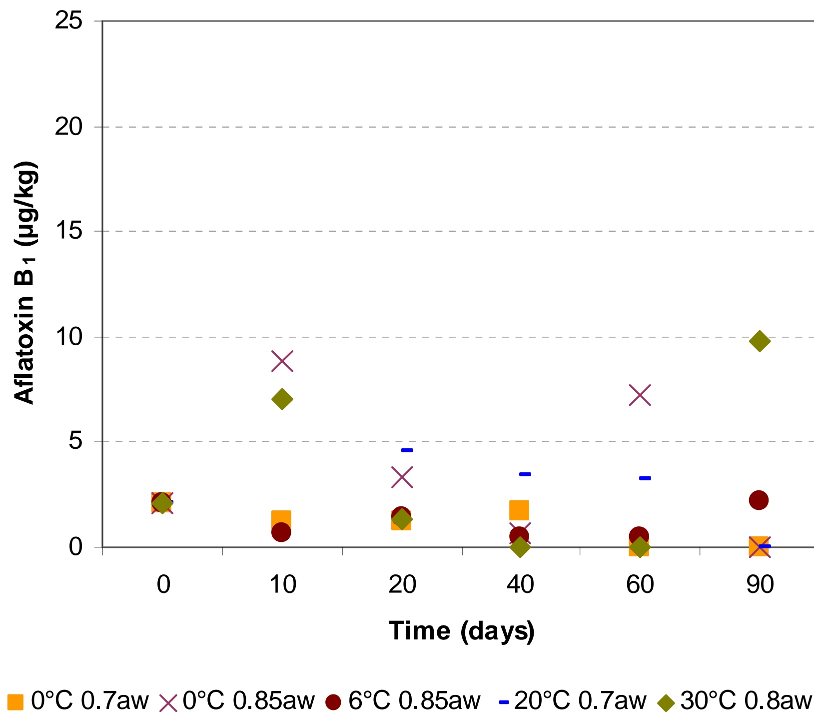

Fig. 3.4 – Concentration of AFB_1 (µg/kg) in *A. flavus* infected maize samples after harvesting from different samples incubated at five subsequent times (10,20,40,60, 90 days).

3.4 Discussion

Fusarium and *Aspergillus* species can infect maize pre-harvest and the mycotoxin contamination can increase if storage conditions are poorly managed (Lauren et al., 2004). The step before drying is critical because in wet kernels an important production of toxins is possible. During maize harvest, it is possible that maize kernels are amassed in the harvest drying centres waiting for drying process with the probable risk of toxins accumulation (Blandino et al., 2004). Results from this study showed that temperature, time, moisture contents and initial fungal content are all important in determining likely spoilage of maize kernels during pre-storage and storage. The effects observed included sometimes decreased fungal infection and increased mycotoxin content and a change in mycoflora. This is not surprising, being known that optimal conditions for fungal growth are not conducive for mycotoxin production, maximised by the

stress suffered by the fungus (Magan and Aldred, 2007). These effects can be observed within relatively short time as shown by our results.

In this study, *F. verticillioides* showed a negative correlation between fungal population and storage time in dried maize during storage. The decreasing trend associated with *F. verticillioides* percentage and storage time was also reported by Orsi et al. (2000).

The pre-storage time tested in this study significantly influenced *F. verticillioides* infection in maize and FUM production. The decrease trend in FUM level observed during the pre-storage period may be reassuring with respect to the stability of the toxin in contaminated kernels. Instability of FUM was also found in previous studies. Orsi et al. (2000) observed an overall decrease of FUM content in stored maize after 140 days of a one year storage period in Brazil. *Fusarium* spp. showed significant negative correlations with mean temperature and relative humidity of the air. Higher temperatures and relative humidity at the end of the study and high moisture content at the beginning of the study were observed. In contrast, Ngoko et al. (2001) found FB_1 to increase with storage time in maize collected in different zones of Cameroon where the rainfall distribution is bimodal and ranges from 1,000 to 1,500 mm per annum with temperatures ranging from 18 to 35 °C.

Our results showed that the incidence of A. flavus and AFB1 content in maize were lower and did not change significantly during the pre-storage time. The population of *Fusarium* and *Aspergillus* and FB_1 contamination did not change significantly based on store type, horizontal or vertical cells. Only AFB_1 content was significantly lower in vertical than in horizontal stores. This is in agreement with previous studies on AFs found that storage systems significantly influenced aflatoxin contamination in maize (Hell et al., 2000; Udoh et al., 2000). First post harvest operations and especially pre-storage of wet kernels before drying process demonstrated a critical phase, with a significant *Fusarium* toxin increase if the drying process is delayed too long, but the usual times of pre-storage in the drying units do not increase risk for mycotoxins accumulation in kernels.

Further studies are necessary to thoroughly explain this situation. Some factors including environmental conditions, intrinsic characteristics of stored products and chemical reactions can be responsible for this. Munkvold and Desjardins (1997) argued against the view that FUM concentration increase in maize stores during storage, as long as conditions of kernels moisture content and temperature are maintained at recommended levels. It is also suggested that FUM molecules might bind with the starch of the product during storage to form a complex, which is not detectable (Kim et al., 2002). Keeping FUM at undetectable levels at post-harvest to drying interval is a challenge (Marin et al., 1999a). Therefore adequate post-harvest management is advantageous for assuring the quality of stored kernels.

The ability of *F. verticillioides* strains isolated from maize to produce fumonisins under controlled environmental conditions was evaluated. It is known that both growth of *F. verticillioides* and fumonisin production are affected by temperature and a_w (Le Bars et al., 1994; Cahagnier et al., 1995; Marin et al., 1999b). The temperature is one of the most important parameter influencing the growth rate of fungal colonies inside kernel tissue: 0 and 40 °C are minimum and maximum temperatures for growth (Battilani et al., 2003).
Our results showed that visible mould infections were lower at 0 °C and 0.70-0.85 a_w than at natural conditions in horizontal store and greenhouse when environmental RH and T were kept constant.
It was observed that mycotoxins did not increase in standard drying process when temperature conditions in the storage were properly controlled and kernels moisture was below 14% (Blandino et al., 2004). Our results displayed that mycotoxins accumulation, also by *Aspergillus* toxins, which are able to grow with low moisture, can be prevented by correct drying process in order to reduce and keep the storage temperature uniform.

CHAPTER 4

EFFECT OF DIFFERENT FUNGICIDES: ROLE OF ACTIVE INGREDIENTS *IN VITRO*

International developments in mycotoxins regulation (EC, 2006 and 2007) have increased the pressure to find strategies for the mitigation of mycotoxins in maize. Fumonisins (FUM) and aflatoxins (AFs) are produced during maize cultivation and their mitigation has been approached with focus on the cropping system (Munkvold and Desjardins, 1997). Direct control of mycotoxin producing fungi has recently been included among good agricultural practices in small cereals in order to control fusaria, but little information is available on the effects of synthetic fungicides on *Fusarium* ear rot and FUM contamination on maize (De Curtis et al., 2008). The use of chemical fungicides is a controversial practice that entails undesirable environmental effects. An alternative strategy to reduce AFs and FUM accumulation in maize ears involves the biological interaction among toxigenic fungi and natural bio-competitive agents. The use of certain bacteria or yeasts to control pre- and post-harvest pathogens and pests of agricultural commodities has been studied *in vitro* with encouraging results (Cavaglieri et al., 2005a; Etcheverry et al., 2009).

4.1 Aim

The aim of this study was to determine *in vitro* the temporal efficacy of different chemical fungicides and a biocontrol bacterium, *B. subtilis*, available as a commercial product for field use, in reducing growth and toxin production by isolates of *F. verticillioides* and *A. flavus* under different water availability regimes.

4.2 Material and methods

4.2.1 Fungal strains

One FUM producer strain of *F. verticillioides* (MPVP 294; Etcheverry et al., 2009) and one AFB_1 and AFB_2 producer strain of *A. flavus* (MPVP A 2092;

Giorni et al. 2007) were used in the experiments. The strains were isolated from maize kernels grown in Northern Italy and stored in the fungal collection of the Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore in Piacenza.

These fungal strains were inoculated in 9 cm Petri dishes containing Potato Dextrose Agar (PDA: Oxoid®) incubated at 25°C for 7 days and used as an inoculum. Two kinds of inoculum were prepared: (1) Petri dishes were washed with 10 ml of sterile water and the fungal suspension was adjusted to a concentration of 10^4 spore per ml and (2) agar discs were cut from the margin the fungal colony (Ø 2mm).

4.2.2 Fungicides

All the available fungicides with confirmed efficacy against trichothecene producing fusaria were included: tebuconazole (Folicur SE®, 43.1 g l^{-1} of active ingredient (ai)); prothioconazole (Proline®), 250 g ai l^{-1}) and procloraz (Sportak® 45EW, 450 g ai l^{-1}). Media were modified by the addition of 0.1, 0.5, 1.0 and 5.0 µg Kg^{-1} respectively of Folicur SE® and Proline® and 0.01, 0.05, 0.1 and 0.5 µg Kg^{-1} of Sportak® 45EW.

4.2.3 Inoculation and measurement

Petri dishes (Ø 9 cm) with PDA were used for the *in vitro* studies. The media were modified with fungicides, shaken vigorously for mixing before being poured into Petri dishes (approx. 45°C). Three a_w levels (0.99, 0.98 and 0.95) were considered in the study; these treatments were obtained by the addition of 0, 9.2 and 23.0 g of glycerol per 100 ml of distilled water (Dallyn and Fox, 1980).

The inoculums spore suspension or mycelial plug from the growing margin of the colony were used to centrally inoculate the fungicide treatments. Three replicates were prepared for each treatment. All plates were incubated at 25°C for 21 days for *F. verticillioides* and for 14 days for *A. flavus*.

The biological control agent *B. subtilis* (Serenade, strain QST713, $5*10^9$ CFU/g, Agraquest, formulated powder) was also included in this study. An aliquot of 10 g of the powder formulation was blended with 90 ml of PDA medium, maintained at 45°C, to obtain a suspension of 10^8 cells ml^{-1}. Serial dilutions were carried out between 10^{-3} until 10^{-8} and finally poured into Petri dishes.
The diameter of the fungal colonies was measured along two perpendicular diagonals crossing the inoculum point after 7, 14 and 21 days for *F. verticillioides* and after 7 and 14 days for *A. flavus*.

4.2.4 Mycotoxins analysis

Mycotoxins were analysed from selected sample sets. Only those inoculated with a spore suspension as inoculum, the colonies grown on unmodified control media or the following treatments were considered: 0.5 and 5 µg Kg^{-1} for tebuconazole and prothioconazole, 0.05 and 0.5 µg Kg^{-1} for prochloraz and 10^4 and 10^8 CFU g^{-1} of *Bacillus subtilis.*

4.2.4.1 Fumonisins

An aliquot of the content of Petri dishes was weighed and transferred to a flask. Fumonisins were extracted with 10 ml of methanol for 45 min using a magnetic stirrer; then the solution was poured into a glass vial and centrifuged at 3000 *g* for 5 min; the solution was diluted (0.1 ml brought to 1 ml) with acetonitrile:water (30+70 v/v) and filtered (HV 0.45 µm, Millipore Corporation, Bedford, MA, USA) before HPLC analysis. The analysis was carried out using a LC-MS/MS system, consisting of a LC 1.4 Surveyor pump (Thermo-Fisher Scientific, San Jose, CA, USA), a PAL 1.3.1 sampling system (CTC Analitycs AG, Zwingen, Switzerland) and a Quantum Discovery Max triple-quadrupole mass spectrometer; the system was controlled by an Excalibur 1.4 software (Thermo-Fisher). Fumonisins were separated on a Betasil RP-18 column (5 µm particle size, 150x2.1 mm, Thermo-Fisher) with a mobile-phase gradient of acetonitrile-water (both acidified with 0.4% acetic acid) from 25:75 to 55:45 in 9 min, then isocratic for 3 min; the flow rate was 0.2 ml min^{-1}. Ionisation was carried out with an ESI

interface (Thermo-Fisher) in positive mode as follows: spray capillary voltage 4.0 kV, sheath and auxiliary gas 35 and 14 psi, respectively, temperature of the heated capillary 270°C. The mass spectrometric analysis was operated in selected reaction monitoring (SRM). For fragmentation of [M+H]+ ions (722 m/z for FB_1, 706 m/z for FB_2), the argon collision pressure was set to 1.5 mTorr and the collision energy to 36 V. The selected fragment ions were: 704, 352 and 334 m/z for FB_1, 688, 336 and 318 m/z for FB_2. Quantitative determination was performed using LC-Quan 2.0 software (Thermo-Fisher). Fumonisins production was quantified in ng g^{-1} of culture medium. The limit of detection was 20 ng g^{-1} for FB_1 and FB_2.

4.2.4.2 Aflatoxins

An aliquot of the content of Petri dishes from the same treatments as analysed for FUM, was weighed and transferred to a flask. Aflatoxins were extracted for 60 min with 20 ml of methanol using a magnetic stirrer; then, the solution was poured into a glass vial and centrifuged at 3000 *g*, for 5 min; the solution was diluted (0.1 ml brought to 1 ml) with acetonitrile:water (25+75 v/v) and filtered (HV 0.45 µm) before HPLC analysis. The analysis was performed using an HPLC instrument consisting of two PU-1580 chromatographic pumps, an AS 1555 sampling system, a FP 1520 fluorescence detector and a post-column derivatization system (Jasco Corporation, Tokyo, Japan); the instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 mm particle size, 125x4 mm i.d., Merck) was used at ambient temperature with a mobile phase of water:methanol:acetonitrile (64:23:13, v/v/v) at 1.0 ml min^{-1}. A solution of pyridinium bromide perbromide (25 mg in 500 ml of HPLC-grade water) was used as a derivatizing agent. The flow of the postcolumn derivatizing solution was set at 0.1 ml min^{-1} and the reaction tubing was 1000x0.5 mm i.d.. The detector was set at λ=365 nm excitation and λ=440 nm emission wavelength (Stroka et al., 2003). Aflatoxins production was quantified in ng g^{-1} of culture medium. The limit of detection was 0.5 ng g^{-1} for each aflatoxin.

4.2.5 Data analysis

Data on FUM and AFs production (values+1) were logarithmically transformed before statistical analysis. This was required because of the wide variability of the data (Clewer and Scarisbrick, 2001). Analysis of variance was performed considering all factors (fungicide types and dosage, a_w); the randomized complete block design of the statistical package SPSS was applied to data collected on fungal growth and mycotoxin production (Statistical Package for Social Science, ver.15.0.1, 2006. SPSS Inc., Chicago, IL, USA). Means were compared using Tuckey test.

4.3 Results

4.3.1 Effect of fungicides on fungal growth

All the factors considered in the trial with chemical compounds influenced significantly fungal growth, except the inoculum type (Table 1).

The effect of the ai used explained 53% of total variance for *F. verticillioides* and 20% for *A. flavus*. All the fungicides significantly ($P < 0.05$) inhibited mycelial growth compared to the control and the most effective was prochloraz, both against *F. verticillioides* and *A. flavus*. The inhibitory effect of all fungicides generally improved with increasing concentration. However, even the lowest concentrations tested resulted in a 35 and 44% decrease in fungal growth compared to the controls, respectively for *F. verticillioides* and *A. flavus*.

Mycelial growth of both considered fungi was slower with decreasing a_w and increased with time.

Tab. 4.1 – The effect of active ingredients (Tebuconazole, Prothioconazole and Prochloraz), different inoculum type (spore suspension or agar disc), and water activity (0.99, 0.98 and 0.95) on *in vitro* growth of *Fusarium verticillioides* and *Aspergillus flavus* at 25°C after 7, 14 and 21 days of incubation for *F. verticillioides* and 7 and 14 days for *A. flavus* measured as colony radius (mm).

Factors	***F. verticillioides*** **radius (mm)**		***A.flavus*** **radius (mm)**	
Active ingredients				
Tebuconazole	34.14	b	20.71	b
Prothioconazole	36.38	a	23.76	a
Prochloraz	8.06	c	9.04	c
Inoculum				
Spore suspension	25.76		17.59	
Agar disc	26.63		18.09	
*Dosage (µg/kg)**				
0	38.58	a	31.46	a
0.1 0.01	25.15	b	17.51	b
0.5 0.05	23.85	bc	15.82	b
1 0.1	22.99	c	13.59	c
5 0.5	20.39	d	10.80	d
Water activity				
0.99	27.36	a	17.96	b
0.98	28.67	a	27.18	a
0.95	22.55	b	14.37	c
Time (days)				
7	18.84	c	12.24	b
14	28.62	b	23.43	a
21	31.12	a	nm	

Different letters indicate significantly different growth of fungi (P≤0.05)

*First number represent the dosage of tebuconazole and prothioconazole and second number of prochloraz.

nm=not measured

Fungal growth was also significantly influenced by all factors considered in the experiments carried out with the biocontrol agent *B. subtilis*. The type of inoculum was an important factor, with fungal growth limited when the spore suspension was used as an inoculum. All the concentrations of *B. subtilis* decreased fungal growth (Table 2); the lowest concentration (10^3) limited growth to 48 and 65% of the control growth for *F. verticillioides* and *A. flavus,* respectively. Concentrations from 10^6 and 10^4 gave the most significant effects, decreasing fungal growth of both mycotoxigenic species by 70-75%. Interestingly, the inhibitory effect of *B. subtilis* decreased with a decrease of a_w of the media.

Tab. 4.2 – Effect of *Bacillus subtilis* (different concentrations) and water activity on the *in vitro* growth at 25°C of *Fusarium verticillioides* and *Aspergillus flavus* after 7, 14, 21 and 7, 14 days of incubation, respectively.

Factors	***F. verticillioides*** **radius (mm)**	***A.flavus*** **radius (mm)**
Inoculum		
Spore suspension	15.40 b	14.99 b
Agar disc	22.22 a	19.43 a
Concentration (CFU)		
0	37.58 a	40.56 a
10^3	18.09 c	26.23 b
10^4	19.90 c	11.69 c
10^5	24.99 b	11.67 c
10^6	11.81 d	10.68 c
10^7	9.98 d	10.19 c
10^8	9.31 d	9.47 c
Water activity		
0.99	16.02 b	11.49 b
0.98	17.11 b	12.46 b
0.95	23.30 a	27.69 a
Time		
7	13.24 c	14.86 b
14	19.76 b	19.57 a
21	23.43 a	nm

nm=not measured

4.3.2 Effects of fungicides on FUM and AFs

The fungicide treatments show a significant effect, reducing both FB_1+ FB_2 and AFB_1 production (Table 3). All the fungicides significantly ($P \leq 0.01$) inhibited mycotoxin production when compared to the control at the end of the incubation period.

The inhibitory effect of all fungicides was very similar at the 2 concentrations considered. Prochloraz and *B. subtilis* gave the best control of FB_1+ FB_2 and AFB_1 production with a reduction of 95% compared to the control. A threshold concentration inoculums of at least 10^4 CFU per g of *B. subtilis* was required to achieve a significant control of mycotoxin production.

Tab. 4.3 – Effect of different active ingredients and dosages on mycotoxins production by *Fusarium verticillioides* and *Aspergillus flavus*, inoculated as a drop of spores suspension on spiked PDA and incubated at 25°C for 21 and 14 days, respectively.

Factors	**FB_1+FB_2 (µg/Kg)**		**AFB_1 (µg/Kg)**	
Active ingredients				
Tebuconazole	4539.0	a	275.4	a
Prothioconazole	4284.3	a	263.8	a
Prochloraz	4201.1	b	210.9	b
Bacillus subtilis	4201.6	b	211.2	b
Dosage (µg/kg)*				
1	12590.3	a	630.6	a
2	205.3	b	53.6	b
3	124.0	b	36.7	b
Fungicides X Dosage (µg/kg)				
1*	1259.3	a	630.6	a
Tebuconazole				
2	657.7	b	86.3	b
3	369.0	b	109.3	b
Prothioconazole				
2	151.3	bc	57.5	b
3	111.3	c	103.2	b
Prochloraz				
2	12.0	c	0.3	b
3	1.0	b	1.9	c
B. subtilis				
2	0.0	c	2.9	b
3	14.7	b	0.0	c

*1=Test

2= 0.5 µg/kg for tebuconazole and prothioconazole; 0.05 µg/kg for prochloraz and 10^4 CFU/g for *B. subtilis*;

3= 5 µg/kg for tebuconazole and prothioconazole; 0.5 µg/kg for prochloraz and 10^8 CFU/g for *B. subtilis*.

4.4 Discussion

Mitigation of mycotoxins in maize is crucial all over the world, with focus on different toxins depending on the maize growing area. Southern Europe has commonly problems with FUM, associated with AFs in recent years (Battilani et al., 2005 and 2008; Pietri et al., 2009; Piva et al., 2006). Genetically resistant hybrids towards Fusaria and Aspergilli are still in development and their commercial availability is not reliable in next few years (Berardo et al., 2005). Guidelines with indications to optimise the cropping system and minimize mycotoxin contamination are available (Scudellari et al., 2008), but the direct control of fungi with chemical or biological agents is considered important , mainly in high risk conditions (Rossi et al., 2007).

Few studies have demonstrated the importance of direct chemical control with fungicides or biological control on maize and little information is available on the effects of synthetic fungicides on *Fusarium* ear rot and FUM contamination (De Curtis, 2008; Folcher et al., 2009). Several citations refer to ECB control that is confirmed as a useful indirect action for reducing mycotoxin levels, even if with variable results in term of percentage mycotoxin reduction (Blandino et al., 2008; Saladini et al., 2008).

More information is available regarding the usage of fungicide on wheat, with the first trials managed in the 1980s (Moss, 1985; Moss and Frank, 1985; Magan and Lacey, 1986). Numerous studies have documented the effect of fungicides application on *Fusarium* head blight, but reports on the efficacy are often conflicting. Triazole fungicides, in particular prothioconazole and tebuconazole, were confirmed to be the most effective against *Fusarium* species in field (Simpson et al. 2001; Vanova et al., 2004, Pascale et al., 2008).

In our *in vitro* study all the compounds tested in the laboratory significantly reduced pathogen development when compared with the control, but prochloraz was more effective than triazoles. Also very good results were obtained with the biocontrol agent *B. subtilis.* All the compounds had an inhibitory effect on mycelial growth and mycotoxin production, at all the concentrations used against both *F. verticillioides* and *A. flavus*. A decrease of fungal growth of

approx. 40% and 70-75% was observed with chemical and biological control, respectively.
These results have not been confirmed by recent in field trials by Folcher et al. (2009), where the application of tebuconazole had no effect on the mycoflora. In contrast, prochloraz significantly reduced *F. culmorum* on small grain in field (Doohan et al., 1996). Considering *A.* section *Flavi*, conventional methods of plant disease control with the use of fungicides (benomyl, thiabendazole, carboxine) were reported as ineffective in corn when applied at environmentally safe concentrations (Bhatnagar et al, 1993). However, in some *in vitro* studies prochloraz and imazalil seem to be two ergosterol biosynthesis inhibitors effective in reducing growth and aflatoxin formation by *A. flavus* and *A. parasiticus* (Delen and Tosun, 1999). This is supported by the results in our study.

There have been several reports that show growth inhibition of fungal pathogens following treatment with bacterial strains like *Bacillus amyloliquefaciens*, *Microbacterium oleovorans*, (Cavaglieri et al., 2005b; Pereira et al., 2007; Etcheverry et al., 2009), *Amphibacillus xylanus* and *Sporolactobacillus inulinus* (Nesci et al., 2005; Etcheverry et al., 2009). Our results suggest that *B. subtilis* is competitive and can inhibit *F. verticillioides* growth and FUM production. This suggests that the effective competition may be the way that this bacteria may function as has been shown by Motomura et al. (1996) in use of *Bacillus* spp. to control plant pathogenic fungi on maize. Because this bacterium occupies the identical ecological niche within the plant as *F. verticillioides*, it is considered an ecological homologue and the inhibitory mechanism, regardless of the mode of action, may operate on the competitive exclusion principle (Bacon et al., 2001). Furthermore *B. subtilis* has been shown to control *A. flavus* (Kimura and Hirano, 1988; Nesci et al., 2005), by inhibiting growth and aflatoxin production in grains in the field or when stored in warehouses.
B. subtilis is however more sensitive to low water activities than fungi. This may be an important factor which need to be taken into account when

examining relative competitiveness and environmental stress factors (Magan, 2006). Environmental stress factors are important because it has been observed that several interactions were influenced by a_w, temperature and substrate. Change in environmental factors cause an impact that can be decisive in determining the co-existence level or dominance of species in a particular ecological niche (Giorni et al, 2009).

In conclusion, our results suggest that applications of proper fungicides or *B. subtilis* may contribute in reducing *F. verticillioides* and *A. flavus* presence, a useful tool to protect maize quality, mostly in European areas where these fungi are widespread.

CHAPTER 5

EFFECT OF WATER ACTIVITY AND FUNGICIDES ON COMPETING ABILITIES OF COMMON MAIZE FUNGI

Maize is colonised by a mixture of spoilage fungi pre- and post-harvest. The dominant species depends on several abiotic and biotic factors, with water activity (a_w) and temperature (T) conditions which determine the dominance of fungal genera in the maize grain ecosystem. *Fusarium* spp. need to compete effectively with other colonisers including a range of *Aspergillus* and *Penicillium* spp. to establish on maize. To understand when *Fusarium* spp. are able to dominate the maize ecosystem it is required to understand the complex interactions which occur between abiotic and biotic factors and their impact on growth and interactions between *Fusarium* spp. and other fungi and their influence on mycotoxin production (Marin et al., 1998a).

5.1 Aim

The aims of this study were 1) to determine the competitiveness of *F. verticillioides* against a range of fungi, commonly growing on maize, on artificial media under different a_w levels and 2) to verify how the presence of sub-optimal concentrations of commercial fungicides affect interspecific interactions.

5.2 Materials and Methods

5.2.1 Strains

Some fungal strain isolated from maize were included in the study (Table 5.1).

Table 5.1 – Strains and isolate information for fungi used during this study.

Species	Isolates	Origin
Fusarium verticillioides	MPVP 294	Italy
Fusarium proliferatum	ITEM 7595	Italy
Aspergillus flavus	MPVP A 2092	Italy
Aspergillus ochraceus	LKN 14027	Denmark
Aspergillus niger	MPVP 2313	Italy
Penicillium verrucosum	BFE 500	Germany

5.2.2 Medium

A maize-based agar medium (MA) was utilised in this study. This was prepared by milling maize kernels into a fine powder and adding 20 g/l to a 2% agar (Oxoid® technical agar n° 1). This was then shaken and autoclaved at 120 °C for 15 minutes and poured into 90 mm Petri dishes. Dark sterilised polyester sheets were put onto the Petri plates surface.

The a_w of agar-based media was adjusted to 0.99, 0.98 and 0.95 a_w by the addition of glycerol as determined by Dallyn and Fox (1980). An Aqualab Series 3 (Labcell Ltd., Basingstoke, Hants, UK) was used to measure the a_w levels of the substrates prior to use.

Prior to pouring, after the media had cooled to approximately 50 °C, 3 fungicides were incorporated into the media to result in ED_{50} amount of active ingredient (Table 5.2).

Table 5.2 – List of fungicides, commercial products and amount of active ingredient (g/L) and ED_{50} dose used in this study.

Fungicide	**Active ingredient**	**Dosage of a.i.** g/L	**ED_{50}** µg/kg
Folicur SE®	Tebuconazole	43,1	6
Sportak® 45EW	Procloraz	450	0.0025
Proline®	Prothioconazole	250	6

5.2.3 Inter-specific interactions between fungi

Spore suspensions (approximately 10^6/ml) were prepared from 14 day old colonies and 0.25 ml of suspension for each strain were put on plates with MA and incubated at 25 °C for 24 hours. These plates were used as inoculum source.

An agar disc (Ø=5mm) was cut from fungal colonies and used as inoculum. The inoculation point for each species was approximately 4 cm apart *F.*

verticillioides. The controls were cultures inoculated with a single inoculum disc at the Petri plate centre.

The trial was managed both with normal MA and with MA spiked with the 3 fungicides.

Plates were grouped based on a_w, sealed in plastic bags and incubated at 25 ºC for 14 days. Each treatment and condition was carried out in triplicate.

Every day during the incubation period, colony diameter was measured by taking two diameters at right angles and crossing the inoculation point.

The interactions between mycelia of dual cultures were determined by macroscopic and microscopic analysis. A score was given to each interaction based on mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (dominant specie 4-0 inhibited species) and dominance at a distance (5-0). In the case of the dominant interactions the higher score was always awarded to the more competitive fungus (Magan and Lacey, 1984c).

For instance, if *F. verticillioides* was dominant over *F. proliferatum* upon contact this resulted in a 4 and 0 respectively being awarded to the fungal species.

5.2.4 Data analysis

Scores to each interaction were summed to obtain an overall Index of Dominancy (I_D) as a measure of competitiveness of each fungal species. The diameters of all colonies were measured for a maximum of 14 days. These data were used to determine the growth rate (mm/day) for each growth treatment. Linear regression between incubation time (x) and colony radius (y) was run; regression coefficient obtained was considered as growth rate.

5.3 Results

5.3.1 Growth rate of fungi in different ecological conditions

F. verticillioides, *F. proliferatum* and *A. flavus* had rising growth rates when a_w increased from 0.95 to 0.99 at 25 °C after 9 days of incubation while growth

rates of *A. ochraceus* and *P. verrucosum* did not change. *A. niger* was able to grow faster at 0.98 a_w compared to the other a_w tested. *F. verticillioides* and *F. proliferatum* had growth rates higher than those of the other species at 0.99 a_w (Figure 5.1).

Both *F. verticillioides* and *F. proliferatum* growth rates markedly decreased in the presence of sub-optimal levels of fungicides, regardless of different a_w (Figure 5.1).

A. flavus growth rate was influenced by the presence of fungicides in particular at 0.98 a_w.

The growth rates of *P. verrucosum* was not affected by fungicides at all a_w tested. Tebuconazole was the most effective towards *A. niger* and the less effective towards *A. ochraceus*.

A. niger growth rate was clearly influenced by the presence of sub-optimal levels of fungicides regardless of different a_w (Figure 5.1).

Considering different active ingredients, they did not influence significantly the growth rate; the trend of growth rates of all the fungi tested was similar when they grew in media spiked with different active ingredients.

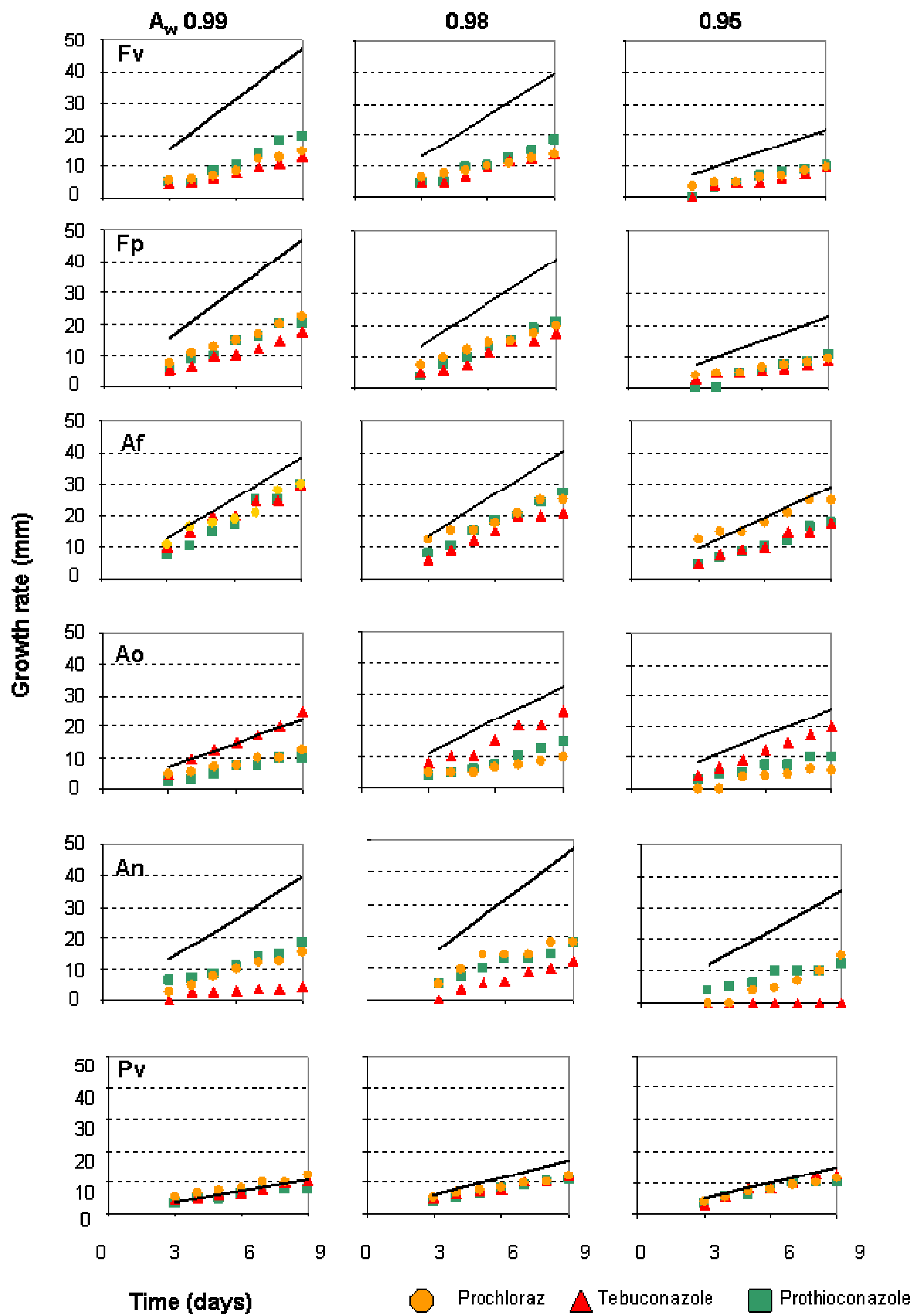

Fig. 5.1 – Growth rate of *F. verticillioides* (Fv), *F. proliferatum* (Fp), *A. flavus* (Af), *A. ochraceus* (Ao), *A. niger* (An) and *P. verrucosum* (Pv) when they were grown on normal maize agar medium (control (▬)) and modified with sub-optimal levels of 3 fungicides and at different a_w levels (0.99; 0.98;0.95) at 25 °C.

5.3.2 Inter-specific interactions between fungi

As the a_w and fungicides treatment changed, species interaction changed. When *F. verticillioides* and *F. proliferatum* were grown together at 0.99 and 25°C, *F. verticillioides* and *F. proliferatum* aggressively defend their resources by preventing progression of each other at their colony perimeter. This is an example of inhibition on contact which is reflected in the interaction score of 2 for both fungi (Figure 5.2 A). In figure 5.2 B, *F. verticillioides* overcome *A. ochraceus* defences and continue growing into the region occupied by *A. ochraceus*. This is an example of dominance on contact which is reflected in the interaction score of 4 for *F. verticillioides* and 0 for *A. ochraceus*.

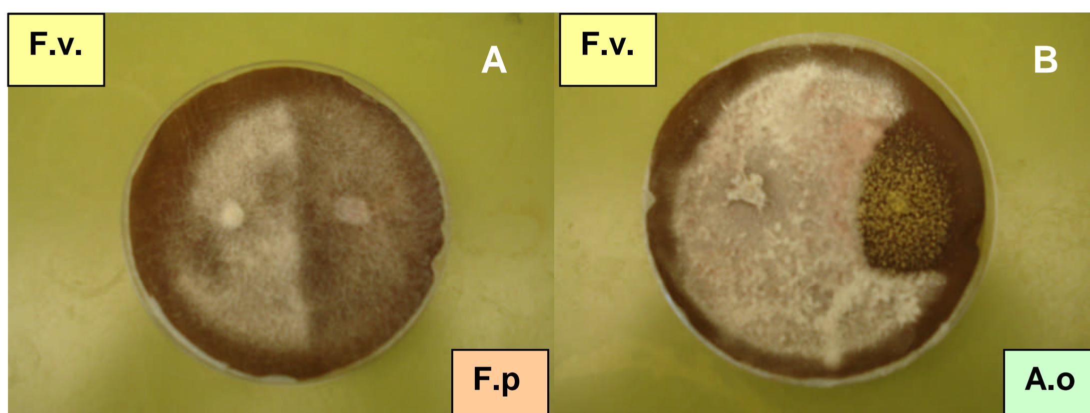

Fig 5.2 – Examples of interactions: A) between *F. verticillioides* and *F. proliferatum* and B) *F. verticillioides* and *A. ochraceus* grown on maize agar with a_w 0.99 and incubated at 25°C. Species key: F.v.=*F. verticillioides*, F.p.= *F. proliferatum* and A.o.= *A. ochraceus*.

Table 5.3 shows the interaction scores for *F. verticillioides* and all the other fungi considered. The first scores always represent *F. verticillioides*; *F. verticillioides* dominated as mutual antagonism on contact at most of the a_w levels at 25°C against the non-*Fusaria* with the exception of *A. ochraceus* and *P. verrucosum*. Overgrowth by *F. verticillioides* was the reaction for *A. ochraceus* at 0.99 and *P. verrucosum* at 0.98 a_w. These dominancy interactions changed to inhibition on contact with drier conditions.

F. verticillioides was dominate by *A. niger* at 25°C and 0.95 a_w levels.

The sum of dominance indexes indicates that *F. verticillioides* was competitive on *A. ochraceus* and *P. verrucosum*, was dominated by *A. flavus* and *A. niger* and mutual antagonism was observed with *F. proliferatum* at 25°C across all the a_w levels tested.

Table 5.3 – Interaction and Index of Dominance (I_D) scores for *F. verticillioides* versus other fungi frequently isolated on maize. Fungi were grown on maize agar at three a_w levels and incubatied at 25°C for 14 days.

aw/spp.	**0,99**	**0,98**	**0,95**	**I_D**
F. proliferatum	2/2	2/2	2/2	**6/6**
A. flavus	2/2	2/2	0/4	**4/8**
A. ochraceus	4/0	2/2	2/2	**8/4**
A. niger	2/2	2/2	0/4	**4/8**
P. verrucosum	4/0	4/0	2/2	**10/2**
Total (I_D)	**14/6**	**12/8**	**6/14**	**32/28**

5.3.3 Growth rate of fungi in spiked media with fungicides

Fusarium and *Aspergillus* species grew more rapidly than *P. verrucosum* and F. verticillioides showed the highest growth rate in the control. The interaction with *F. proliferatum* did not reduce the relative growth rate of *F. verticillioides* also in presence of sub-optimal levels of fungicides and prochloraz, tebuconazole and prothioconazole decreased growth rates of both *Fusarium* species. Data collected at 25°C and 0.99 a_w are shown in figure 5.3.

At 25°C *F. verticillioides* grew slower than *A. flavus* in presence of all the fungicides tested, in particular tebuconalzole and prothioconazole. *A. flavus* had the highest growth rate between *Aspergillus* spp.; growth rate of *A. niger* and *F. verticillioides* was very similar only on MA added with prochloraz while *A. ochraceus* grew faster than *F. verticillioides* in presence of tebuconazole.

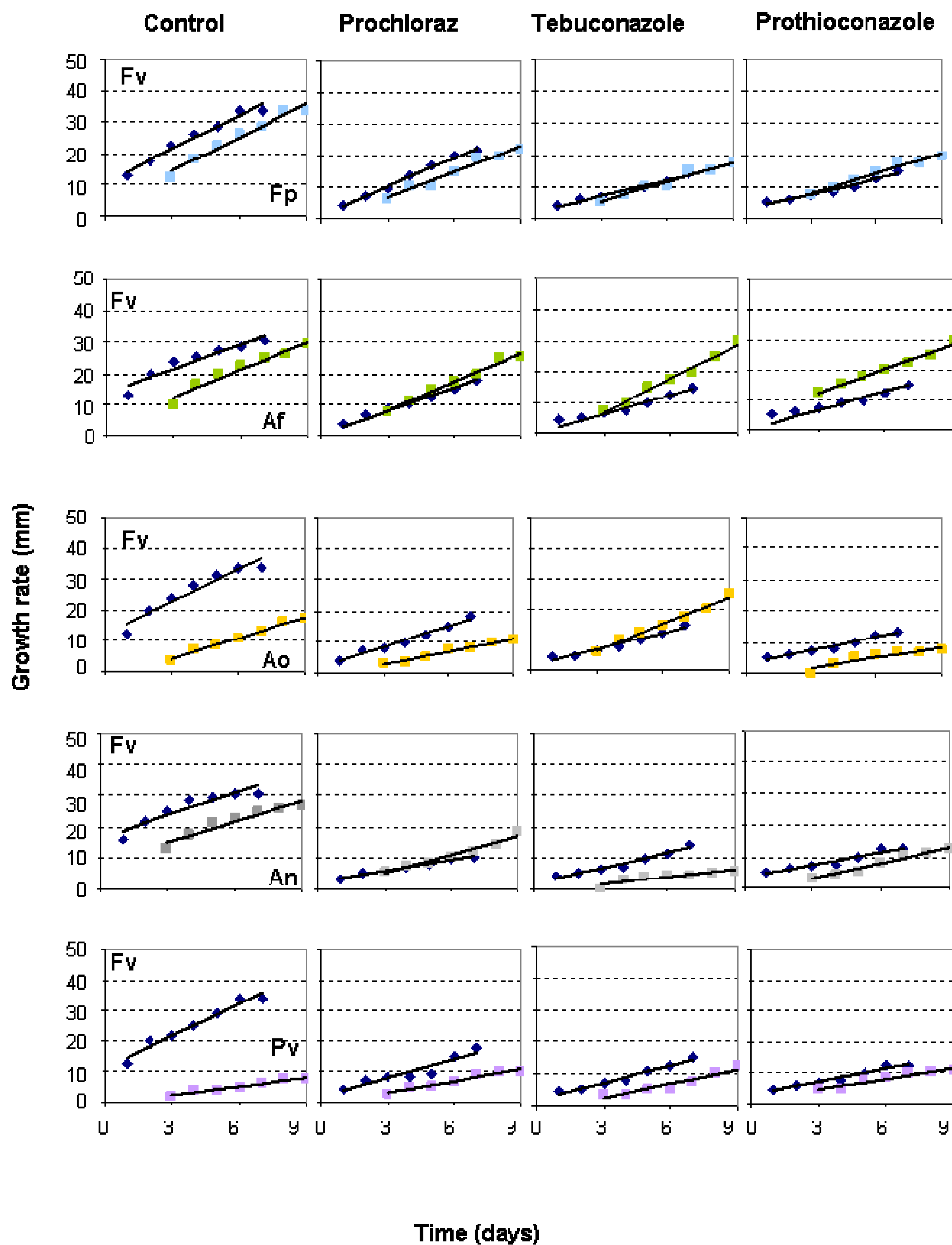

Fig. 5.3 – Growth rate of *F. verticillioides* (Fv) when growing on maize normal agar medium (control) and spiked sub-optimal levels of fungicides and interacting with *F. proliferatum* (Fp), *A. flavus* (Af), *A. ochraceus* (Ao), *A. niger* (An) and *P. verrucosum* (Pv) at 25°C and 0.99 a_w on the same medium spiked.

Growth of *F. verticillioides* and its interaction with the other considered fungi changed in MA spiked with fungicides compared to the unspiked media: mutual antagonism on contact and inhibition and overgrowth by other species were the most common reactions at 0.99-0.95 a_w (Table 5.4). Interestingly, *A. flavus* was able to dominate and overgrow *F. verticillioides* at 25 °C regardless of a_w levels and fungicides added, while *A. ocracheus* dominated *F. verticillioides* at all a_w levels in presence of tebuconazole. *F. verticillioides* was dominated by *A. niger* at 0.99 a_w when prochloraz or tebuconazole were added to MA. The same interaction was obtained in treatments with tebuconazole and prothioconazole at 0.98 and 0.95 a_w for *P. verrucosum* that was able to inhibit *F. verticillioides* growth. When incubated at 25°C, regardless of a_w level, in spiked media, *F. verticillioides* was unable to compete as effectively as without fungicides.

Fusarium verticillioides behaviour changed to mutual antagonism on contact or overgrowth with addition of fungicides and interactions mostly resulted in dominance on contact of the other species on *F. verticillioides*, except for *F. proliferatum* (Table 5.4).

Table 5.4 – Interaction between *F. verticillioides* and other common maize grain fungi, on maize agar at various a_w levels and spiked with tebuconazole, prochloraz and prothioconazole. Plates were incubated at 25°C for 14 days.

	Tebuconazole			**I_D**	**Prochloraz**			**I_D**	**Prothioconazole**			**I_D**
spp./a_w	0,99	0,98	0,95		0,99	0,98	0,95		0,99	0,98	0,95	
F.proliferatum	2/2	2/2	2/2	**6/6**	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**
A. flavus	0/4	0/4	0/4	**0/12**	0/4	0/4	0/4	**0/12**	0/4	0/4	0/4	**0/12**
A. ochraceus	0/4	0/4	0/4	**0/12**	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**4/6**
A. niger	2/2	2/2	–*	**4/4**	0/4	0/4	0/4	**0/12**	0/4	0/4	0/4	**0/12**
P.verrucosum	2/2	0/4	0/4	**2/10**	2/2	2/2	2/2	**6/6**	2/2	0/4	0/4	**2/10**
Total (I_D)	**6/14**	**4/16**	**2/14**	**12/44**	**6/14**	**4/16**	**2/14**	**20/44**	**6/14**	**4/16**	**2/16**	**12/46**

*–= missed data

5.4 Discussion

The present work give a general idea of how a range of fungal species from maize may interact and their potential competitiveness also in presence of sub-optimal levels of fungicides. Some patterns were observed when the interactions between species were examined. For example, interaction between *Fusarium* species were always mutually antagonistic upon contact. *Fusarium* species exhibited dominance on contact towards *A. ochraceus* and *P. verrucosum* at 0.99 a_w, but at lower a_w (0.98 and 0.95) mutual antagonism was more common and sometimes they were dominated by other species, mainly by *A. flavus* and *A. niger* at 0.95 a_w in agreement with other reports (Marin et al., 1998a).

This study has shown that *F. verticillioides* was able to compete effectively in dual culture with other fungal species commonly isolated in maize, although the dominance against some species was modified by a_w and presence of fungicides. This is in contrast with results obtained by Wicklow et al. (1988) that indicated *F. verticillioides* as particularly effective in inhibiting the infection by *A. flavus* and other fungi common in maize.

In our results, *A. flavus* was the most competitive species, with the highest total I_D score. This is not surprising because the tested products are considered as effective mainly towards Fusaria (Folcher et al., 2009). *A. flavus* was also able to reduce the growth rate at sub-optimal levels of active ingredients tested. This is in agreement with previous studies by Marin et al., (1998a) that reported some *Aspergillus* species as able to inhibit the growth of some *Fusarium* species in a range of natural conditions. In our study the dominance of *F. verticillioides* without the presence of sub-optimal active ingredients could be predominantly due to its ability to grow rapidly and invasively.

P. verrucosum did not have high numerical scores under the conditions tested, and grew significantly slower than the other fungi considered.

Previous studies demonstrated that *F. verticillioides* and *F. proliferatum* shared their niches with *A. ochraceus* based on both interaction experiments and niche

overlap (Marin et al., 1998b). Our study showed that when *F. verticillioides* shared its niches with *A. ochraceus* it was able to compete effectively in dual culture for the maize medium domain, although this dominance disappeared when fungicides active against Fusaria were added.

CHAPTER 6

EFFECT OF DIFFERENT FUNGICIDES: ROLE OF ACTIVE INGREDIENTS IN FIELD

6.1 Aim

The aim of this study was to verify the fungicides and biological agents efficacy in controlling *F. verticillioides* and fumonisins (FUM) in maize kernels.

6.2 Materials and Methods

6.2.1 Field trials

Two hybrids, PR34A15 (FAO class 500, 125 days growth period length) and PR33T56 (FAO 500, 128 days) Pioneer Hi-bred INC. Des Moines, Iowa, USA, were grown in Ravenna, a relevant maize growing area of North-East Italy, respectively in 2007 and in 2008.

Maize was seeded on 12 and 18 March and harvested on 30 August and 16 September, in 2007 and 2008 respectively. Common agricultural practices for the area were applied in both years. Irrigation was done only in 2008

The experimental fields were organised as randomized completed blocks with 4 replications; parcels size was 7.2 m x 45 m (315.2 m^2).

Growth stages were observed weekly and the crop was sprayed at female flowering (BBCH 67; Weber and Bleiholder, 1990) and around 2 weeks later (Table 6.1).

Tab.6.1 – Date of spray and meteorological condition monitored at each application in 2007 and 2008.

	2007		**2008**	
Application Date:	29/06/2007	11/07/2007	10/07/2008	26/07/2008
Air Temperature (°C):	26,5	17.5	29	30
Relative Humidity (%):	70	70	70	65
Wind Velocity (m/s):	0-2	0-2	1-2	1-2
Cloud Cover (%):	0	0	0	0

[1] 67 = Female: stigmata drying

[2] 69 = End of flowering: stigmata completely dry

Chemical active ingredients and biological control agents (BCA) considered to control *F. verticillioides* were reported in Table 6.2.

Tab. 6.2 – List of active ingredients and biological control agents used in field trials to control *F. verticillioides* and fumonisins production in maize, commercial products, content of active ingredients and dosage distributed per ha.

Active ingredient	**Commercial product**	**Dosage a.i.**	**Dosage/ha**
Prothioconazole	Prosaro	125g/L	1L
Metconazole	Caramba	0.33l/hl	1L
Prochloraz	Sportak	450g/L	1.1L
Tebuconazole	Folicur	43.1g/L	5L
Bacillus subtilis	Serenade	$5*10^9$ CFU/g	900g
Trichoderma harzianum	Root Shield	250g/hl	750g

The active ingredients were applied alone or in combination and unsprayed plots were included.

During harvest, samples of 7-9 kg of kernels were taken from each plot. A sub-sample of 1 kg was randomly sub-sampled for the mycological and mycotoxins analysis.

In 2007 two type of trials were considered. The first trial was done considering mostly the chemical active ingredients, while the second trial was focused on BCA (Table 6.3). In 2008 only the trial on BCA was repeated with the adding of a new chemical a.i. and a higher dosage for *B. subtilis* (Table 6.3).

Tab. 6.3 – List of active ingredients used in trials in 2007 and 2008 and their dosage.

Thesis	2007 Trial 1		2007 Trial 2		2008 Trial 1	
	a.i.	Dosage	a.i.	Dosage	a.i.	Dosage
1	Untreated	-	Untreated	-	Untreated	-
2	*Bacillus subtilis*	900g/ha	*Bacillus subtilis*	900g/ha*	*Bacillus subtilis*	900g/ha*
3	*Trichoderma harzianum*	750g/ha	*Bacillus subtilis*+ Nufilm	900g/ha+ 300ml/ha	*Bacillus subtilis* + Nufilm	900g/ha+ 300ml/ha
4	Prothioconazole	1l/ha	*Bacillus subtilis* + Tebuconazole	900g/ha +5l/ha	*Bacillus subtilis* + Tebuconazole	900g/ha +5l/ha
5	Prochloraz+ Metconazole	1.1l/ha+ 1l/ha	*Bacillus subtilis* (15.65%)	900g/ha	*Bacillus subtilis* (15.65%)	1800g/ha
6	Metconazole+ *Bacillus subtilis*	1l/ha+ 900g/ha	*Bacillus subtilis*	900g/ha	*Bacillus subtilis*	900g/ha
7	Metconazole	1l/ha	-	-	Prothioconazole	1l/ha

* Time of application BBCH 67 and 69

Hourly data on temperature, relative humidity and rain were collected from a meteorological station placed close to the field during the period January-October

The incidence of infected kernel (%) and the number of colony forming units (CFU) of *F. verticillioides* and *A. flavus* per g of flour (CFU/g) and FUM were carried out as described in Annex 1 and Annex 2.

6.2.2 Statistical analysis

Data on the incidence of *F. verticillioides* and *A. flavus* were arcsine transformed, while FB_1 and FB_2 content in kernels was Ln transformed before statistical analysis. Arcsine transformation is appropriate for observations which are proportions (Fowler and Cohen, 1990), while log transformation is always required for data that cover a wide range of values from single-digit numbers to numbers in hundreds or thousands (Clewer and Scarisbrisck, 2001). The analysis of variance was performed considering all factors involved; a

randomized complete block design of the statistical package SPSS was applied (Statistical Package for Social Science, ver.15.0.1, 2006. SPSS Inc., Chicago, IL, USA). Means were compared using the Tuckey test.

6.3 Results

6.3.1 Meteorological conditions

Meteorological conditions during maize growing season in 2007 were very dry (Figure 6.1), while in 2008 the season was very wet in spring and beginning of summer and very dry and hot during crop maturation (August and September) (Figure 6.2).

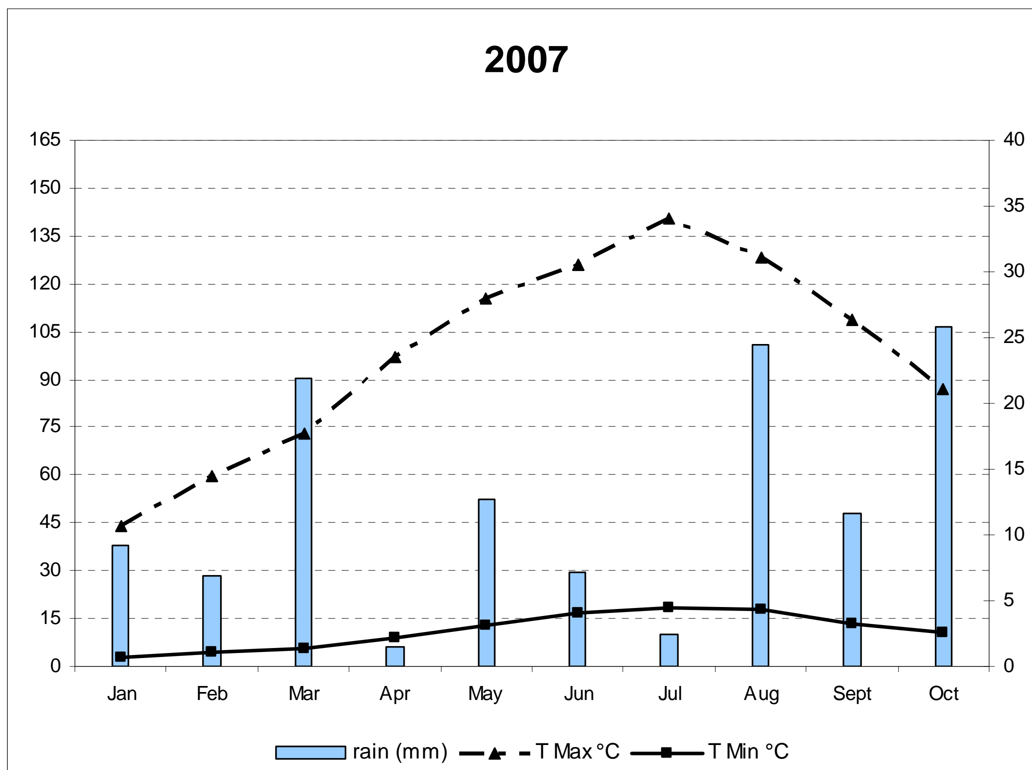

Fig. 6.1- Meteorological data collected in 2007

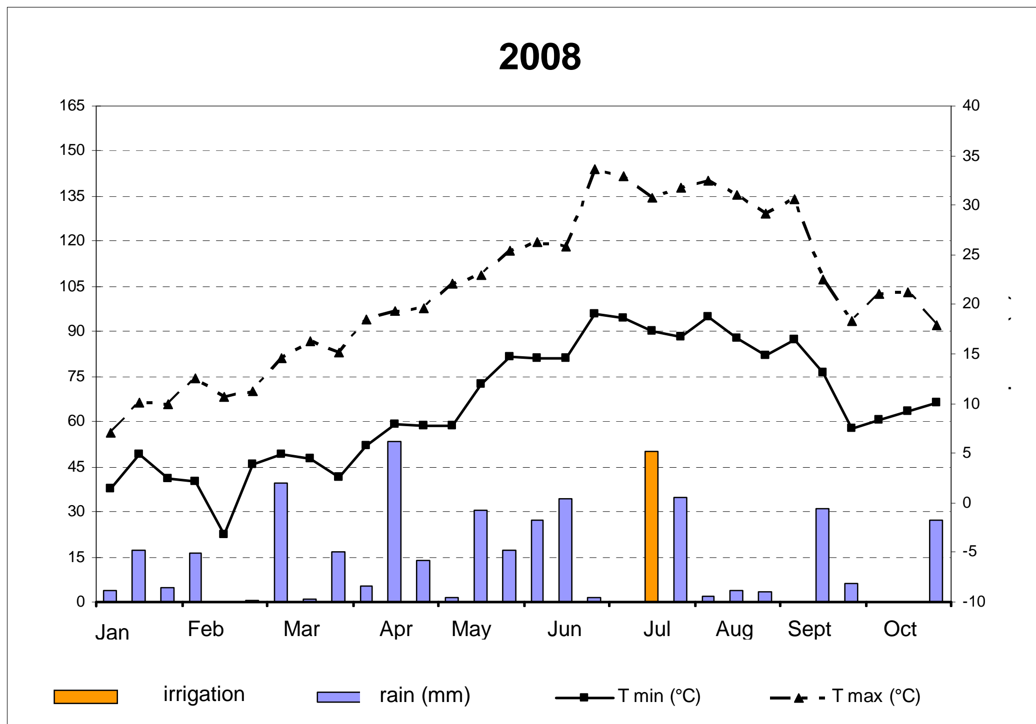

Fig.6.2 - Meteorological data collected in 2008

6.3.2 Field trials 2007

6.3.2.1 Trial 1 - 2007

The incidence of kernel infected by *F. verticillioides* was statistically significant but only *T. harzianum* caused a significant reduction compared to the test, while *A. flavus* presence resulted highly reduced by the treatment with Metconazole together with *B. subtilis* but it was enhanced in 4 of the thesis considered. In fact, *A. flavus* incidence was higher than in the untreated sample in treatments with *B. subtilis*, *T. harzianum*, Prochloraz together with Metconazole and above all in treatments with only Metconazole (Table 6.4).

Regarding CFU, significant reductions were noticed among all treatments considered for *F. verticillioides* while not important variation were found for *A. flavus* (Table 6.4).

FUM were found only in low concentrations and statistical analysis did not show significant differences among values (Table 6.4).

AFB_1 was not found.

Tab. 6.4 – Effect of different fungicides application on incidence of *F. verticillioides* and *A. flavus* (% and CFU/g) on maize in field and on fumonisins production in 2007 (two different trials: 1- chemical active ingredients and 2- biological control).

Thesis	*F. verticillioides (%)*		*A.flavus (%)*		*CFU_fus/g*		*CFU_asp/g*		FB_1 (µg/Kg)	FB_2 (µg/Kg)
1	14.5	a	27.5	abc	1.3E+07	a	2.5E+02	b	97.5	18.2
2	12.5	ab	29.5	abc	7.7	c	6.6	b	209.3	48.1
3	8.0	b	34.0	ab	0.0	c	1.7E+05	a	111.8	18.2
4	13.0	ab	4.0	bc	6.4	c	5.7	b	36.7	0.0
5	12.5	ab	31.5	abc	0.0	c	2.6E+02	b	67.2	18.2
6	9.5	ab	1.5	c	3.3	c	7.9	b	71.4	0.0
7	12.5	ab	44.0	a	7.7E+03	b	1.3E+01	b	179.4	59.7

Different letters indicate significant differences according to the Tuckey test ($P \leq 0.01$)

6.3.2.2 Trial 2 - 2007

Both biological and chemical treatments did not produce significant reductions in *F. verticillioides* presence while for *A. flavus*, only B. subtilis resulted able to reduce significantly its presence in comparison with the control (Table 6.5).

Regarding CFU, all the treatments significantly reduced *F. verticillioides* but the highest reduction was obtained with *B. subtilis* together with Tebuconazole (99,9%); while none of treatments considered was able to reduce significantly *A. flavus*. (Table 6.5).

Fumonisins were found only in traces and differences among theses were impossible to find.

AFB_1 was not found.

Tab. 6.5 – Effect of different fungicides application on incidence of *F. verticillioides* and *A. flavus* (% and CFU/g) on maize in field and on fumonisins production in 2007 (two different trials: 1- chemical active ingredients and 2- biological control).

Thesis	***F. verticillioides* *(%)***		***A.flavus* *(%)***		***CFU_fus/g***		***CFU_asp/g***	**FB_1 (µg/Kg)**	**FB_2 (µg/Kg)**
1	10.5	ab	16.5	a	1.3E+07	a	5.9	74.7	71.4
2	6.0	b	2.5	ab	4.5E+02	b	0.0	74.7	0.0
3	10.0	ab	1.5	ab	4.6E+02	b	2.3E+01	55.5	0.0
4	11.5	ab	1.5	ab	5.7	b	0.0	55.5	0.0
5	15.0	a	0.0	b	6.3	b	2.7E+01	55.5	0.0
6	11.0	ab	2.5	ab	5.4E+02	b	0.0	241.1	0.0

6.3.3 Trial 1 - 2008

Incidence of *F. verticillioides* resulted very influenced by all the treatments considered; in fact, respect to the untreated plot, all the other thesis resulted more contaminated. Probably the treatments enhanced the *Fusaria* population. The same was not observed for the presence of A. flavus that was not significantly modified by treatments considered (Table 6.6).

Only the thesis with *B. subtilis* sprayed both at BBCH 65 and at BBCH 67 and with prothioconazole resulted able to reduce highly the CFU of *F. verticillioides* (99%), while no significant differences were found for *A. flavus* (Table 6.6).

Regarding FUM, only FB_1 resulted significantly influenced by treatments. In particular only the treatment with *B. subtilis* at BBCH 67 and 69 did not produce significant differences, while all the other treatments considered were able to reduce the mycotoxin level from a minimum of 6% to a maximum of 32%. However, the level of FUM found was under the legal limits.

AFB_1 was not found.

Table 6.6 – Effect of different fungicides application (see following table) on incidence of *F. verticillioides* and *A. flavus* on maize in field and on fumonisins production in 2008.

Thesis	*F. verticillioides* (%)		*A.flavus* (%)	*CFU_fus/g*		*CFU_asp/g*	FB_1 (µg/Kg)		FB_2 (µg/Kg)	
1	17.0	c	7.0	3.1E+04	a	0.0	239.8	ab	66.1	b
2	38.0	a	2.5	1.5E+01	c	5.5	237.3	ab	65.0	b
3	21.5	abc	4.5	3.0E+03	ab	0.0	186.4	c	30.4	b
4	24.5	bc	3.0	1.3E+02	bc	9.5	163.0	c	24.2	b
5	24.5	abc	10.0	7.4E+04	a	0.0	202.0	c	24.2	b
6	24.5	abc	4.0	1.4E+05	a	0.0	226.3	c	34.5	b
7	40	a	5.5	4.8	c	0.0	198.4	c	104.1	ab

6.3.4 Comparison among common applications sprayed in 2007 and 2008

Considering only application common in both years, it has been possible to analyse significant differences among the main factors (year and thesis).

The year resulted not significant for *A. flavus* presence (both incidence and CFU), F. verticillides (CFU) and fumonisins content but it resulted important for *F. verticillioides* incidence (Table 6.7).

In 2007 contamination was significantly lower than in 2008; in particular the incidence of *F. verticillioides* was 42% lower than previous year (Table 6.7).

Differences among thesis resulted not significant for fungal presence (both Fusarium and Aspergillus) and FUM production. However, when thesis are considered linked to a single year, they resulted significantly different for *F. verticillioides* presence (both incidence and CFU). In particular, all the treatments considered were able to increase *F. verticillioides* incidence but, considering CFU, all thesis resulted reduced (Table 6.7).

Regarding FUM, none of the main factors considered neither their interaction resulted able to significantly reduce their presence (Table 6.7).

Table 6.7 – Analysis of variance considering only biological control in 2007 and 2008 on incidence of *F. verticillioides* and *A. flavus* on maize in field and on fumonisins production (FB_1+FB_2).

Main Factor		*F. verticillioides* (%)		*A.flavus* (%)	*CFU_fus/g*		*CFU_asp/g*	FB_1+FB_2 (µg/Kg)
Year	**2007**	18.5	b	n.s.*	n.s.		n.s.	n.s.
	2008	32.0	a	n.s.	n.s.		n.s.	n.s.
Thesis	**All thesis considered (from 1 to 6)**	n.s.		n.s.	n.s.		n.s.	n.s.
Year x Thesis								
2007	**1**	10.5	cd	2.5	4.4E+07	a	0.0	72.3
	2	6.0	d	2.5	6.6E+03	bcd	0.0	72.3
	3	10.0	cd	1.5	8.9E+03	bcd	3.3E+02	54.2
	4	11.5	bcd	1.5	5.0E+02	d	0.0	54.2
	5	15.0	abcd	0.0	7.1E+02	d	5.8E+02	54.2
	6	11.0	cd	16.5	3.7E+03	bcd	0.0	285.6
2008	**1**	17.0	d	7.5	3.7E+04	b	1.1E+03	249.1
	2	38.0	ab	4.5	1.8E+04	cd	0.0	275.5
	3	31.0	abc	3.0	3.8E+03	bc	1.0E+02	201.5
	4	21.5	c	10.0	8.8E+03	cd	0.0	175.2
	5	24.5	bcd	5.5	1.2E+05	ab	0.0	207.3
	6	40.0	a	2.5	2.8E+02	d	4.6E+02	244.7

*n.s.= not significant

6.4 Discussion

Results obtained show low fungal presence and low mycotoxins contamination in maize. Probably this was the consequence of the very favourable weather conditions had during maize maturation (no stress and dry conditions) in the two years considered (2007 and 2008).

However, populations of *F. verticillioides* and *A. flavus* were found on corn, even in low quantities, but fungicides treatments used in trials did not show any effect on their presence on kernels. These results corroborated some previous observations pointing out the inefficiency of fungicides treatment alone on

maize in the experimented areas (Folcher et al., 2009); however, our studies showed a decrease in CFU in all the thesis compared to control and the main reduction in fungal CFU was in the thesis sprayed with *Trichoderma harzianum* and prochloraz with the addition of metaconazole.

Interestingly, in our trials prothioconazole never showed relevant efficacy; this is in contrast with several field experiments carried out by Pascale et al. (2008) where application of fungicides containing prothioconazole provided a strong reduction of the main fungal diseases in small grain, mainly those caused by *Fusarium* spp.

Fungicides used in our studies were neither able to modify significantly *A. flavus* incidence. Probably they have no direct effect on aflatoxin producers, and only a limited influence on the ecological equilibrium between species naturally present on maize.

Unfortunately, we found no indications regarding the efficacy of the different active ingredients used on FUM because of the limited FB_1 and FB_2 contamination of maize in the two years considered.

In our studies, fungicides were sprayed following the suggested dosage for wheat and barley. Probably, considering the bigger surface of vegetation of maize, compared to that of wheat and barley, the dosage needs to be increased to improve the efficacy.

Mycotoxin levels at harvest are generally considered the consequence of fungal presence on grain during the growing season, consequently some methods involving agronomical practices like reducing the inoculum destroying residues or crop rotations can be useful for controlling *Fusarium* spp. (Reid et al., 2001; Schmidt and Mitzsche, 2004). Another important method to reduce dangerous fungal infections is the usage of biological agents like *Bacillus subtilis* (Bacon et al., 2001) .

Also in our studies, *B. subtilis* seems to be a biological agent able to control *F. verticillioides* on corn. A key point to obtain good results is the time of its application that could be correctly positioned, as resulted in our trials, at the beginning of flowering and anthesis (BBCH 65-67). As well, 900 g/ha seem to be the optimal dose to use.

In the second year of biological treatments, *B. subtilis* alone or plus its adjuvant Nufilm was confirmed suitable to be a product able to control *Fusarium* spp. on corn but, because of the low level of contamination, it was not possible, as for chemical treatments, to evaluate a reduction of FUM on kernels.

CHAPTER 7

RELATIONSHIP BETWEEN KERNEL A_W, HUMIDITY AND DIFFERENT MAIZE HYBRIDS

7.2 Material and Methods

7.2.1 Field trials

Field trials with maize were carried out in North Italy, Lodi and Venice in 2007 and 2008 and Pavia in 2008. A strip plot experimental design with 3 replicates was applied; the plot included 8 rows (around 5 m width), 5 m long.

Maize seeding and harvest time are summarised in table 7.1.

Table 7.1. Maize seeding and harvest time in the field trials managed in 2007 and 2008 in different places in North Italy.

	2007		2008		
	Lodi	Venice	Lodi	Venice	Voghera
Seeding	09/April	16/April	28/March	02/May	02/April
Harvest	04/October	04/October	01/October	08/October	09/October

The crop was managed according to the ordinary cropping system for the area and the control of *Ostrinia nubilalis* Hübner (European Corn Borer, ECB) was not applied.

Ten maize hybrids belonging to FAO class 500-700, with a growing period between emergence and ripening of 128-132 days (medium-late season) and representative for kernels characteristics variability found in commercial hybrids, were included (Table 7.2). The extractable starch, measured in percentage of dry matter (dm), and the indexes of kernel density, kernel dent, horny endosperm and pericarp removal were considered.

Tab.7.2 – List of hybrids included in the field trials carried out in Lodi and Venice in 2007 and 2008 and in Voghera in 2008.

Hybrid	FAO class	Days (n°)	Extractable Starch (% d.m.)	Index* of kernel density	Index of kernel dent	Index of horny endosperm	Index of pericarp removal
A	500	128	67.1	4.8	6.1	5.6	5.8
B	500	128	66.9	5.4	6.4	5.3	5.8
C	500	130	65.6	5.2	6.1	5.7	5.7
D	600	130	64.3	5.8	6.8	6.1	5.9
E	600	130	64.8	5.6	7.1	6.0	5.5
F	700	130	67.1	5.7	6.6	5.6	5.7
G	700	130	65.4	6.1	6.7	6.0	5.8
H	700	132	64.9	5.4	6.5	5.4	6.1
I	700	132	66.3	4.9	5.9	5.5	5.5
L	700	132	65.7	5.8	6.5	5.8	5.7

*Indexes values can vary between 0 and 9.

Data on temperature, relative humidity and rain were collected from meteorological stations placed close to the experimental fields.

Growth stage was weekly detected from early July to harvest.

Starting from mid August, around early dough growth stage, ten ears were weekly collected in each plot. They were de-husked and ECB attack was scored according to a reference scale (Table 7.3); it considers 6 levels of attack based on the presence of visible symptoms in various parts of the ear.

Tab.7.3 – Scale for the ECB attack assessment. The score was based on the presence of visible symptoms in different parts of the ear.

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

Ears were hand shelled and 250 g of kernels were randomly sampled from each 10 ears sample; humidity (H), water activity (a_w) and FB_1 and FB_2 content were determined in each sample.

7.2.2 Samples analysis

7.2.2.1 Water activity and humidity in kernels

AquaLab LITE (version 1.3 © Decagon devices Inc.) equipment was used to determine water activity (a_w) in kernels content. This equipment uses a dielectric humidity sensor to measure a_w and temperature of the sample and its accuracy is ±0.015 a_w. For each sample 20 kernels, about 6 g weight, were considered for the measure.

The determination of kernels moisture content was carried out following the method reported in the Official Journal of Italian Republic for human food and livestock feed (Gazzetta Ufficiale della Repubblica Italiana n°145, 21st June 1985). The maize kernels were dried and, in case of kernels with moisture content above 17%, pre-drying was performed. Mass loss was determined by weighting the sample before and after drying with an analytical balance.

7.2.3 Fumonisins analysis

For the FUM analysis maize kernels were grounded at 1 mm by Retsch ZM200 mill.

<u>7.2.3.1 Sample Extraction</u>

12.5 g ground sample and 1.25 g of salt were added with 50 ml of methanol: water (80:20). It was mixed for 45 minutes and filtered.

10 ml of the filtered extract were diluted with 40 mL Phosphate Buffer Solution (PBS: 8 g/l NaCl, 1.2 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl; pH 7.0 with HCl 1:1), mixed and filtered through microfibre filter (Vicam part # 31955).

<u>7.2.3.2 Column Chromatography</u>

10 ml filtered extract were passed completely through FumoniTestTM WB affinity column at a rate of about 1-2 drops/second until air comes through column.

10 ml of PBS were passed through the column at a rate of 1 -2 drops/second until air comes through the column.

Glass cuvette were place under FumoniTestTM WB column and 1.5 ml HPLC grade methanol was added into glass syringe barrel. FumoniTestTM WB column was eluted at a rate of 1 drop/second or slower. The column was dryed by a vacuum pump. The eluate was diluted with 1,5ml of ultrapure water.

400 µl of OPA (100 mg OPA, 2.5 ml methanol, 12.5 ml Na tetraborate 0.1 M, 125 µl mercaptoethanol) was added into a vial, 100 µl of diluted eluate were added, and mixed. After 1 minute it was injected into HPLC.

7.2.4 Statistical data analysis

All data analysis was carried out with SPSS (Statistical Package for Social Science, ver.15.0.1, 2006. SPSS Inc., Chicago, IL, USA). ANOVA was applied to all data collected: severity of ECB attack, a_w, H and FB_1+FB_2 content in maize kernels.

Based on meteorological data collected, degree day (DD) were computed from female flowering (BBCH 65; Weber and Bleiholder, 1990) to harvest. Regression analysis was applied to DD, a_w and H data.

Fumonisins contamination was related to all parameters measured (ECB, a_w and H) and kernels characteristics using Pearson correlation analysis.

Binary logistic regression was applied to determine the probability to have kernels samples with $FB_1 + FB_2$ content higher than 4000 µg/kg (legal limit for maize destined to human consumption; EC, 2007) in relation to a_w or H of kernels at harvest. The function used is as follows:

$$P= 1-EXP(-a*EXP(-b*X/100))$$

where P is the probability (0-1 scale), and a_w or H were used as independent variable (x).

7.3 Results

7.3.1 Meteorological conditions

In summer 2007 mean daily temperature was higher in 2007 in July and in 2008 in August, Mean daily temperature in July ranged between 22.9°C in Venice in 2008 and 24°C in Lodi in 2007, while in August 21.9 °C was the lower mean calculated in Venice 2007 and 23.5 °C the maximum computed in Voghera (Figure 7.1). Taking into account the growing period of maize, between March and October, rainfall was more abundant in 2008 compared to 2007; in fact, in all places more than 400 mm of rain were measured in 2008, while in 2007 389 mm and 248 mm were measured in Lodi and Venice respectively (Figure 7.1). During the female flowering, considering 1 week before and 1 after, Venice 2007 was the wettest place, with 36.9 mm of rain, and Lodi 2008 the driest, with only 0.6 mm; less than 10 mm of rain fall in the other places (Figure 7.1).

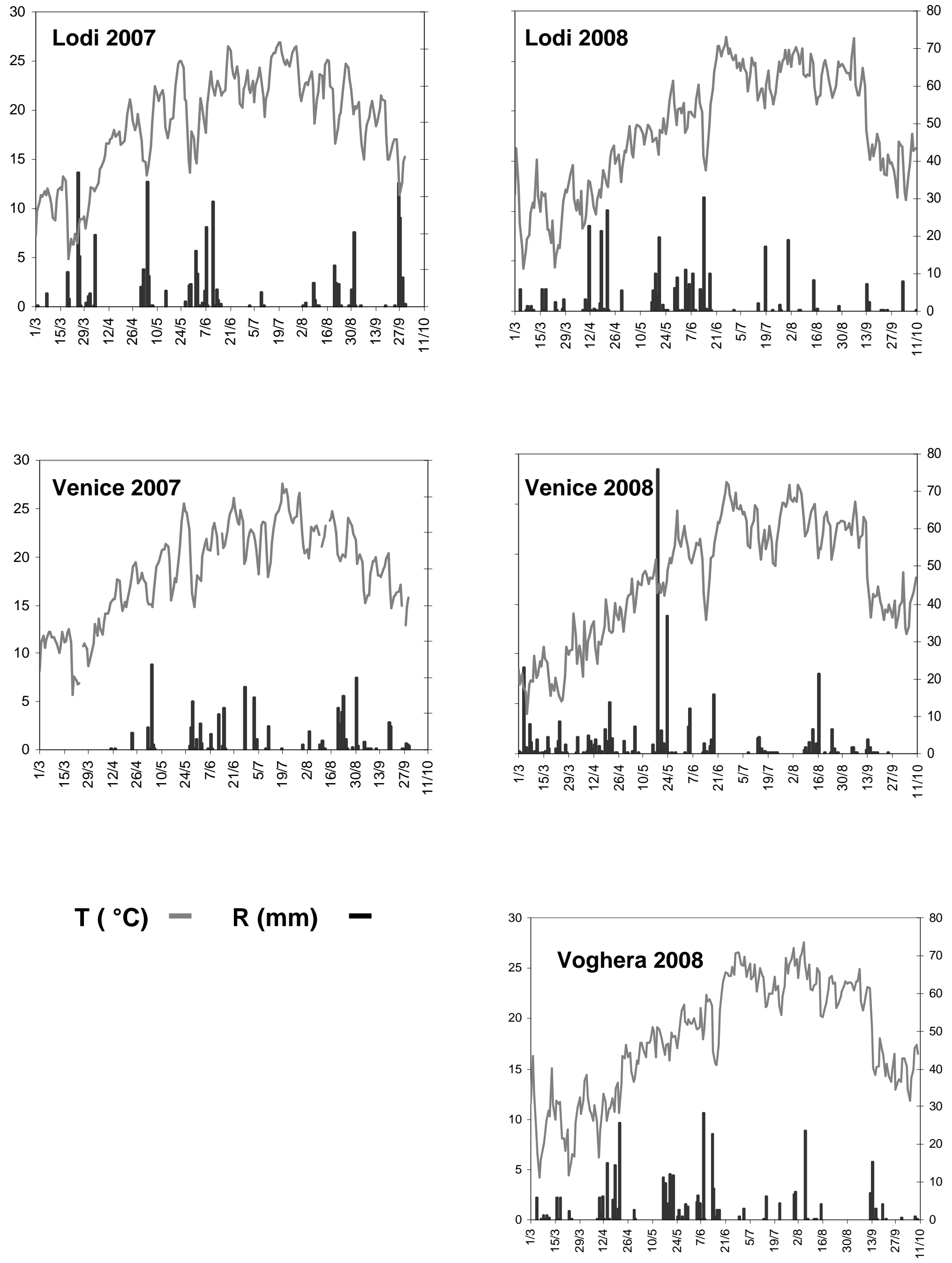

Fig. 7.1 –Dynamic of daily mean temperature (T) and total rain (R) in Lodi and Venice in 2007 and 2008 and Voghera in 2008.

7.3.2 European Corn Borer

ECB severity varied significantly between years and maize growing area, moving from low incidence of the attack, limited to top/medium ear (Lodi 2007) to 100% incidence and symptoms visible in different ear parts (Lodi 2008). The growing year resulted more relevant than the growing place; in fact, the most and least severe attacks were observed in the same growing area in the 2 considered years (Table 7.4).

Significant differences were noticed between hybrids, with mean attack score varying between 1.73 and 2.12, respectively in hybrid L and E. ECB susceptibility was not related to the FAO class of hybrids; in fact, very similar ECB severity was detected in 3 hybrids belonging to 3 different FAO classes (C, E, F belonging respectively to FAO class 500, 600 and 700).

ECB severity increased in later growth stages, but differences were not significant in September (Table 7.4).

7.3.3 Water activity

Mean a_w values were very similar in all the maize field considered except Lodi 2008, significantly lower (a_w =0.87). Differences were noticed between hybrids, mainly in those belonging to FAO class 500; the lowest a_w were measured in hybrids A and B and the highest in C. Water activity decreased going towards ripening, as expected, and differences were significant between each following sampling date (Table 7.4).

7.3.4 Humidity

Mean H was significantly different between field and followed a similar behaviour if compared with a_w. Relevant difference were observed between hybrids; the highest values were report in hybrid E, not different from I, and the lowest in A and B, both included in FAO class 500. Humidity decreased with ripening, as observed for a_w (Table 7.4).

7.3.5 Fumonisins

Fumonisins B_1 and B_2 content in kernels was significantly higher in Lodi in 2008, compared to all the other field trials, while contamination detected in Lodi 2007 and Venice 2008 was lower and below the legal limit of 4000 µg/kg.

Between hybrids, A was the less contaminated and E, not different from G and I, was the most contaminated (Table 7.4).

Tab. 7.4 – Analysis of the variance on the data of FB_1 and the FB_2 (µg/Kg) kernels contamination, the severity of the ECB attack, kernels humidity and a_w considering location, hybrids and sampling dates.

	ECB		**a_w**		**H**		**FB_1+FB_2**	
Location								
Lodi 2007	0,71	d	0,94	b	28.4	c	3,72	d
Venezia 2007	1,25	c	0,94	b	28.6	c	9,43	b
Lodi 2008	3,05	a	0,87	c	22.7	d	11,99	a
Venezia 2008	2,17	b	0,96	a	36.4	a	3,15	d
Pavia 2008	2,28	b	0,94	b	32.2	b	5,91	c
Hybrids								
A	1,85	bc	0,91	c	26.6	e	4,44	d
B	1,79	bc	0,90	c	26.4	e	6,87	bcd
C	2,01	ab	0,95	a	30.6	bc	8,49	ab
D	1,81	bc	0,93	ab	29.1	d	5,17	cd
E	2,12	a	0,93	b	32.2	a	10,61	a
F	1,82	bc	0,93	b	28.7	d	6,87	bcd
G	1,93	abc	0,94	ab	30.8	bc	7,68	abc
H	2,05	ab	0,94	ab	30.2	c	5,88	bcd
I	1,86	abc	0,94	ab	31.3	ab	8,20	ab
L	1,73	c	0,94	ab	30.6	bc	4,69	cd
Sampling dates								
25 August	1,48	c	0,98	a	38.2	a	5,00	c
1 September	1,78	b	0,96	b	33.9	b	6,08	b
8 September	2,0	a	0,93	c	28.9	c	8,32	a
15 September	2,09	a	0,91	d	25.5	d	8,06	a
22 September	2,14	a	0,88	e	21.8	e	6,98	ab

7.3.6 Trend of a_w and H as related to degree day

The trend of a_w as related to DD, well described by a non linear regression function (R^2 between 0.61 and 0.87), varied between hybrids. The minimum value at 2000 DD was around 0.7 (hybrids A,B,D,E,F and G), while the maximum was 0.80 (hybrid L; Figure 7.2).

Linear regression described well the decrease of H related to DD increase (R^2 between 0.88 and 0.96). The minimum H value of 20% was always observed with DD between 1750 and 1850, except for hybrid G (Figure 7.2).

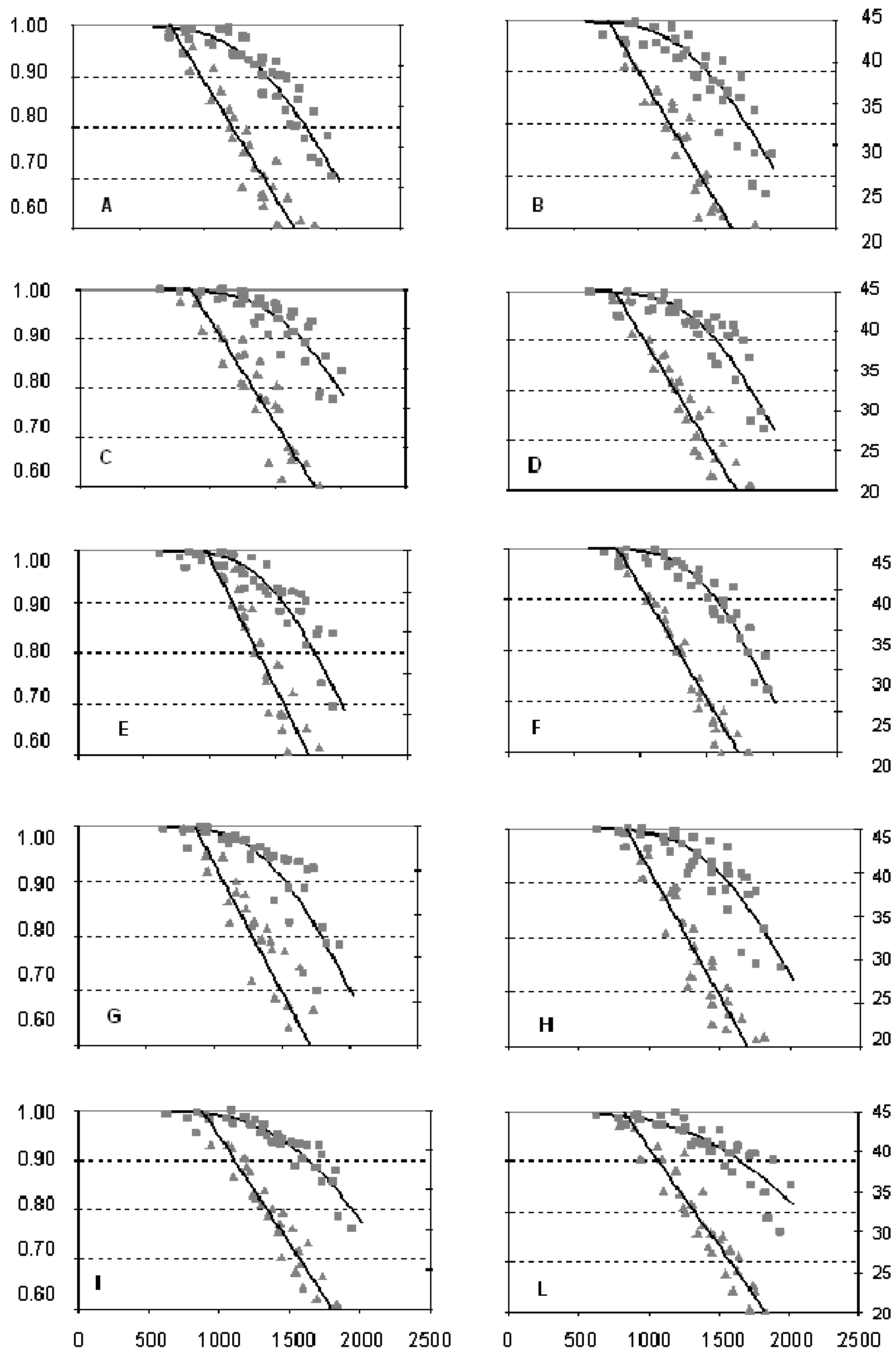

Fig. 7.2 – Non linear and linear regression describing respectively a_w and H trend in relation to degree day computed starting from female flowering (BBCH 65).

7.3.7 Fumonisins and kernels parameters

FUM content in kernels was significantly and positively correlated to ECB attack ($r=0.414$, $P \leq 0.01$), and negatively with a_w ($r=-0.485$, $P \leq 0.01$) and H ($r=-0.509$, $P \leq 0.01$). No correlation was found between FUM content and kernels characteristics.

The probability of kernel contamination with FUM above the legal limit of 4000 µg/kg was well described by the binary logistic regression (Table 7.5).

Tab. 7.5 – Parameters of the binary logistic regression applied to predict the probability of having maize samples contaminated above 4000 µg/kg as function of a_w and H.

	a	b	R^2
a_w, mean function	-17.26	16.01	
H, mean function	3.96	-0.147	
H, hybrid E	0	1.17	
H, hybrid I	0	1.60	

The probability, as function of a_w, follows the same behaviour in all the hybrids considered; a 50% probability is associated to 0.93 a_w, according to the experimental conditions considered (Figure 7.3).

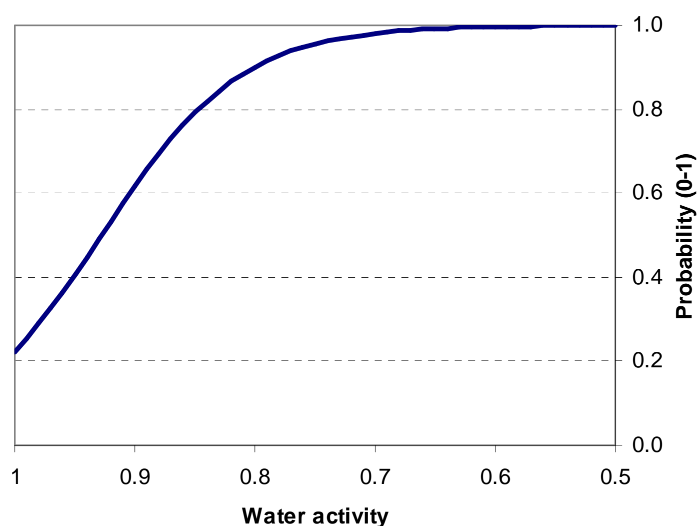

Fig. 7.3. – Probability of kernels contamination above 4.000 µg/Kg in relation to a_w decrease

Regarding H, 27% determines a 50% probability to have kernels above the limit according to the binary logistic regression. It represent almost all the hybrids considered, but E and I have a threshold limit of H at 37% and 34% respectively, which means that they are more exposed to FUM risk compared to the others (Figure 7.4).

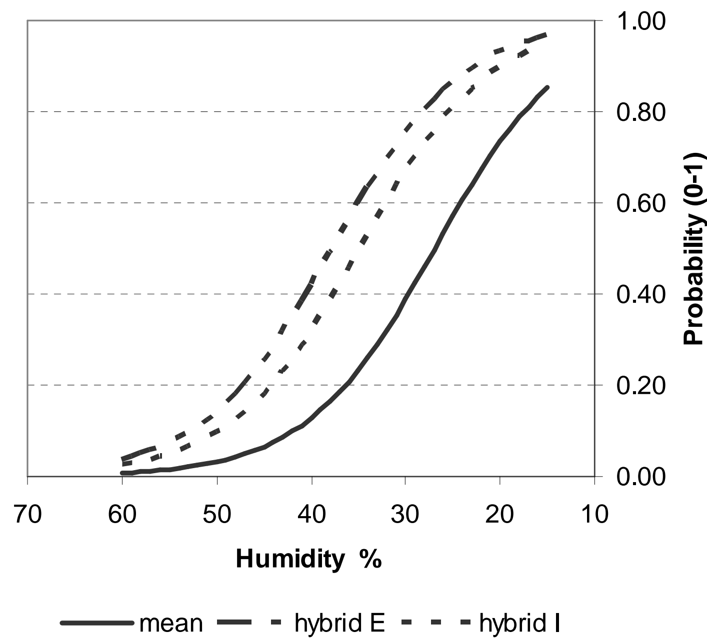

Fig. 7.4. – Probability of kernels contamination above 4.000 µg/Kg in relation to humidity decrease.

Regarding the percentage of fumonisin B_1 and B_2 content in maize kernels of the 10 hybrids considered compared to the maximum amount produced in the field at harvest, the analysis of variance considering the 5 different locations compared to the hybrids resulted significantly and positively correlated to FUM content in maize kernels. In fact, the data showed that hybrids, in particular A and C had a similar content of FB_1 and FB_2 in maize kernels in different geographic areas and years, while a wide variability was observed for the other hybrids (Figure 7.5).

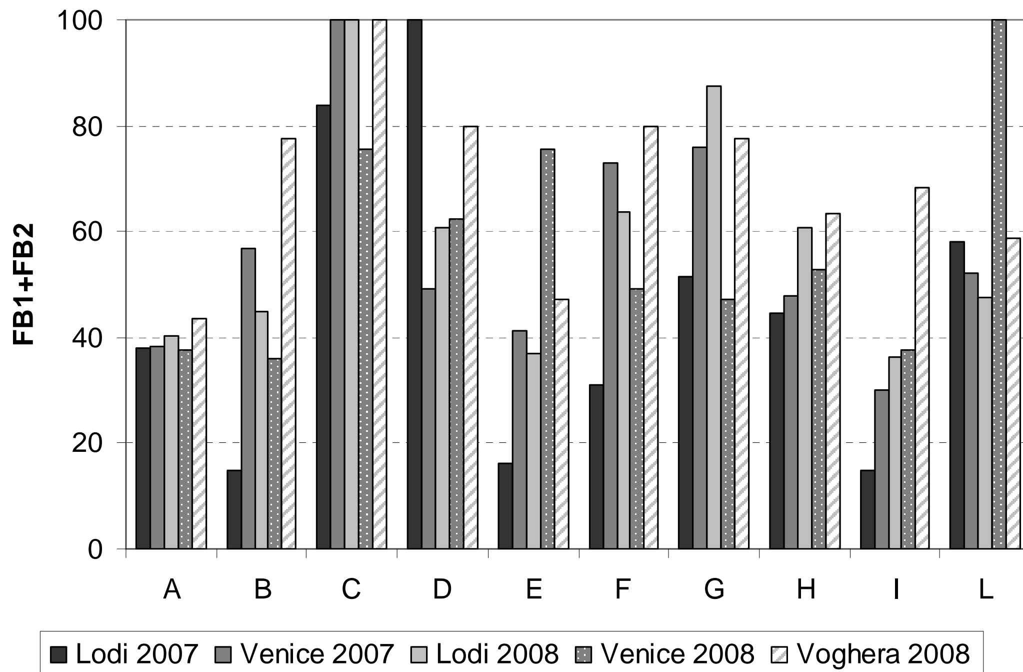

Fig. 7.5 – Percentage of fumonisin B_1 and B_2 content in maize kernels of the 10 hybrids considered in the study, compared to the maximum amount produced in the field at harvest in the 5 different location considered in the study.

7.4 Discussion

The study managed in north Italy, in 3 very different geographic areas, with 10 different commercial hybrids confirmed the relation between some variables and FUM contamination at harvest.

Meteorological conditions resulted more relevant than the growing area. In fact, the biggest differences in ECB attack and FB_1 + FB_2 content were detected in Lodi in maize harvested in 2007 and 2008. The relevance of these results can be considered as a general result, applicable to all geographic areas, being those considered very different.

The role of ECB attack in enhancing FUM contamination, reported by several authors (Alma et al., 2005; Mazzoni and Battilani, 2007; Saladini et al., 2008), is surely confirmed: in fact, the higher ECB score corresponded to the highest FUM contamination. Nevertheless, high contamination levels were detected

also with low ECB attacks, so as low contamination with severe ECB attacks. The lowest FUM contamination was detected in hybrid A, where also ECB attack was limited, while E was the most contaminated, also highlighted because of the hard ECB attack. The worst place for FUM contamination was Lodi in 2008, where the strongest ECB attack for FUM contamination were Lodi in 2007, where ECB attack was light, and Venice in 2008, with the highest a_w and H values measured.

Based on these results, it is stressed that any tool able to limit ECB attacks is a good preventive actions for FUM contamination, but it is not necessarily a solution. In fact, it is well know that the first infection way of *F. verticillioides* is through the silks at female flowering (Battilani et al., 2003).

Late season hybrids are considered as more prone to FUM contamination (Berardo et al., 2005), but this is not totally confirmed by this research. In a previous work where 3 maize hybrids were included (Battilani et al., 2007) the highest FUM contamination of hybrids FAO class 700 was attributed to the long lasting of a_w values suitable for fungal activity in FUM synthesis. In this research differences between hybrids seem not related to FAO class. The relevance of a_w is confirmed, but its trend in time and related to DD can be very similar in hybrids belonging to different FAO classes; the highest a_w value was detected in a FAO class 700 hybrid (L), but very similar was hybrid C (FAO class 500).

It was cited by several authors that late harvest, with a longer maize stay in field, are associated to higher levels of mycotoxin contamination (Berardo et al., 2005; Battilani et al., 2008; Scandolara et al., 2008). The different behaviour noticed in this study between hybrids regarding water loss, both in term of a_w and humidity, suggests that the relevance of harvest time could be different in different hybrids.

Fumonisin storage resulted negatively correlated to a_w and H; it suggests that “stay green” hybrids are more prone to FUM accumulation. This can be explained by the longer lasting of ecological conditions favourable for *F. verticillioides* activity (Battilani et al., 2007). a_w and H at harvest are suitable to describe the risk of FUM contamination above the legal limit, as shown by the

logistic regression, but several other factors have to be taken into account to obtain reliable predictions of FUM contamination in maize kernels at harvest..

This study suggests that ECB control and a rationale harvest based on the accurate evaluation of kernels a_w and H represent a crucial aspects to reduce the risk of FUM accumulation in kernels. Hybrids can play a major role, surely in relation to their rapidity of water loss.

Other genetic aspects of hybrids are surely relevant; in fact, some hybrids have a coherent behaviour in different geographic areas and years, while a wide variability was sometimes observed. The identification of the genetic base of these aspects will provide a good support in plant pathogen interaction understanding.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

Aspergillus and *Fusarium* species can infect maize during pre-storage and the mycotoxins contamination can increase during storage if conditions are poorly managed. To reduce or prevent production of mycotoxins, drying should take place as soon as possible and rapidly after harvest. It is also important to avoid kernel damage before and during drying and in storage.

Another important point in the FUM prevention is linked to the post-harvest time before maize drying. Among post harvest operations, the pre-storage of wet kernels before drying process resulted critical. In fact, we observed a significant *Fusarium* toxins increment when the drying process was delayed too long. In our study, it has been demonstrated that the level of FUM was higher in samples left more that 48 hours before the drying stage and it increased with time. However, from 12 to 36 hours before drying process, that are the normal times of pre-storage, do not increase the risk of mycotoxin contamination in kernels.

Mitigation of mycotoxins in maize is crucial all over the world, with focus on different toxins depending on the maize growing area. Guidelines with indications to optimise the cropping system and minimize mycotoxin contamination are available, but the direct control of fungi with chemicals or biological agents is considered relevant, mainly when environment conditions are favourable for fungal growth.

It could be possible to reduce FUM presence in maize using CO_2 or O_3, but it has been impossible to do studies on this because instruments to maintain modified atmosphere were not available. We have, then, taken into account another methodology to prevent mycotoxins accumulation based on biological control or on the use of fungicides.

In our studies, all the compounds tested *in vitro* significantly reduced fungal development. In particular, prochloraz has been more effective than triazoles both on mycelial growth of *F. verticillioides* and *A. flavus* and mycotoxins production and very good results has been obtained also using *B. subtilis*.

However, those chemical compounds did not produce similar results in field while *B. subtilis* confirmed its ability to inhibit *F. verticillioides* growth and FB_1 production. This confirm that *Bacillus* spp. strains can be used as biological control agents on maize.

Other important points in *F. verticillioides* and FUM control is the ECB control together with a careful check of harvest period in relation to kernels H and a_w as suggested by our study. These represent two important practises to prevent an high accumulation of fumonisins.

It is also important the selection of the suitable hybrid, in particular for its capacity to loose water during the growing season and secondary for its season lenght, even if this latter characteristic is not so relevant as commonly considered Probably other genetic characteristics can have a role in the presence of FUM in maize, but these are not yet known.

Some hybrids show a behaviour similar in different growing areas, they are constantly more or less contaminated respect to other hybrids cultivated in the same conditions. Relations between genotype and fumonisins presence could be better understood when differences among hybrids characteristics will be better defined.

During the last years, several studies contributed significantly to improve the agricultural practices for maize and reduce risks for human and animal health. In fact, it has been observed a general reduction trend in FUM presence.

This research can contribute to the prevention of fumonisin contamination adding knowledge to some aspects of plant-pathogen interaction still unknown.

Acknowledgements

I thank my supervisors Prof. Paola Battilani and my co-supervisors Prof. Naresh Magan and Prof. Amedeo Pietri for their support and help during the whole period of my research.

Many thanks also to colleagues of Cranfield University (Neus, Angel, Kalli) for their suggestions and help during experiments.

I would like to thank the reviewers Prof. Vittorio Rossi and Prof. PierLuigi Meriggi for the availability and attention to my research

The research was supported by Cooperativa Terremerse and Pioneer Hi- Bred Italy.

Un grazie speciale alla Dottoressa Paola Giorni che oltre ad essere una collega è per me un' amica, una guida e un esempio. GRAZIE di tutto.

Ringrazio le mie amiche-colleghe (Paola, Monica, Benedetta ed Irene) che mi hanno ascoltato e supportato-sopportato nei momenti difficili.

Ringrazio la mia famiglia che mi ha sorretto, consolato e spronato sempre.

Ringrazio mia nonna che mi ha sempre dimostrato il suo appoggio

Ringrazio Paolo e nonno, siete sempre con me!

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ANNEX 1

MYCOLOGICAL ANALYSIS

In the experiments the incidence of kernels infected by *Fusarium* and the count of units forming colony (CFU) on flour were carried out. Analysis were done as follow.

Incidence of kernels infected by Fusarium

Fifty kernels were randomly selected for each sample. They were surface disinfected with a solution of 1% sodium hypochlorite and 90% ethylic alcohol for 2 minutes and washed with sterile distilled water. Kernels were plated in Petri dishes (9 cm diameter) with Peptone PCNB agar (PPA:15 g l^{-1} peptone, 1g l^{-1} KH_2PO_4, 0.5g l^{-1} $MgSO_4$*7H_2O, 750 mg l^{-1} pentachloronitrobenzene, 20g l^{-1} agar and H_2O to 1l) as medium and incubated at 25°C for 7 days. Colonies with white mould, looking like *Fusaria*, were transferred on Petri dishes with Potato Dextrose Agar (PDA: 1l potato broth 600g l^{-1}, 10g l^{-1} sucrose, 15g l^{-1} agar) and identified according to Burgess et al. (1994). The result was expressed as percentage of infected kernels

Count of CFU

Forty grams of each corn sample were milled. Sub-samples (10 g) of corn flour were blended with 90 ml of sterile 0.1% peptone:water (w/v) and serial dilutions were carried out with 9.0 ml from 10^{-2} until 10^{-7}. One ml of conidial suspension from each dilution was plated in a Petri dish with PDA added with 50 mg of chloramphenicol and incubated at 25°C for 6 days.

The total number of fungal colonies was counted and the genera identification was carried out (Ono et al, 2002). The result was expressed as CFU/g.

ANNEX 2

MYCOTOXINS ANALYSIS

The determination of fumonisins was carried out with liquid chromatography to high resolution (HPLC), after purification of the extracts employing immunoaffinity column based on the specific antibody for every mycotoxin or group of mycotoxins. Extraction and purification of samples were done following methodical applications of the immunoaffinity column adopted as reported in the VICAM handbook (www.vicam.com). The quantification was obtained considering the means of the method using calibration curves prepared in a range of concentrations taking into account the limit values established by the Italian norm, or set in literature and considered limit values in other states.

Extraction of Fumonisin

A portion of milled sample, added with sodium chloride, was extracted with a solution methanol:distilled water (80:20 v:v) for 1 hour on stirrer.

Purification and analysis of Fumonisin

An aliquot of the extract, obtained after filtration with filter paper was diluted with PBS (1:4) and leaked; 10 ml of the extract were introduced in the immunoaffinity column Fumonitest VICAM (HPLC), with elution flow (1drop/second); successively the column was washed with 10 ml of PBS and the toxin eluted with methanol (1,5 ml) with the same flow. Distilled water (1,5 ml) was added to the extract and after mixing, 25 μl were captured and joined to 225 μl of DEVELOPER A+B. Then, after mixing, a portion was injected in HPLC instrument and the following chromatographic conditions were used:

HPLC (SHIMADZU, Kyoto, JAPAN), detector set up: λ ass of 335 nm and λ em of 440 nm;

Column Phenomenex Synergi 4 max-RP80 To (150x4,6 mm);

Phase mobile: methanol-0,1 M sodium monobasic phosphate (77: 23 v: v) carried to pH 3,3 with orthophosphoric acid;

Flow: 1 ml/min

Loop of injection: 50 μl

Quantification carried out on calibration chart to 5 concentrations of fumonisins FB_1 and FB_2 standard, range 0,1-4 μg/g

Limit of determination 0.1 μg/g.

Extraction of Aflatoxins

A portion of milled sample, added with sodium chloride, was extracted with a solution methanol:distilled water (80:20 v:v) for 12 hour.

Purification and analysis of Aflatoxins

An aliquot of the extract, obtained after filtration with filter paper was diluted with distilled water (3:10) and leaked; 20 ml of the extract were introduced in the immunoaffinity column Aflatest-P VICAM (HPLC), with elution flow (1 drop/second); successively the column was washed with 10 ml of distilled water and the toxin eluted with methanol (1 ml) with the same flow. Distilled water (1 ml) was added to the extract and after mixing, an aliquot was injected in HPLC instrument and the following chromatographic conditions used:

HPLC (SHIMADZU, Kyoto, JAPAN), detector set up: λ ass of 360 nm and λ em of 440 nm;

Column Phenomenex Synergi 4 μ max-RP80 To (150x4,6 mm);

Phase mobile: water+acetonitrile+methanol (6+2+3 v+v+v) +350 μl with 4M nitric acid and 120mg of potassium bromide/l of mobile phase;

Flow: 0.8 ml/min

Loop of injection: 50 μl

Post-column derivatization with Kobra cell setting of 100 μA with quantification performed on the calibration curve to 5 concentrations of B_1, B_2, G_1, G_2 standard Supelco, range 0,2-5 μg/Kg.

Limit of determination: 0.2 μg/Kg for B_1 and G_1 , 0.06 μg/Kg for G_2 and B_2.