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Role of the plant-pathogen cross talking in *Fusarium* mycotoxin production and masking in maize

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Summary

Fusarium verticillioides is one of the most important maize pathogen worldwide.
This fungus is known to produce secondary metabolites able to produce species specific toxic effects in case of ingestion of highly contaminated food or feed. For this reason, the International Agency for Research on Cancer (IARC), after the evaluation of toxicity of these metabolites, classified these compounds in class 2B as possible carcinogenic substance for humans.
These secondary metabolites, called fumonisins, are produced in field during the fungal infection of maize plant, but they can continue to be produced also during post-harvest storage, if environmental conditions are favourable. Furthermore, recently a new form of these toxins have been discovered. This form is called hidden because a bound, between fumonisins and matrix components, is formed It is demonstrated by the fact that these hidden fumonisins are not recovered with standard analysis but it is detected if an alkaline hydrolysis managed before the standard analysis. There are different hypothesis on how this bound is formed, through a chemical bound or through a superstructure with macromolecules of the matrix; the latter is considered more reliable for masking phenomena *in planta*.
In this work we investigated the *in vivo* and *in vitro* ecological conditions that can favour the fumonisin production, both the free and hidden forms, and we also try to investigate the mechanisms behind the masking effect. Samples of different maize hybrids have been harvested from dough stage to the harvest maturity to follow the trend of fungal incidence and both fumonisin forms contamination, but also of changes in chemical composition during the sampling period. Differences in the level of contamination have been found among hybrids during the growing season. Furthermore, the production of fumonisins has been found correlated to the total lipids content, another parameter that changed during the growing season. This finding underlined the existence of a relationship between toxin contamination and fatty acids composition of the hybrid.

Recently the existence of a cross talk between plant and pathogen has been demonstrated, based on some oxidized signal molecules (oxylipins) produced from fatty acid precursors. This result was also confirmed by the molecular analysis on the *in vitro* ecosystem that showed the activation of the genes involved in plant oxylipins production during the incubation time.

Also post-harvest contamination of maize was investigated, with particular attention to the effects of the drying treatment, a common post-harvest practice aimed at decreasing the water availability, and to the storage capacity of a new low cost storage system, silo bag. The drying treatment was showed to affect fumonisins content, in particular an increased fumonisins contamination was detected after heat treatments. This increment seemed to be produced by chemical changes of matrix components, caused by high temperature, that produced the release of hidden fumonisin in free form.

Silo bags were shown to be an effective system to store cereals because no significant change occurred in fungi or toxins contamination during a 9-month storage. Therefore, being more flexible and less expensive than traditional store houses, they should be very useful for farmers.

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CHAPTER 1

Introduction

1. Introduction

1.1 Maize: origins, diffusion and uses

The term "maize" (*Zea mays L.*) originated from the Arawak "*mahiz*", which literally means "that which sustains life" (McCann, 2001). The name corn was subsequently introduced by the Europeans that used to refer to this American grain as "Indian corn", denoting its connection with the Native Americans. The plant of maize was native of the tropical and subtropical areas of the South American continent; in particular, it was just cultivated in Mexico from prehistory, between 5200 and 2500 B.C. It was exported to Spain in 1493, thanks to Cristoforo Colombo, after the discovery of the American continent. After that, maize diffusion took place rapidly in all the European countries, firstly as ornamental plant and then as alimentary resource. The rapid diffusion interested especially the Balkan areas, in the XV century, where the environmental conditions were so favourable to assure a double return with respect to traditional cultivars.

Many hypotheses were proposed on the origin of this plant. Even if it is nowadays accepted that maize is the domesticated variant of teosinte (*Zea diploperennis*) (Beadle, 1939), this hypothesis was contrasted by Mangelsdorf and Reeves hypothesis (1939) concerning an extinct wild maize from which modern maize arose. The two plants have dissimilar appearance, maize having a single tall stalk with multiple leaves while teosinte being a short, bushy plant. Despite that, they show the same number of chromosomes (2n=20) and they can simply hybridise, they are both monoecious and they both have a terminal male inflorescence while the female ones are lateral (Fig. 1.1).

More recently another hypothesis was proposed by Eubanks (Eubanks, 1995; Eubanks, 1997a; Eubanks, 1997b) about the possibility that maize arose from teosinte and another grass (*Tripsacum dactyloides*), in confirmation of this her team obtained the cross between these two plants.

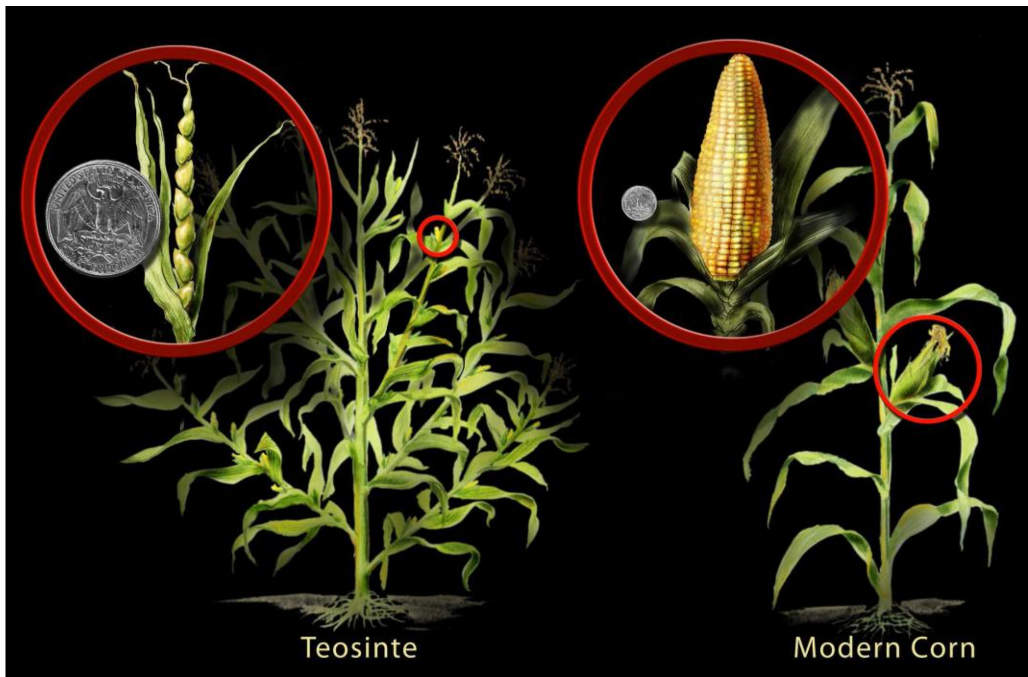

Fig. 1.1 Maize and Teosinte plants, source Nicolle Rager Fuller, National Science Foundation.

Maize is the first cereal for production in the world with 817 million tons (tons), followed by rice (685 million tons) and wheat (over 600 million tons) (FAO, 2009). The United States of America are the most important producers, with more than 20% of the world area dedicated to this crop. Among European countries, Ukraine and France reach the highest values of production (Table 1.1).

Table 1.1 Top ten countries for maize production, source FAO 2011.

Countries	Production (tons)
1. USA	313918000
2. China	192904232
3. Brazil	55660400
4. Argentina	23799800
5. Ukraine	22837900
6. India	21570000
7. Mexico	17635400
8. Indonesia	17629000
9. France	15703000
10. Romania	11717600

Italy is present at the 13th position with a production of 9752590 tons in 2011 (FAO,2011), and the area, dedicated to this cultivation corresponds to 970.000 ha (USDA GRAIN Report, 2012). There's a great difference between the production in the three areas in which Italy is divided, North, Centre and South areas (Table 1.2).

Table 1.2 Total production area grown with maize in different Italian areas, source ISTAT 2012.

Italian geographic areas	Production (quintals, %)	Area (ha, %)
North	72815602 *92.30*	1424.645 *50.15*
Centre	3818147 *4.84*	56.98 *2.01*
South and Islands	2252928 *2.86*	1359.068 *47.84*

In Italy, this cultivation is concentrated especially in the North regions, on the contrary in South areas, where the water sources are limited, durum wheat is primarily cultivated.

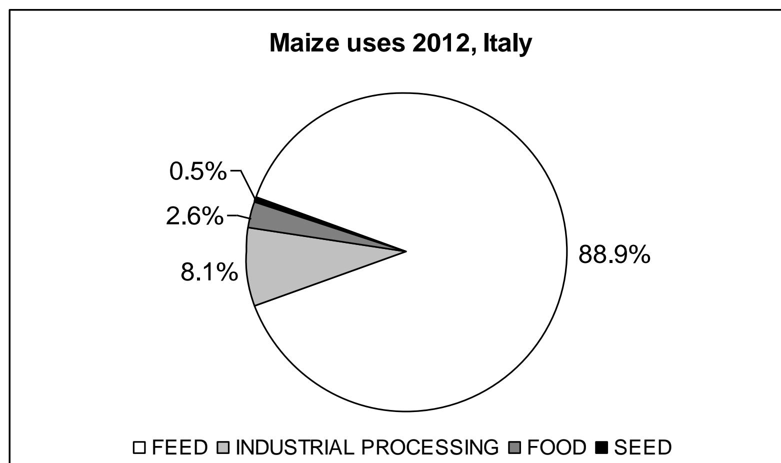

Fig. 1.2 Maize utilisation in 2012 in Italy, source USDA GRAIN Report 2012.

Maize is used for three possible destinations: food, feed for livestock and raw material for industry (Fig. 1.2). Maize grown in subsistence agriculture continues to be used as a basic food crop. In developed countries most of the production is used in

compounded feeds for poultry, pigs and ruminant animals. In recent years, even in developing countries, where maize is a staple food, more of it has been used as an animal feed ingredient.

As a food, the whole grain, either mature or immature, may be used; or the maize may be processed by dry milling techniques to give a relatively large number of intermediary products that, in turn, have a great number of applications in a large variety of foods. Other by-products of dry milling include the germ and the seed-coat. The former is used as a source of edible oil of high quality, while the seed-coat or pericarp is used mainly as a feed, although it is considered as a source of dietary fibre (Earll et al., 1988). Wet milling is a process applicable mainly in the industrial use of maize, although the alkaline cooking process used in manufacturing tortillas is also a wet milling operation that removes only the pericarp (Bressani et al., 1990). Wet milling yields maize starch and by-products such as maize gluten meal, an improper term defining maize proteins that are neither gliadin nor glutenin, used as a feed ingredient. The maize germ processed to produce oil gives as a by-product, maize germ meal, used as an animal feedstuff. Some attempts have been made to use these by-products for humans in food mixes and formulations.

Although the technology has been available for a long time, the increase in fuel oil prices resulted in much research on the fermentation of maize to produce alcohol, popular in some states of North America. Fermentation also provides some alcoholic beverages.

Finally, maize plant residues also have important uses, including animal feeds as well as a number of chemicals produced from the cobs, such as furfural and xylose. These residues are also important as soil conditioners.

1.2 Maize: botanical characters

Zea mays is an annual grass of the *Maydeae* family of the genus *Gramineae*. The maize stem is erect; it could reach 2-3 metres of height and is divided into internodes of about 44.5 centimetres. The leaves, generally 50–100 centimetres long and 5–10

centimetres wide, develop from each internode alternatively. The nodes are full while the internodes empty and they show a wider circular section.

Each corn plant contains both male and female reproductive organs (Fig. 1.3). The tassels, the terminal flowers situated at the apex of the stem, ordinarily develop only male spikelets which grow in pairs with one being sessile, having no stalk, and the other pedicellate, a single blossom on a lean stalk. Each tassel contains more than twenty million pollen grains. The lateral organ or female inflorescence is the ear and they are situated under the leaves and close to the stem. Each ear of corn contains upwards of one thousand potential kernels. Like the male tassels, the ears also bear spikelets, with only one of the flowers developing. Each of these flowers has one ovary terminated by a long protrusion known as the silk.

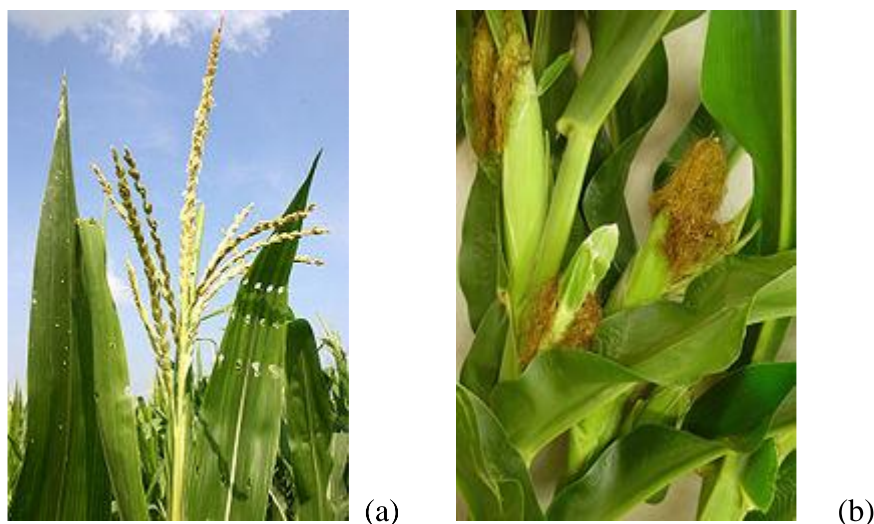

Fig. 1.3 Maize plant: (a) male inflorescence, called tassel; (b) stem, ear or female inflorescence, silks and leaves.

1.3 Maize: growth stages

According to BBCH-scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie), a scale used to identify the phenological development stages of a plant, maize cycle is divided into 10 principal growth stages.

The initial stage is called germination; it starts with the seed inbibition, a phenomenon through which the seed rehydrates and reactivates its tissues. This

phenological phase lasted until the emission of the radicle, followed by the emission of the coleoptile. After that, the coleoptile penetrates the soil surface, a step called emergence, and the germination finishes.
The germination is then followed by vegetative stages. Leaves development takes place, starting with the leakage of the first leaf through the coleoptile. During the other leaves emission, also the stem elongation occurs with the appearance of the nodes.
The last part of vegetative stages could take place contemporaneously with the heading or male inflorescence emergence. The emission of the male inflorescence and their maturation anticipates the female ones, because the female inflorescence must be ready to receive the pollen released during the anthesis. Flowering ends with the drying of the stigmata, the female inflorescences.
After pollination, the seeds start their development. The first step is called blister stage and the seeds show a 16% of dry matter content. This is followed by the milky stage in which the content of dry matter increases to 40%. During ripening, the seeds continue to accumulate dry matter, the starch, and to decrease the water content with a consequent hardening of the seeds and change in the colour that is variety-dependent. Ripening is divided in three steps: dough stage, in which the dry matter rise to 55%; physiological maturity, with a 60% of dry matter, full ripe, where the content of dry matter reaches the 65%.
The last stage of plant life is called senescence and the dried plant dies and collapses.

1.3.1 Seed composition

Many factors can affect kernel composition within and between maize varieties, mainly the genetic background, but also the environmental conditions, the age of the plant and the geographic location. Edible kernels, or *caryopses*, show a high incidence on the total dry weight (DW) of the maize plant, about 42% (Barber, 1979). The kernel can vary in colour (white, yellow, orange, red and black) depending on environmental and genetic parameters but also on cultivation methods (Fig. 1.3). The

quantity of kernels per ear could vary from 300 to 1000, the weight from 190 to 300 g per 1000 kernels and also the spatiality vary from 12 to 16 kernels per row (FAO, 1992).

The kernel is divided in three main compartments showing different percentage of DW (Fig. 1.4): the endosperm correspond to 83%, the embryo (germ) to 11% and the pericarp only to 6% (Earle et al., 1946).

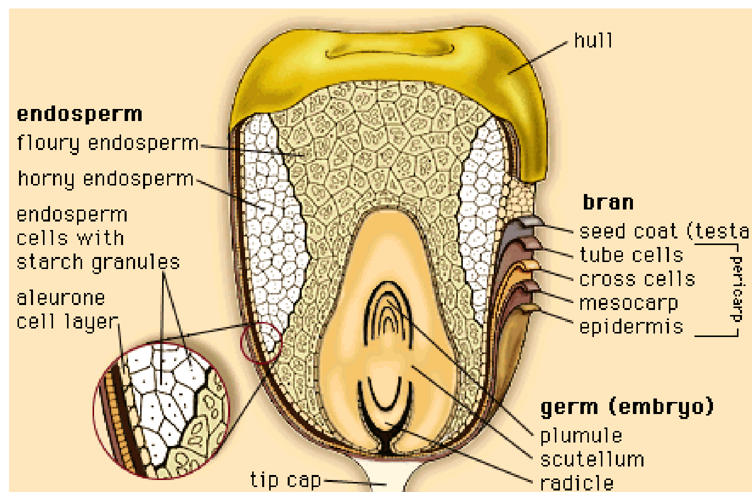

Fig. 1.4 Maize kernel structure, source Encyclopaedia Britannica Inc. (1996).

The carbohydrates are mainly represented by starch, which corresponds to 72% of DW, and they are located in the endosperm. It is composed by two glucose polymers, amylose and amylopectin, with a ratio amylose/amylopectin approximately around 25/75. However, some genetic modification could lead to different percentages of these two constituents. For example amylose extender mutants show a ratio of 80/20 (Inglett, 1970), while waxy mutants have a decreased amylose/amylopectin ratio as low as 0/100 (Bates et al., 1943). Also free sugars are present in maize kernel, less than 3%, with sucrose, as the main component, and maltose, glucose, fructose, and raffinose in traces (Boyer and Shannon, 1987). These free sugars are primarily located in the germ (Inglett, 1970).

The proteins are distributed between germ and endosperm with a greater concentration in the germ (Table 1.3), but the highest amount is included in the endosperm (Wilson, 1987). Maize proteins consist of a mixture of prolamins,

glutelins, albumins, and globulins, which are differentiated by solubility properties. Prolamins, or zeins, are the major fraction, followed by glutelins (35% total protein), both of which are endosperm specific proteins. Albumins contribute about 7%, globulins 5%, and non-proteins 6%, that consist mostly in free amino acids, in particular asparagine, glutamine, proline, and alanine (Osborne, 1924; Wilson, 1983). The germ contains some lysine and tryptophan, which are essential amino acids for human survival, nevertheless total protein quality decreases because zeins are low in lysine and tryptophan, and this is not sufficient for maize to be considered as a high-quality protein source.

The lipids fraction is present in kernel primarily in oil form, ranging from 3.5 to 6%, and mainly located in the germ being the germ itself one third oil (Table 1.3). Some mutants, as floury-2, sugar-2 and opaque-2 show higher oil percentages because of the increased ratio germ/endosperm (Arnold et al., 1974; Flora and Wiley, 1972; Roundy, 1976). The lipids are present in greater part as triacylglycerols, the major storage form, but additionally as phospholipids, sterols, waxes, alcohols and hydrophobic micronutrients (Reiners and Gooding, 1970). Maize oil contains predominantly unsaturated fatty acids, as linoleic (C18:2) and oleic (C18:1), linolenic followed by a saturated fatty acid, the palmitic acid (C16:0).

The fibre is the main component of the kernel coat, the pericarp, but it can be found also in other seed parts in smaller amounts (Table 1.3). Pericarp fibres include 67% hemicellulose, 23% cellulose, and 0.1% lignin (Burge and Duensing, 1989). The total fibres represent the 7% of the total kernel composition, with a major percentage of the insoluble fraction with respect to the soluble one (USDA Nutrient Database, 2009).

The minerals are also present in maize kernel, especially in germ, with the prevalence of phosphorus, in the form of phytate, potassium and magnesium (Miller, 1958). They are followed by sulfur, contained in the amino acids methionine and cystine (Watson, 1987a; Watson, 1987b), zinc, primarily present in the endosperm (Inglett, 1970), calcium and iron, bound to phytate and less bioavailable (Wright, 1987).

Finally mineral present in traces are manganese, copper, selenium and iodine (Mertz, 1970).

Table 1.3 Chemical composition of the main kernel parts, adapted from Watson (1987b).

Chemical components	Germ (%)	Endosperm (%)	Pericarp (%)
Starch	8.3	87.6	7.3
Sugar	10.8	0.6	0.3
Protein	18.4	8.0	3.7
Ether extract	33.2	0.8	1.0
Fibre	8.8	2.7	86.7
Ash	10.5	0.3	0.8

1.3.2 Starch synthesis

Starch is the form of stored energy in seeds endosperm which generally consists of D-glucose homopolymers, called amylose and amylopectin. Amylose is a linear polymer composed of 1,4-α-D-glucose chains while amylopectin is a branched glucan that shows α-1,6 glucosidic bonds connected to the linear chains. Amylopectin is also the relatively most abundant polymer in starch and is formed by repeated amorphous and crystalline lamellae (Fig. 1.5).

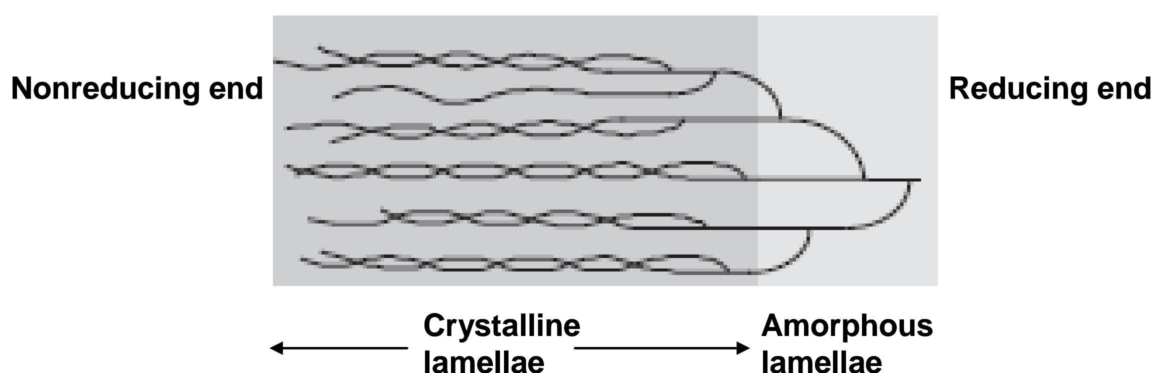

Fig. 1.5 Amylopectin structure, source Jeon et al. (2010).

The starch coming from different species of cereals shows different degrees of polymerization in the periodic waves of the polymodal chain-length. These chains are in fact divided in four different groups in which the degrees of polymerization varies

in the intervals: (A) 6-12; (B_1) 13-24; (B_2) 25-36 and (B_3) more than 37 (Hanashiro et al., 1996). The maize endosperm is approximately composed by amylopectin chains belonging to A and B_1 groups (Hannah and James, 2008; Myers et al., 2000).

Starch biosynthesis requires the coordinated activities of several enzymes. The first regulatory step is catalyzed by ADP glucose pyrophosphorylase (AGPase). This enzyme, composed by 4 subunits, uses the glucose-1-phosphate (Glc-1-Pi), as substrate, and ATP to produce ADP-glucose (ADP-Glc) and pyrophosphate (PPi). In maize, its activity is primarily localized in cytosol but, it can also act in plastids (Denyer et al., 1996).

The synthesis proceeds with the elongation of linear glucan chains by the transfer of ADP-Glc units to the non-reducing ends of glucan chains. The enzymes that have been thus far identified in cereals endosperm are granule-bound starch synthases (GBSSs) and soluble starch synthases (SSs) and their isoforms (James et al., 2003).

Of the GBSSs two isoforms have been identified: GBSSI that is mostly confined to the seed endosperm or in storage tissues, while GBSSII acts primarily in non-storage tissues where the transitory starch accumulates. In maize mutants, where GBSSI production is suppressed, the total starch content does not vary, but the amylose component is reduced or completely absent (Tsai, 1974). GBSSs isoforms also show an involvement in amylopectin synthesis, in particular in the formation of the extra-long unit chain fraction (ELC) that completely lacks when GBSSI decreases its activity (Franco et al., 2002; Yoo and Jane, 2002).

The other enzymes involved in starch elongation are SSs and they are grouped into three classes: early, late and steady expressers. Early expressers act primarily during pre-storage phase while the late expressers, which are also most abundant than the early ones, are more active during the starch filling phase. The various isoforms of these enzymes are shown to use different substrates of amylopectin, for example SSIIIa, a late expresser, demonstrates to synthesize amylopectin chains with DP$\geq$30 (Cao et al., 1999). On the contrary, SSI, whose isoforms are not known, acts in the elongation of short amylopectin chains, belonging to A and B_1 groups, up to a critical

length, and then it is tightly bound to longer chains and becomes an inactive protein within the starch granule (Commuri and Keeling, 2001).

The amylopectin branches are produced by branches enzymes (BEs) that act by cleaving α-1,4 linkages and reattaching via an α-1,6 glucan linkages. Two classes are known of these enzymes that present different biochemical and physicochemical properties: BEI produces longer chains with DP≥16 while BEII generates shorter chain of DP≤12 (Nakamura, 2002). Also debranching enzymes (DBE) act in controlling the number of branches of amylopectin and they are isoamylases (ISAs) and pullulanases (PULs). ISAs act also on phytoglicogen but their role is important in hydrolyzing excessively or improper chains of amylopectin (Ball et al., 1996; Nakamura, 2002), whereas PULs, that act also on pullulan, express their primary function of starch degradation during the seed germination (Beatty et al., 1999).

Finally, the plastidial starch phosphorylases (Pho1 and Pho2) catalyze the transfer of the glucosyl units from Glc-1-P to the non-reducing ends of the glucan chains. Pho1 is localized in the plastid and act at the surface of the granule phosphorylating it and modifying its structure (Tetlow et al., 2004). On the contrary Pho2 primarily acts in the cytosol.

1.4 *Fusarium verticillioides*: taxonomy and morphological characters

Fusarium is one of the most heterogeneous and difficult to classify fungal genus, but it contains many species of economic importance due to their capability to cause plant disease. These pathogens are also ubiquitous, they can be found from arctic to tropical areas, and this widespread distribution could be attributed to their capacity to grow on a wide range of substrates and to their efficient dispersal mechanisms. This genus belongs to the kingdom of Eumycota, phylum Dikaryomycota, subphylum Ascomycotina and order Hypocreales. The genus *Fusarium* has been also divided in sections. This special classification is used for those genera that are formed by a great number of species, and every section includes some species with similar

morphological characteristics. In particular, *F. verticillioides* belongs to the section *Liseola*.

The *Fusarium* genus can cause two different diseases on maize that could be differentiated by epidemiological conditions. The "red ear rot" (Gibberella ear rot) is prevailing in cooler areas, like in North Europe, that are interested by higher precipitation during the growing time, especially during flowering (Bottalico, 1998). It is mainly caused by *Fusarium graminearum* (teleomorph *Gibberella zeae*) and it could be distinguished by the development, from the tip of the ear, of a red or pink mould that covers a large proportion of the ear (Koehler, 1959; Miller, 1994).

Fusarium verticillioides (Sacc.) Nirenberg (synonym: *Fusarium moniliforme*, teleomorph: *Gibberella moniliformis* Wineland; synonym *Gibberella fujikuroi* mating population A) is the most important maize pathogen causing "pink ear rots" (Fusarium ear rot) (Munkvold and Desjardins, 1997). This species can infect kernels randomly, with the development of a white or light pink mould, but sick kernels could also be symptomless (Koehler, 1959; Miller, 1994).

The *Fusarium* species have traditionally been identified based on morphological criteria. Using these criteria, it must be considered both physical and physiological characters, like presence, shape and size of macro- or micro-conidia, their supporting structures, but also the presence of chlamydospores is an important character.

Fusarium verticillioides microconidia appear small, hyaline and mostly single-celled; they are abundantly produced in long chains arising from a base cell, called phialide. These chains of xenospores are well adapted for wind, rain, and vectored dispersal and this suggests an important role in the infection process of the corn plants. The macroconidia are less abundant than the microconidia and they are produced in specialized structures, which are called sporodochia, where the entire spore mass could be encased in slime. They appear orange or tan in colour, relatively long and thin, slightly falcate or straight and thin walled, with 3- or 5- septa. Their terminal cells, apical and basal, are also considered relevant characters for fungi identification because of their different shape. The apical cell is curved and often tapered while the

basal cell is notched and foot shaped. This specie does not produce chlamydospores, even if some swollen cells produced in the hyphae can be mistaken for pseudo- or chlamydospores (Leslie and Summerell, 2006).

1.5 *Fusarium verticillioides*: ecology

F. verticillioides grows from 3-5°C to 32-37°C, with an optimal growth in the range of 22-27°C, and a maximum linear growth at 25°C. *F. verticillioides* can also grow until the minimum level of water activity a_w=0.88, with an optimum that varies between 0.96 and 0.98 a_w (Marín et al., 1996).

Many environmental factors affect FB_1 production. The main abiotic parameters that affect *F. verticillioides* activity are temperature (T) and water activity (a_w). Temperature and a_w ranges at which FB_1 can be produced are very narrow to those values at which the fungus can grow. The optimum of temperature for FB_1 production is around 20-30°C. In particular their synthesis starts at 20-25°C, reaching the maximum production in correspondence to a low fungal growth rate (Marín et al., 2004). Optimal a_w conditions are 0.95-0.99 a_w and no production was observed below 10°C and 0.93 a_w (Marín et al., 1999b).

Marìn *et al*. (1995) also observed that *F. verticillioides* growth was inhibited by water stress, whereas FBs production was enhanced.

Quite low levels of CO_2 and vacuum, as well as nitrogen, have an inhibitory effect on both FB_1 production and *F. verticillioides* growth, while O_2 has no impact on FB_1 synthesis. Both CO_2 and O_2 carry on their effect in synergy with a_w that was confirmed as the main affecting factor (Samapundo et al., 2007).

Some studies reported that FB_1 is best produced in ripe maize kernels (Battilani et al., 2011) than in earlier ripe stages because the presence of starch improves FB_1 synthesis, in particular amylopectin plays this key role (Bluhm and Woloshuk, 2005; Chulze et al., 1996; Warfield and Gilchrist, 1999).

Fumonisin B_1 is especially produced when the fungus is stressed and during all the vegetative stages of maize plant, in particular when a dry period precedes or is

contemporary to grain filling (Bacon et al., 2008). Symptomless infections on kernels due to *F. verticillioides* seems to be a contributing factor for FB production under favourable conditions (Bacon and Hinton, 1996).

1.6 *Fusarium verticillioides*-Maize: cycle of infection

Fungi are able to produce many different structures to facilitate survival and spatial distribution, like hyphal fragments, or more resistant, like sclerotia or rhizomorphs, and both sexual and asexual spores. In particular, *F. verticillioides* produces thickened hyphae, highly dehydrated, with survival capabilities (Nyvall and Komendahl, 1968). Many sources of inoculum are present in field but the primary one, causing the survival of these specific pathogens, is the colonization of plant debris. *Fusarium* species survive on maize residues in field (Cotton and Munkvold, 1998), but they are also able to colonize other crop residues that usually are not considered as hosts for these pathogens (Parry et al., 1995).

The species belonging to this genus are heterothallic, but the sexual reproduction is less important from an epidemiological point of view, while it has an important role in the genetic recombination. The asexual spores are the most efficient reproduction mechanism, thanks to the large number of conidia produced (Munkvold, 2003). Another, even if less important, source of inoculum for symptomless infection is represented by infected kernels. Finally some experiments demonstrated the possibility of a symptomless systemic infection through the seedling roots caused by *F. verticillioides* strains originated from the rhizosphere, where toothpicks inoculated with a strain of this fungus were posed near seed and that caused as later recovery of the toxins in the kernels (Desjardins et al., 1998).

As mentioned before, *Fusarium* microconidia are primarily dispersed by wind and are the most efficient in the infection process, even if also macroconidia can have a role. These conidia were found in the air near the field but they were also found at 300-400 Km of distance (Ooka and Kommedahl, 1977).

Many studies have shown that spore production, under field conditions, is affected by some environmental factors. Among these, temperature plays an important role together with water activity (Hsieh, 1979), humidity (Indira and Muthusubramanian, 2004; Tonapi et al., 2007) and alternate light and dark (Devi and Singh, 1994). Sporulation was demonstrated to occur between 5°C and 45°C, with a maximum production at 27°C (Rossi et al., 2009).

During a study on spores germination two different ways of germination were observed, depending on the different phenotype of the strains. In one of the two systems used by *F. verticillioides*, spores formed the first hypha that grows along the silks (surface germination), whereas in the other way, spores produce an invasive germ tube (invasive germination) (Glenn, 2006).

A theoretic and conceptual model for the dynamic simulation of the life cycle of *F. verticillioides* was proposed by (Battilani et al., 2003) (Fig. 1.6). In this model, *F. verticillioides* showed to be able to invade maize grains via three pathways: (i) systemic growth through seed transmission or in roots, stalks, or leaves; (ii) ear infection through the air borne spores or (iii) insects as vectors of conidia. A variety of insects, in particular Lepidoptera or Coleoptera, could cause the dispersal of the spores depending on the area where the cultivation grown. In Europe, the dispersion of *F. verticillioides* microconidia was primarily ascribed to the European corn borer (ECB) *Ostrinia nubilalis* (Mazzoni et al., 2011). The larvae of these insects were able to directly damage kernels by reaching the pericarp and giving the fungus a point of entry or the same larvae can act as vectors of the inoculum (endogenous or exogenous) and carry it directly inside the kernels (Sobek and Munkvold, 1999).

The infection of kernels is detected around the 4th or 5th week after the pollination (milk stage) and rapidly increases until 9th week that corresponds to harvest maturity. A week later the beginning of kernels infection also fumonisin contamination is detected and the peak of this contamination occurs 2 or 3 weeks later (dent stage) (Bush et al., 2004). These results on fumonisin contamination have previously been confirmed in a study conducted by Warfield and Gilchrist (1999). They demonstrated

through the inoculation of kernels at different stage (blister, milk, dough and dent) that the dent stage is the most conducive for fumonisin contamination, while the blister is the least conducive.

The fumonisin production could sometimes have phytotoxic effects but is not required for the plant pathogenesis (Desjardins and Plattner, 2000; Marasas, 1996).

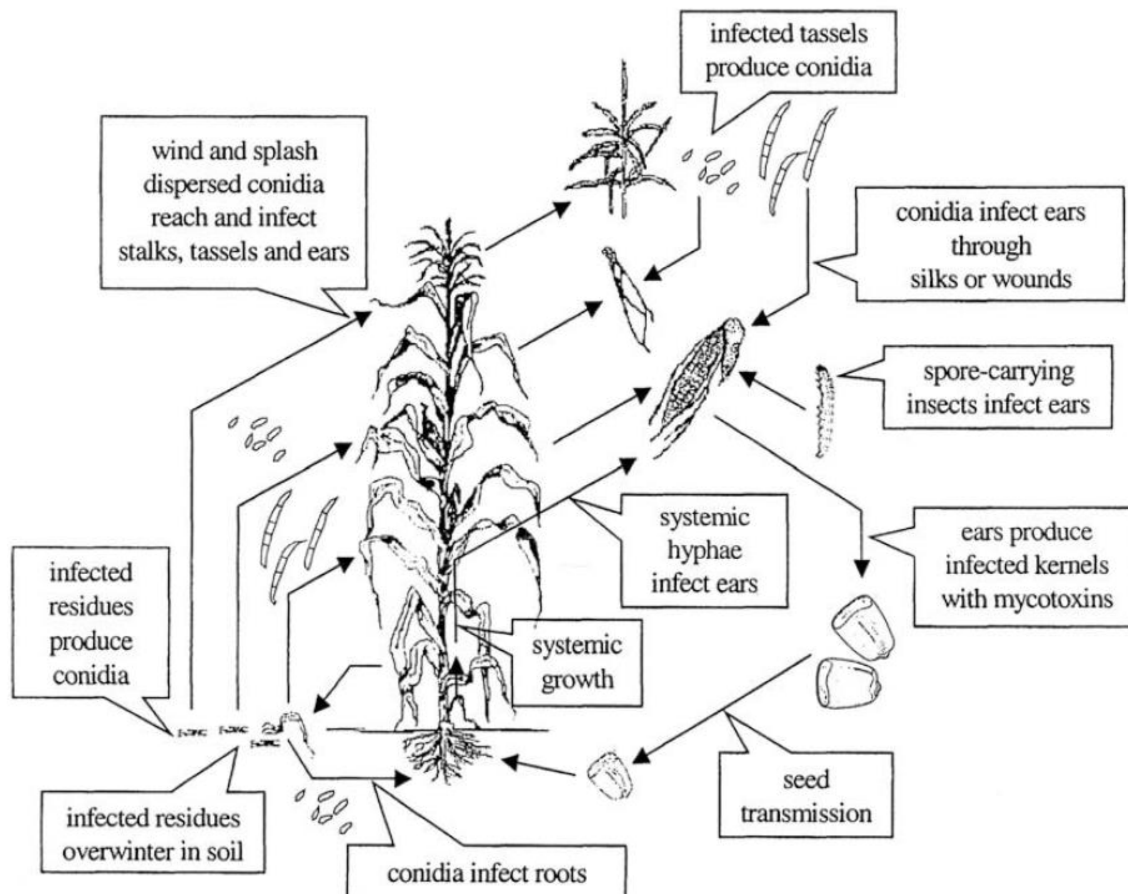

Fig. 1.6 Cycle of infection of *Fusarium verticillioides* on maize, source Battilani et al. (2003).

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1.7 *Fusarium verticillioides*-Maize: oxylipins production

The family of molecules called oxylipins are formed by oxidized fatty acids and their derived metabolites. They are produced by plants (Blée, 2002; Koo and Howe, 2009), mammals (Funk, 2001), fungi and bacteria (Andreou and Feussner, 2009a). They exhibit a crucial biological role as signal of intra- and inter-cellular communication in plants and fungi, but also in vertebrates and invertebrates.

In plants, oxylipins stimulate signals involved in the organization of plant defence against pathogens, they show also antimicrobial effects providing building units against pathogens attacks, they are involved in the regulation of plant cells death and they have a role in the formation of phytohormones during the senescence (Camera et al., 2004; Shah, 2005). In fact, some genes, encoding oxylipins biosynthetic enzymes, are induced by the inoculation with plant pathogens and, as a result, the production of oxylipins increases (Gobel et al., 2001; Hamberg et al., 2003; Ponce de Leon et al., 2002; Weber et al., 1999).

Plants oxylipins act in two different ways against these pathogens: some of these molecules show antimicrobial effects (Blee, 1998a; Blee, 1998b; Graner et al., 2003; Seo et al., 2001a; Weber et al., 1999), while others contribute to plant defence as signaling molecules inducing defence genes expression or regulating cells death (Camera et al., 2004). These effects are overlapping in oxylipins groups; in fact, the production of antimicrobial compounds is not separated from the production of plant signals. Moreover, some of the most active antimicrobial products were previously identified as signaling molecules or cells death inductors.

Plant oxylipins derive from linolenic, linoleic and hexadecatrienoic acids, while fungal oxylipins from oleic, linoleic and linolenic acids (Fig. 1.7). Phyto-oxylipins are produced during the metabolism of unsaturated fatty acids by their oxidation with one, two or four oxygen atoms, through three different pathways. These oxidations could be catalyzed by the action of (i) endoplasmatic reticulum localized cytochrome P450, (ii) lipoxygenase (LOX) or (iii) cyclo-oxygenase like activity made by α-dioxygenase. On the other side fungal oxylipins production primarily involves enzymes, recently identified, with strong homology to mammalian prostaglandine H synthases or cyclooxygenases (COX) (Tsitsigiannis and Keller, 2007).

In plants, in particular, LOXs catalyze the incorporation of molecular oxygen into free fatty acids, primarily linoleic and linolenic acids, either at position 9 or 13 of their carbon chains, and, therefore, are referred to as 9-LOXs or 13-LOXs (Howe and Schilmiller, 2002). The expression of 9-LOX gene, *ZmLOX3*, is induced by *F.*

verticillioides in lines that show a high accumulation of fumonisin (Wilson et al., 2001). This positive regulation has been confirmed by (Gao et al., 2007), whose experiments show that the inactivation of maize *ZmLOX3* resulted in drastically reduced levels of conidia and fumonisin production on kernels by *F. verticillioides*, although it did not lead to any detectable reduction of fungal vegetative growth.

Fig. 1.7 Overview of oxylipins production in plants, source Andreou et al. (2009b).

In fungi, a mixture of oxylipins, called Psi factor, is known to regulate the ratio of asexual and sexual spores produced. For this reason, oxylipins producing genes are named Ppo (psi-producing oxygenases) and their presence is demonstrated in numerous filamentous fungi, also in *Fusarium* spp. They are involved in the modulation of metabolic function as growth and maturation, and affect the pathogenesis abilities. The Ppo null strains of *A. nidulans* grow and sporulate poorly and have a decreased production of degradative enzymes, the toxin sterigmatocystin is not produced, while the penicillin increases. The fungal oxylipins, in fact, demonstrate to affect also the secondary metabolism, both in the production of pharmaceutical (antibiotics) and toxins. In *F. sporotrichioides* the disruption of a Ppo orthologue causes the impairment of the T2 toxin production, while in *Cercospora zeae-maydis* the homologue gene upregulation occurs when the conditions are favourable for cercosporin production.

The fungal oxylipins pathway regulation is complex and tight. The temporal and spatial activity of the enzymes, involved in oxylipins production, is of fundamental importance for fungal growth when the environmental conditions change. An organized network of signaling cascade that synchronize oxylipins products is demonstrated to exist in fungal tissues as a response to the different environmental conditions; this fact could conduce to a "oxylipin signature profile" typical for every pathogens. The same happens in plants where the oxylipin pools could lead to specific profiles of the tissues, the organelles and plants they belong to.

The production of PUFAs and oxylipins by plants and fungi can act like a cross-talk between the two kingdoms (Fig. 1.8). Fungi oxylipins are able to modify plant's response to the infection, and vice versa plant's oxylipins can alter the fungi secondary metabolism, in particular they can affect the mycotoxins production (Christensen and Kolomiets, 2011; Reverberi et al., 2012). Gao et al. (2007), found that the silencing of the 9-lipoxygenase pathway in maize leads to the decrease of conidia and fumonisin B_1 production in kernels inoculated with *F.verticillioides* strain.

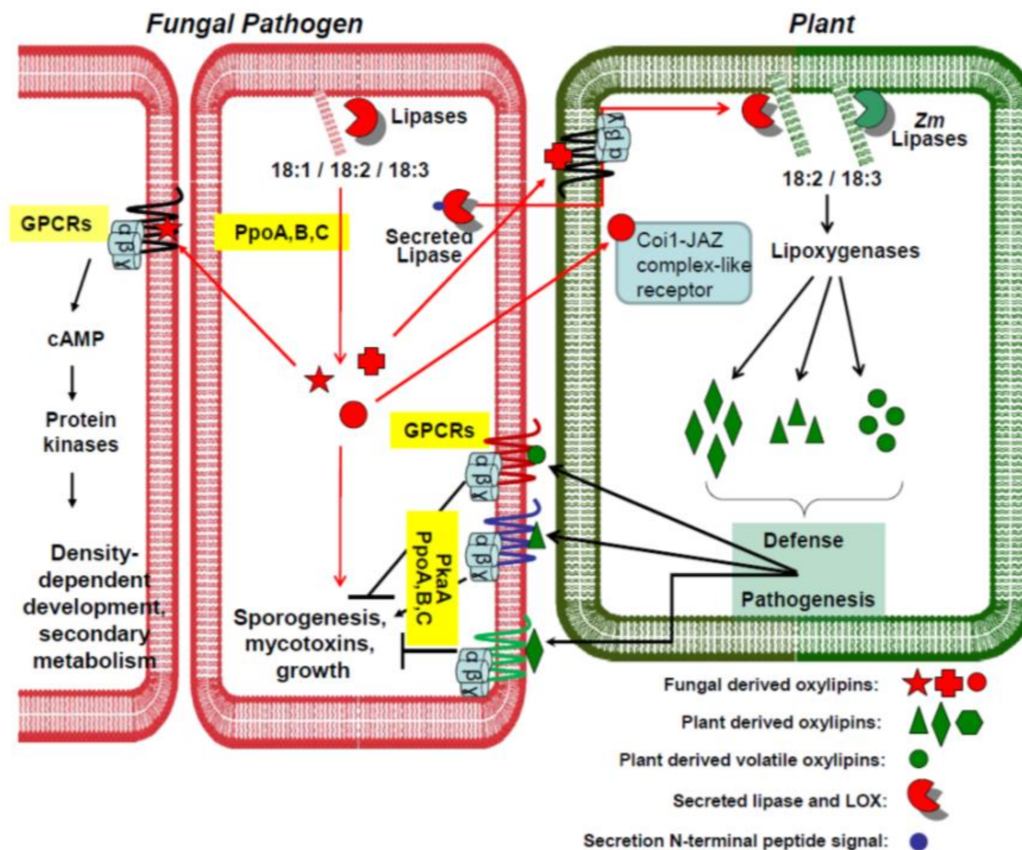

Fig. 1.8 Proposed mechanisms of oxylipins cross-talk between plant and pathogen, source Christensen and Kolomiets (2011).

1.8 Fumonisin biosynthesis

The FBs-like structures, with exception for the C-series, are formed by a 20 carbon linear backbone with 3 or 5 hydroxyl groups, two methyl groups, an amino group and two tricarballilyc esthers (TCA) (Fig. 1.9). The mechanism of production of these molecules is very similar to the cycle of condensation to synthesize fatty acids, but it was traced back to the action of a polyketide synthase because the β-carbonyl groups are left totally or partially unprocessed. The enzyme acts not only on acetyl and malonyl, but also on different substrates; this mechanism involves also further processing after carbon chain is formed, like cyclization or addition of side groups (Hopwood and Sherman, 1990; Katz and Donadio, 1993).

The C_3-C_{20} carbons were demonstrated to come from acetate molecules, through isotope feeding experiments (Blackwell et al., 1996), while C_1 and C_2 were shown to derive from alanine (Branham and Plattner, 1993a). The two methyl groups, located

at C_{12} and C_{16}, derive from methionine amino acid (Plattner and Shackelford, 1992). The hydroxyl groups show two different origins, the group located at C_3 derived from the carbonyl group of acetate molecules, while the C_5, C_{10}, C_{14} and C_{15} are provided from the molecular oxygen (Caldas et al., 1998). Finally, the tricarballylic esthers are considered to derive from the citric acid cycle (Blackwell et al., 1996).

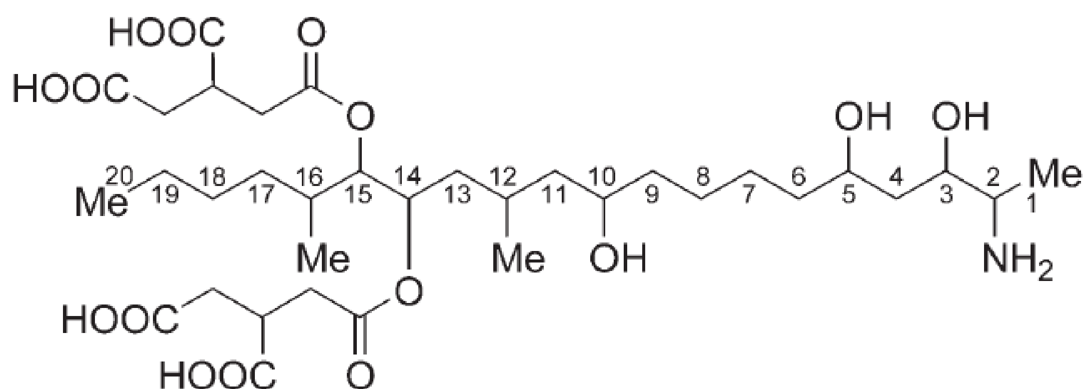

Fig. 1.9 Fumonisin B_1 structure, source Bartok et al. (2010).

Proctor et al. (2003) and Seo et al. (2001b) defined a cluster of 15 genes necessary and co-expressed for FBs biosynthesis. These genes forming the FUM cluster are FUM1 (formerly FUM5), FUM6, FUM7, FUM8, FUM3 (FUM9), FUM10, FUM11, FUM2 (formerly FUM12), FUM13, FUM14, FUM15, FUM16, FUM17, FUM18 and FUM19. Brown et al. (2005; 2007), more recently, found other two additional genes, FUM20 and FUM21, in the cluster.

The FUM1 gene product is a highly-reducing polyketide synthase (HR-PKS), a protein with seven domains:

1. β-ketoacyl synthase (KS)
2. Acyltransferase (AT)
3. Dehydratase (DH)
4. Methyltransferase (MT)
5. β-ketoacyl reductase (KR)
6. Enoylreductase (ER)
7. Acyl carrier protein (ACP).

The domains KS, AT, AC participate in the condensation of the chain, while KR, DH, ER participate in the β-carbonyl processing. The domains are located on two different subunits; the α-subunit shows the presence of KS, KR and AC, while AT, ER and DH on β-subunit (Hopwood and Sherman, 1990). The disruption of this gene causes a decrement of fumonisin production by over 99%.

The result obtained by the action of this enzyme is the C_3-C_{20} carbon chain, that is released by the introduction of a C-C bond through the condensation of the alanine amino acid, that correspond to the C_1 and C_2 of the FBs backbone. This two carbon elongation also results in the introduction of an amino group into the final product, obtaining a 3-keto intermediate. This result is obtained by the action of FUM8 product that corresponds to 2-oxoamino synthase, a class II α-aminotransferase. This class of enzymes is able to catalyse the condensation of amino acids and acyl-CoA thioester substrates (Seo et al., 2001b). The fum8p is, for these reasons, the key factor to determine the chain size that determine if the toxin produced belongs to B- or C-series. The difference existing between the two series is the lack of C_1 in the C-series. The *F. verticillioides* FUM8 mutants have demonstrated to produce predominantly FCs.

Both the B- and C-series toxin undergoes to some tailoring steps after the release of the carbon chain. This has been demonstrated by the ability of the enzymes involved to work on free substrates (Bojja et al., 2004). These steps include oxyreductions and esterifications of the 3-keto intermediate. The first step is the reduction of the ketone group, for which is responsible the FUM13p. FUM13 encodes for a NADPH-dependent ketoreductase able to reduce substrates like alcohols and aromatic compounds. The disruption of this gene produces two new analogues, the 3-keto forms of FB_3 and FB_4 (Butchko et al., 2003).

The vicinal diol, present at the C_{14} and C_{15}, comes from the action of an enzyme, P450 monoxygenase encoded by FUM6. FUM6 mutants do not produce any detectable intermediate, this fact also suggest that the 3-keto intermediate is not stable. Furthermore other 2 genes encode for a P450 monoxygenase FUM2 and

FUM15 (Proctor et al., 2003). The fum2p encodes for the P450 monoxygenase that catalyze the C_{10} hydroxylation (Proctor et al., 2006), while the role of fum15p is still unclear and it is not essential for FBs biosynthesis.

The esterification of the two tricarballylic side chains to the vicinal diol involves the action of four genes, FUM7, FUM10, FUM11 and FUM14 (Proctor et al., 2006; Zaleta-Rivera et al., 2006). FUM7 encodes an enzyme, an iron-containing alcohol dehydrogenase, whose action is still unclear.

FUM11 disruption causes the increase in the production of half-hydrolyzed and keto half-hydrolyzed homologues of fumonisins, indicating that fum11p is not essential for the biosynthesis but for its complete esterification of the structure (Butchko et al., 2006).

FUM10 and FUM14 disruption mutants show the same phenotype, that result in the production of hydrolyzed FB_3 and FB_4 (HFB_3 and HFB_4). In particular, fum14p is a peptidyl carrier protein (PCP) and a condensation domain (C) (Zaleta-Rivera et al., 2006).

Mutants bringing the disrupted FUM3 (FUM9) produce only FB_3 and FB_4 that lack C_5 hydroxyl group. The sequencing of this gene shows that it encodes for a dioxygenase (Gelderblom et al., 2007).

Even if the cluster proposed is made of 17 genes, some of them seemed to be not essential for FBs synthesis; in fact experiments conducted on the disruption of FUM15, FUM16, FUM17 and FUM18 do not show any change in fumonisins production. FUM15 disruption does not affect the presence of hydroxyl groups along the backbone carbon chain. FUM16 and FUM10 both encode fatty acid-CoA synthases but, while the disruption of FUM10 causes the non-esterification of fumonisins, the disruption of FUM16 does not cause any change. The FUM17 and FUM18 encode for ceramide synthases, and are involved in resistance to fumonisins, being this enzyme inhibited by fumonisins.

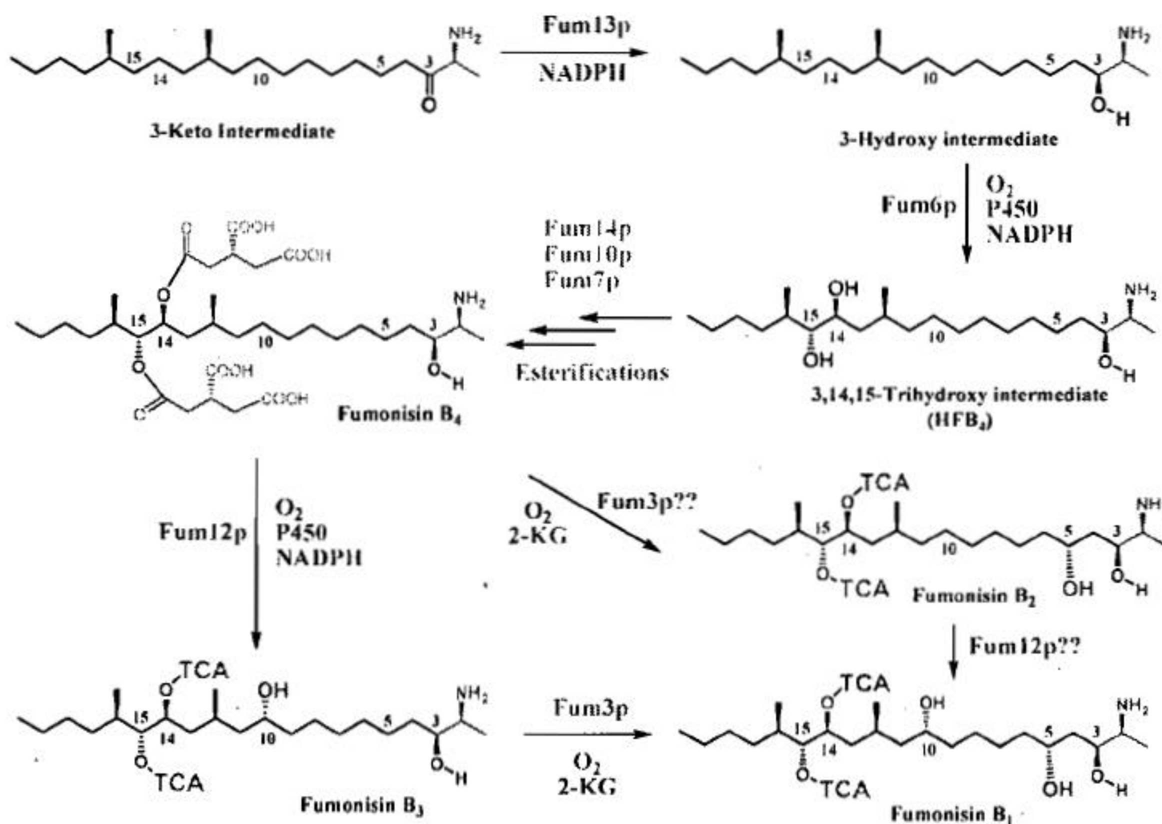

Fig. 1.10 Tailoring of the free 3-keto intermediate, source Gerber et al. (2009).

1.8.1 Regulation in fumonisin biosynthesis

The cluster does not contain any regulatory gene, but the regulation of fumonisin biosynthesis was demonstrated to be very complex, and it involves multiple factors, genetic as well as environmental.

Some environmental factors are known to significantly affect the production of fumonisins, such as moisture content, temperature, host specificity, maize kernels environment and nutritional conditions. With reference to moisture content , it has been found an increase in the production of toxins correlated to an increase in a_w, tested at all temperature between 15-30°C (Samapundo et al., 2005). The same studies also demonstrated that the temperature was observed to have more effects at lower a_w contents than at higher ones. This suggests a more direct effect of a_w on fumonisin production whereas temperature effects depend on a_w value (Marín et al., 1999a; Samapundo et al., 2005).

Other environmental factors that can impact the toxin production are known to be nitrogen media content and carbon nutrient specificity. A positive correlation was found between nitrogen limitation and the fumonisin biosynthesis, while under high nitrogen concentrations the synthesis is inhibited (Shim and Woloshuk, 1999). No effect is showed to be determined by carbon and phosphate contents.

Finally there is another environmental factor that is demonstrated to influence fumonisin synthesis, the pH, but a pH regulatory gene is implied in that mechanism. It has been shown that this gene, called PAC1 because of his high homology to PACC-like genes (pH regulatory genes) present in many filamentous fungi, is required for a reduced growth and fumonisin synthesis under alkaline conditions while in acidic conditions it is showed to cause higher fumonisin concentration (Flaherty et al., 2003; Shim and Woloshuk, 2001).

Another gene is demonstrated to be involved in the regulation of secondary metabolism (fumonisin biosynthesis) and fungal development (conidiation), and it is called FCC1, a cyclin-like C-type gene. Its product is a cyclin, that is an essential activating subunits of a specific cyclin-dependent kinases (CDKs), encoded in that case by FCK1 gene. When the regulatory CDK complex is formed, it was hypothesized it could act linking the receptors that sense the environment or alternatively it could directly regulate the transcription activated as a response in extracellular stress (Shim and Woloshuk, 2001).

The gene AREA is also required for growth and fumonisin synthesis regulation. The activation of this gene is the result of too high presence of nitrogenous compound found in host tissues. It regulates a catabolic pathway whose extent is to diminish the concentration of nitrogenous metabolite (Kim and Woloshuk, 2008).

ZFR1, a zinc binuclear cluster-type gene, is a positive regulator for fumonisin biosynthesis. Mutants lacking that gene are not able to produce detectable amount of fumonisin and no evidence of FUM1 and FUM8 transcripts are found (Flaherty and Woloshuk, 2004). So its suppression decrease the level of FB_1 production but its

overexpression only restores the fumonisin biosynthesis and it is not followed by an overproduction.

GBB1 is a gene encoding an heterotrimetric G protein β subunit and is found to produce a positive regulation on FB_1 synthesis in *F. verticillioides*, but also on conidiation and hyphal development, while it is not required for virulence (Sagaram and Shim, 2007). Its suppression in mutant strains does not produce the complete deletion of FB_1 synthesis or conidiation, but a significant decrease. On the other side, the gene GBP1, encoding the putative monomeric G protein, is a negative fumonisin-specific regulator (Sagaram et al., 2006). It is involved in FB_1 synthesis regulation but not in the conidiation. Its deletion leads to FUM1 and FUM8 repression and to an overproduction of FB_1, while its overexpression does not involve the suppression of the synthesis.

The CPP1, a gene encoding a putative protein phosphatase 2A catalytic subunit, is negatively correlated to FB_1 biosynthesis. In addition to its functional role in hyphal morphogenesis and its influence on the ratio macro/microconidia and spore germination, it has also a specific regulation role on FUM1 gene but not on the expression of other PKS genes. The deletion of this gene, in fact, leads to an increase in FB_1 synthesis as a result of an elevated FUM1 expression (Choi and Shim, 2008).

FST1 expression is mainly induced in endosperm tissues, the maize kernel fraction which supports the highest level of FB_1 production. FST1, a putative sugar transporter gene, has a negligible effect on growth and kernel acidification after colonization, but also on growth on synthetic media containing glucose or maltose as the sole carbon source. Its disruption causes no reduction in sugar uptake but a deficiency in specific aspects of sugar signaling and a significant reduction in fumonisin synthesis (Bluhm et al., 2008).

1.9 Fumonisin analogues and their toxicity

More than 28 fumonisin analogues (Table 1.4) have been identified and have been divided into A, B, C and P series (Rheeder et al., 2002). These molecules showed

different levels of toxicity, but the most abundant and the most toxic compounds belong to the B series (Marasas, 1996).

Table 1.4 Fumonisin side chain structures of the 28 analogues identified, source Rheeder et al. (2002).

Analogues	Side chains to fumonisin backbone						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**
FA_1	TCA	TCA	OH	OH	H	NHCONH3	CH3
FA_2	TCA	TCA	H	OH	H	NHCONH3	CH3
FA_3	TCA	TCA	OH	H	H	NHCONH3	CH3
$PHFA_3a$	TCA	OH	OH	H	H	NHCONH3	CH3
$PHFA_3b$	OH	TCA	OH	H	H	NHCONH3	CH3
HFA3	OH	OH	OH	H	H	NHCONH3	CH3
FAK1	=O	TCA	OH	OH	H	NHCONH3	CH3
FBK1	=O	TCA	OH	OH	H	NH2	CH3
FB1	TCA	TCA	OH	OH	H	NH2	CH3
Iso-FB_1	TCA	TCA	OH	H	OH	NH2	CH3
$PHFB_1b$	TCA	OH	OH	OH	H	NH2	CH3
$PHFB_1a$	OH	TCA	OH	OH	H	NH2	CH3
HFB_1	OH	OH	OH	OH	H	NH2	CH3
FB_2	TCA	TCA	H	OH	H	NH2	CH3
FB_3	TCA	TCA	OH	H	H	NH2	CH3
FB_4	TCA	TCA	H	H	H	NH2	CH3
FB_5							
FC_1	TCA	TCA	OH	OH	H	NH2	H
***N*-acetyl-FC_1**	TCA	TCA	OH	OH	H	NHCONH3	H
Iso-FC_1	TCA	TCA	OH	H	OH	NH2	H
***N*-acetyl-iso-FC_1**	TCA	TCA	OH	H	OH	NHCONH3	H
OH-FC_1	TCA	TCA	OH	OH	OH	NH2	H
***N*-acetyl-OH-FC_1**	TCA	TCA	OH	OH	OH	NHCONH3	H
FC_3	TCA	TCA	OH	H	H	NH2	H
FC_4	TCA	TCA	H	H	H	NH2	H
FP_1	TCA	TCA	OH	OH	H	3HP	CH3
FP_2	TCA	TCA	H	OH	H	3HP	CH3
FP_3	TCA	TCA	OH	H	H	3HP	CH3

Class B. Firstly isolated in 1988 by (Gelderblom et al., 1988) from *F. verticillioides* contaminated maize kernels; these mycotoxins were also found to be produced by other *Fusarium* species, as *Fusarium proliferatum* and *Fusarium nygamai* (Marasas, 2001; Rheeder et al., 2002). Furthermore other genera were able to produce these toxins, as *Alternaria alternata* f. sp. *lycopersici*, and also by *Aspergillus niger* (Frisvad et al., 2007; Marasas, 2001; Rheeder et al., 2002).

FBs have demonstrated to be stable compounds; they were shown to react with reducing sugars, but only if treated at high temperatures. The product was isolated by (Howard et al., 1998) and recognized as *N*-carboxymethyl-FB_1; later it was also

found in raw corn from field, even if in small amounts (Howard et al., 1998). The FBs contamination of food seemed to be reduced in case of a base treatment of the food (nixtamalization) but this treatment leads to the increase of hydrolyzed forms, without change in the level of toxicity (Norred et al., 1991).

Recently new analogues of this class have been found and they are characterized by reversed-phase high-performance liquid chromatography/electrospray ionization ion trap mass spectrometry (Bartok et al., 2006). They had showed a higher molecular weight and more apolar characteristics of the backbone that was ascribed to the esterification of the hydroxyl groups of the chain with organic acids, and for this reason they were called esterified FB (EFB). Also some isomers of these new structures had been isolated (iso-EFB), but their relative quantities (%FB_1) were in the range 0.003–0.036% (Bartok et al., 2010).

Class A. Among the fumonisins structures, originally isolated from *F. verticillioides* infected maize (Bezuidenhout et al., 1988), also the A-series fumonisins, the N-acetylated analogues of the B-series, were identified. They were produced in small quantities and even if previous studies showed a lower toxicity of the N-acetylated forms (Alberts et al., 1993; Tanaka et al., 1993), recently the necessity of a primary amino group for the ceramide synthase inhibition have been demonstrated and the toxicity of FA_1 and FB_1 equalized (Westhuizen et al., 1998). This has been ascribed to a spontaneous rearrangement of N-acetylated to O-acetylated structure that seemed to be more effective in ceramide synthase inhibition (Norred et al., 2001). In the study conducted by Norred et al. (2001), an increase in the ratio sphinganine/sphingosine (biomarker for fumonisins exposure) was induced in rat liver slices by the exposure to fumonisin B_1 or impure FA_1 preparation period. The subsequent analysis of the impure FA_1 preparation identified the presence of 3- and 5-*O*-acetyl FB_1. These metabolites showed a similar retention time as FB_1 indicating the presence of a free amino group, while FA_1 was not retained.

Class C. Ather fumonisins structures extracted from a *F. moniliforme* infected maize were the C_1-forms (Branham and Plattner, 1993b). These new structures are different

from FBs because of the lack of the C_1 terminal methyl group. The toxicity of these new structures is very similar to the one induced by FB_1 because the missing group is not required for this biological activity (Sewram et al., 2005). The fumonisin structure derived from the condensation of the carbon chain with an amino acid that, in case of the B series corresponded to alanine, while in the case of the C series corresponded to glycine. The α-aminotransferase, enzyme codified by FUM 8 gene and responsible for this condensation, seemed to be able to work on both amino acids substrates, because they were simultaneously produced by the same strains, even if C analogues were in smaller amount (Sewram et al., 2005). Furthermore, it had been demonstrated by Sewram et al. (2005), that among the C analogues, FC4 had been produced in higher amount.

Class P. This new class of analogues was firstly isolated by Musser et al. (1996). Their structures had been identified to be identical to the B series with the exception of the replacement of the C2 amino group with a 3-hydroxypyridinium functional group (Musser et al., 1996). This new class of analogues showed a lower phyto- and cytotoxicity than the FBs, and this had been caused by the absence of the free amino group; even if they were produced in significant amount, about 30% of the B form in coltures, they do not contribute to the toxicity produced by contaminated foodstuff (Abbas et al., 1998).

Hydrolysed forms. The hydrolysed forms (HFB_1, HFB_2 or AP_1, AP_2) are characterised by the loss of TCA side chains, caused by strong heat treatment, as roasting or extrusion, or an alkali treatment, called nixtamalization and used in masa preparation (Humpf and Voss, 2004; Scott and Lawrence, 1996; Voss et al., 1998). The studies concerning the toxicity of these forms showed that HFB_1 was less cytotoxic in cell culture and less potent in ceramide synthase inhibition if compared to FB_1 (Schmelz et al., 1998). Those results were in contrast with the *in vivo* studies demonstrating that the administration of a purified HFB_1 solution to female mice and pregnant rats did not cause liver or kidney apoptosis or sphingolipid metabolism disruption (Howard et al., 2002). The apparent contradiction of these results could be

justified by the discovery of presence of N-acyl-HFB metabolites, products of the acylation of HFB operated by the ceramide synthase. This was demonstrated to occur *in vitro* and *in vivo* for HFB_1 and HFB_2, substrates equally well accepted by the enzyme (Seiferlein et al., 2007), and the same study showed, also, cytotoxic effects of these compounds, able to reduce the number of viable cells tested, and their capability to inhibit the ceramide synthase.

***Hidden Fumonisins*.** The existence of other fumonisin derivatives was hypothesized when toxic effects were recorded after the ingestion of low contaminated feed. These compounds demonstrated to be adsorbed and reconverted in active forms inside the organism (Dall'Asta et al., 2010; Falavigna et al., 2012), and they also showed not to be detectable by the traditional analysis because of the low recoveries. A higher recovery of fumonisins was obtained by Shier et al. (1997) using a sodium dodecyl sulphate (SDS) solution hydrolysis on maize meal dough roasted samples, that suggested a possible linkage of fumonisin with proteins or starch.

Seefelder et al. (2003) demonstrated that, upon thermal treatment, FB_1 could react via TCA moieties with methyl-α-D glucopiranoside, simulating starch model, and with protected amino acids, as protein model. This mechanism was supposed to consist in the formation of covalent bonds between TCA side chains and the hydroxyl groups of starch or the amino or sulfidryl groups of amino acids in proteins (Seefelder et al., 2003). The model, proposed for this linkage, needed an initial activation of the molecule with the loss of water from the side chain to produce a cyclic anhydride, which can react with matrix functional groups. This activation could be obtained only by heating, and this did not explain the presence of these binding forms also in raw maize.

It was demonstrated that these phenomena occurred also in the field, as a plant response to the infection, to compartmentalize the target molecules (Poppenberger et al., 2003); the same mechanism has already been proved in the case of deoxynivalenol-3-glucoside, enzimatically produced by wheat to detoxify these

molecules, or in case of zearalenone-4-glucoside (Berthiller et al., 2005; Schneweis et al., 2002).

Many studies suggested the presence of protein binding forms (Kim et al., 2003; Park et al., 2004). This fact was investigated through the purification of maize proteins from carbohydrates, and then using Osborne fractions to divide maize proteins, a significant amount of fumonisins analogues were released by the globulins and prolamins fractions (Dall'Asta et al., 2008). Lazzaro et al. (2012) demonstrated also that the presence of hidden fumonisins is strictly related to the complexity of the matrix, in fact, while hidden fumonisins were detected on maize based medium, no recovery was observed on malt extract medium (Falavigna et al., 2013).

1.10 Maize storage and spoilage control

Independently from its final use, as food, feed and source for processed products or energy, post-harvest maize is stored for variable periods of time before its final utilisation. Fungal spoilage in harvested maize is a major concern; it can infect maize pre-harvest and improper storage conditions, like high temperatures and moisture content, can further favour mycotoxins production and lead to a reduction in grains quality (Chulze, 2010; Marín et al., 2004).

Many ecological parameters are known to affect storage safety, but among them, grain water activity (a_w) is one of the most important to be controlled. Usually, maize harvesting is carried out when the relative humidity is around 25% (~0,93 a_w) (Trucksess et al., 1988), in order to avoid further field contaminations and the increase of mycotoxins content in field (Battilani et al., 2011; Cotty and Cardwell, 1999), while water activity below 0.70 a_w (<14% moisture content), is considered suitable for a safe (Magan and Aldred, 2007; Yılmaz and Tuncel, 2010). Thus, cereal drying is considered necessary.

Fungal pathogens and mycotoxins associated with maize, mainly *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides* and fumonisins, are not inhibited by the ecological conditions of maize at harvest time (Battilani et al., 2008; Giorni et al.,

2008). Furthermore, fungi can continue to develop and produce mycotoxins during storage in a conducive environment. For these reasons, drying should take place soon after harvest, as rapidly as possible (Chulze, 2010), or a delay of grain drying could turn into a significant increase of mycotoxins content (Silva et al., 2008).

Many different technologies can be utilised to dry cereals, from solar radiation (Folaranmi, 2008) or natural and unheated air (Kaaya and Kyamuhangire, 2010) to more sophisticated and expansive dryers using heated air or with mixing systems (Costa et al., 2010; Jittanit et al., 2010; Tuncel et al., 2010). Drying costs increase moving from natural to artificial drying, but it is also strongly related to the grain water content, reason that push the farmers to delay harvest.

After drying, another important variable is air composition of the storage environment. Modified atmospheres and alternative gases, like SO_2, are useful to control aerobe obligate parasites, even if some fungi involved in grain biodeterioration are known to be micro-aerophilic (Magan and Aldred, 2007). Atmospheres with 25% of CO_2 are able to reduce *A.flavus* growth, but 50% of CO_2 is required for aflatoxins synthesis inhibition (Giorni et al., 2008). Also for *Fusarium* species high concentration of CO_2 are needed to inhibit the growth and the mycotoxins production, but a combination of low a_w and anaerobic environment could be sufficient.

Systems used to obtain this change in atmosphere composition could be very expansive, but in the last 20 years in developing countries a cheaper system has been improved. Silo bags are hermetic waterproof bags, with store capacity of about 200 tons of grain, but with a variable size, in which the respiration of the biotic mass lead to an increase of CO_2 and a decrease of O_2 inhibiting the development of fungal and insects spoilage and consequently mycotoxins (Gaston et al., 2009; Gregori et al., 2013; Pacin et al., 2009).

Also the use of preservatives can successfully control and inhibit the growth and mycotoxins production. Research has been carried out on both essential oils and anti-oxidants (Fanelli et al., 2003; Hope et al., 2005). 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).

1.11 Effects of the assumption of fumonisins

Animal effects. Fumonisins are known to cause a range of specie-specific toxic responses, like pulmonary oedema in swine, as well as hepatocarcinogenic, hepatotoxic, nephrotoxic and cytotoxic effects in rats and mice; however horses have demonstrated a higher sensibility to FB_1 toxicity with respect to other species. The target organs, in horses, are the central nervous system, the liver and the heart (Voss et al., 2007). Horses leucoencephalomalacia (ELEM) is caused by the ingestion of these toxins and exhibits two main syndromes , the neurotoxic form or the hepatotoxic form, less frequent, but these two syndromes can also occur simultaneously. ELEM pathological signs are characterized by liquefactive necrotic lesions in the white matter of the cerebral hemispheres of horses and histologically, necrosis with influx of macrophages, oedema, and haemorrhage are the primary findings (Voss et al., 2007). Cardiotoxicity is also documented and cardiovascular dysfunction manifests itself with a decreased heart rate in horses with neurological disease. An association is detected among fumonisin-induced neurologic disease, increased serum and myocardial sphinganine (SA) and sphingosine (SO) concentrations and decreased cardiovascular function in horses (Caloni and Cortinovis, 2010). The clinical course of ELEM is generally short with the acute onset of signs followed by death within hours or days, but if the neurological signs are early recognized, the elimination of contaminated feed and therapeutic support could lead to a decrease in morbidity and mortality (Foreman et al., 2004).

The pulmonary oedema is documented only in swine and the two physiologically main outcomes of this syndrome are ventricular failure, which increases pulmonary capillary hydrostatic pressure and increased vascular permeability after injury to the

alveolar capillary endothelium or alveolar epithelium. The accumulation of membranous material in porcine pulmonary capillary endothelial cells is believed to be specifically induced by FB_1 and caused by altered sphingolipid metabolism (Haschek et al., 2001).

Human effects. (Marasas et al., 2004) The human consumption of high contaminated corn based foods is not unequivocal related to man disease, but an increase in the incidence of oesophageal cancer was noticed in areas like Africa and China (Chu and Li, 1994; Gelderblom et al., 1988; Hussein and Brasel, 2001; Myburg et al., 2002; Seegers et al., 2000; Sun et al., 2007; Yoshizawa et al., 1994). Furthermore, a relationship between high level consumption of contaminated foods and neural tubes defects (NTD) is also documented, especially on Texas-Mexico border (Marasas et al., 2004). For all of these reasons, the International Agency for Research on Cancer (IARC) declared fumonisin B_1 as a class 2B carcinogenic (IARC, 1993).

The mechanism through which these metabolites act is the disruption of sphingolipid metabolism. It starts with the inhibition of ceramide synthase (Merrill et al., 2001; Riley et al., 2001), due to FB_1 structure that is similar to sphingoid bases and fatty acil-CoA's. The free sphingoid bases and other metabolites, as sphingosine, sphinganine 1-phosphate, or N-acetyl-sphinganine and -sphingosine, can accumulate (mainly sphinganine) and implement their growth inhibitory and cytotoxic effects. The ceramide inhibition results in an increase in SA/SO ratio, a useful biomarker to determine fumonisins exposure (Riley et al., 1994). The accumulation of sphinganine has been closely related to liver and kidney toxicity, and in case of high level of consumption, to lesions of these two organs.

Other studies, conducted to determine NTD occurrence causes among Mexican women, showed a potential link among fumonisin consumption, folate deficiency and increased risk for NTD. Epidemiological studies indicate that vitamin supplements containing folic acid can significantly reduce (50–70%) women's risks for an NTD affected pregnancy, and data from clinical trials support the hypothesis that this apparent reduction in risk may be specifically attributable to folic acid, even if it is

not clearly understood. Sphingolipids seem to play an important role in folate receptor function. This high-affinity receptor, a glycosylphosphatidylinositol (GPI)-anchored protein, is responsible for the transport of folate into cells of several tissues with elevated requirements for this vitamin. Fumonisins affect the folate transporters by altering both its endocytic trafficking and the amount of the receptors available for transport. The folate vitamins play an essential role as cofactors in many biochemical reactions including the biosynthesis of purines and thymidine, the regeneration of methionine from homocysteine, and histidine metabolism. Cellular processes dependent upon folate can be compromised if dietary levels of this vitamin are insufficient or its transport into cells is affected. These findings provided a conceptual framework whereby exposure to FB_1 might be a risk factor for NTD by disrupting folate utilization via depletion of cellular sphingolipids needed for normal receptor function (Fig. 1.11).

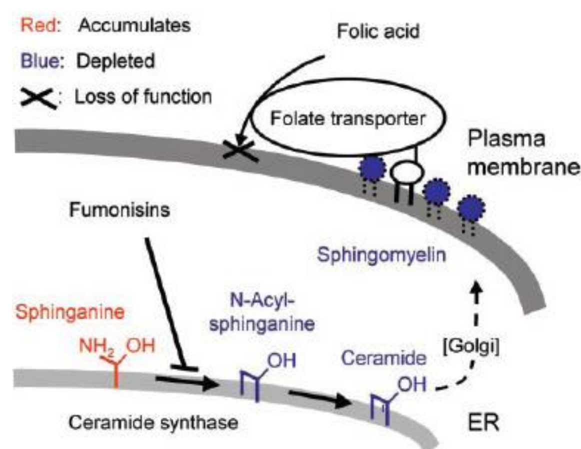

Fig. 1.11 Interference of fumonisin in folate transport, source Marasas et al. (2004).

1.12 Aims of the work

The discovery of this new form of fumonisin was hypothesized to be a plant's response to fungal infection and to the accumulation of toxic metabolites, a mechanism for detoxification that was just demonstrated in plant for some toxic metabolites.

These molecules are linked to matrix components, starch or protein, but there are different hypothesis on how these hidden fumonisins are linked, through a chemical bound or through a super structure.

The correlation of hidden fumonisin presence with some unsaturated fatty acids led to the hypothesis, after the recent discoveries about the plant-pathogen cross talk, that some signal molecules (oxylipins) could be involved in the masking mechanism.

The aims of this work were to:

- study the distribution of fungal infections, with particular attention to those caused by *Fusarium* species, and of fumonisins, both in free and hidden forms, during the growing season of different maize hybrids from different areas of Northern Italy;
- evaluate the changes in chemical composition and in lipidomic profile of different maize hybrids from different areas of Northern Italy, in relation to fumonisin contamination , both in free and masked forms, during the growing season;
- analyze the effects of post-harvest drying treatments on fungal incidence and on fumonisin contamination, both in free and hidden forms;
- evaluate the *in vitro* interactions between *Fusarium verticillioides* and maize kernels with particular attention to conidiation, colonization, fumonisin B_1 synthesis and the expression of oxylipin producing genes, involved in stress response;
- study the presence of fungal infections and of mycotoxin contaminations during storage of cereals in silo bags, a new low cost technology.

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CHAPTER 2

Maize lipids play a pivotal role in the fumonisin accumulation

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Maize lipids play a pivotal role in the fumonisin accumulation

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Abstract

The role of lipids in maize - *Fusarium verticillioides* interaction in field conditions was investigated. Ten maize hybrids were sampled at 4 growing stages from different fields located in North Italy. The chemical composition, the fungal incidence and the free and hidden fumonisin contamination were determined. Data obtained suggested a significant implication of fatty acids content in maize kernels in both fungal infection and toxin accumulation. An untargeted lipidomic analysis of maize kernels along the growing season and at harvest was performed. Combining mass spectrometry with the chemometric analysis, 7 compounds able to differentiate high-contaminated from low-contaminated samples were highlighted (cut-off: 2000 µg/Kg of fumonisin B1). Amongst them, the oxylipin 9-HODE and 3 sphingoid bases were identified. These results suggested that *F. verticillioides* and fumonisins could severely interfere with sphingolipid and oxylipin metabolism even in plants growing in field.

2.1 Introduction

Maize is a worldwide distributed crop, mainly destined to feed and food production, and it is considered one of the most susceptible to toxinogenic fungi, being particularly prone to mycotoxin accumulation (Woloshuk & Shim, 2013). Commonly, *Fusarium verticillioides* Sacc. (Nirenberg), belonging to *Fusarium* section Liseola, is considered the dominant toxigenic fungus in maize in temperate areas. Some of the disease caused by *F. verticillioides* infection in maize include seed rot, root rot, stalk rot, kernel or ear rot, and seedling blight (Cook, 1981). In addition, *F. verticillioides* is a well-known fumonisin producer, toxic compounds able to disrupt sphingoid bases metabolism in animals and plants (Williams et al., 2007). These compounds show a large variety of toxicological effects and are considered of concern for animal and human health (Stockmann-Juvala & Savolainen, 2008). In particular, FB_1 has been included in the class 2B by the International Agency for Research on Cancer because of its possible carcinogenic effect in humans (IARC, 1993). Moreover, the European Union has enforced the legislation to fix a threshold of FB_1+FB_2 content in raw maize and derived products destined to human consumption (EU Commission Regulation No. 1126/2007); recommendations have also been defined for feed intended for different animal species (EU Commission Regulation No. 576/2006).

A number of fumonisin analogues have been identified so far, like fumonisin B, C, A and P series, being among them FB_1, FB_2 and recently FB_3 generally found as dominant in maize (Rheeder et al. 2002). Recently, hidden forms of fumonisins belonging to group B have been detected in raw maize (Dall'Asta et al., 2009). These forms, probably bound or strongly associated with food components (i.e. proteins or starch), are of concern for food safety, since they potentially contribute to the total daily intake of mycotoxins after release during gastrointestinal digestion (Dall'Asta et al., 2010), but they are not detected by analytical methods commonly applied. The hidden forms of fumonisins may be detected only in an indirect way, through the

application of a hydrolysis step before the standard analysis (Dall’Asta et al., 2009; Scott, 2012).

Presence of both free and hidden fumonisins seems to be strongly linked to maize hybrid characteristics, in particular grain hardness (Blandino and Reyneri, 2008), season length (Battilani et al., 2008; Blandino and Reyneri, 2008; Loffler et al., 2010) and a_w dynamic during the growing season (Battilani et al., 2011).

Recent works have shown that the chemical composition of maize kernels can influence fumonisin content (Dall’Asta et al., 2012); according to this study, the distribution and the absolute amount of total fatty acids seem to be strongly related to fumonisin accumulation in maize under in field conditions. In particular, hybrids with higher linoleic acid content showed a higher fumonisin contamination, while hybrids with higher oleic to linoleic ratio showed a higher content in hidden fumonisins (Dall’Asta et al., 2012).

This behaviour was ascribed to the peculiar role played by fatty acids and their metabolites in the plant-pathogen cross-talk. Fatty acids are indeed precursors of oxylipins, compounds involved in plant-fungi interaction: an organized network of signaling cascade that synchronize oxylipin products was demonstrated in fungal tissues as a response to different environmental conditions; this fact could conduce to a “oxylipins signature profile” typical for every pathogen (Tsitsigiannis et al., 2007; Reverberi et al., 2010). Similarly, plants contain phyto-oxylipin pools conferring an oxylipin signature on a given organelle, tissue or plant (Blee', 2002; Camera et al., 2004).

Very recently, a lipidomic approach was applied to find out possible markers of different hybrids, notably referring to the growing season and the amount of fumonisins (Scala et al., 2013).

The aim of this study was thus to confirm the role of maize hybrids in fumonisin production and masking along the growing season and to further investigate the role of lipids in maize-*F. verticillioides* cross talk under in field conditions.

2.2 Materials and methods

2.2.1 Maize samples collection

In 2010, ten different maize hybrids were collected during the growing season in commercial fields grown in diverse districts of Northern Italy: Cremona (CR; 6 hybrids), Modena (MO; 2 hybrids) and Parma (PR; 2 hybrids). Hybrids with different season length, defined as FAO class, were considered: FAO 300 (short season; 1 hybrid), FAO 500 (3 hybrids), FAO 600 (2 hybrids), and FAO 700 (4 hybrids). Sample codification was given according to Dall'Asta et al. (2012): the hybrids already considered in the previous study are named with the same code (H1, H3, H8, H9), other hybrids are reported with increasing numbers (H11 – H16).

Sampling was carried out every two weeks, from early dough maturity up to harvest, 4 times in total; early dough maturity was reached between 4 and 6 weeks after silk emergence. Each field was divided into 3 sectors, each one sampled collecting ten ears following a "X shape" design; a total of thirty ears were sampled from each field and considered as a representative sample for the field.

After husk elimination, ears were shelled, kernels of each sample were mixed and a_w content was measured with an AquaLab lite (version 1.3 © Decagon Devices Inc.). Samples were divided in two sub-samples, destined to mycological and chemical analysis, and then immediately processed or stored at -20°C, respectively.

2.2.2 Incidence of infected kernels

Fifty kernels were randomly selected from each sample and surface disinfected in 1% sodium hypochlorite solution for 2 min and then in 90% ethanol solution for 2 min. Kernels were rinsed with sterile distilled water and dried under a sterile flowhood.

Grains were plated on Petri dishes (Ø 9 cm) containing Potato Dextrose Agar (PDA, Oxoid Ltd., Basingstoke, Hampshire, England) added with 0.1% streptomycin (Sigma-Aldrich) and incubated at 25 °C for 7 days with a 12 hours light/12 hours dark photoperiod.

After incubation, kernels showing mould development were counted. The identification of growing colonies at genus/section level was done for *Fusarium*, *Aspergillus* and *Penicillium* according to Summerell et al. (2003), Raper and Fennell (1965) and Pitt (1979), respectively. Data obtained were used to calculate both the incidence of all fungi and of each genus in plated kernels.

2.2.3 Chemicals

Fumonisins B_1, B_2 and B_3 standard solutions (a mixture of FB_1, FB_2 and FB_3, 50 µg/ml each, in acetonitrile/water, 1:1 v/v) were purchased from Romerlabs (Tulln, Austria). Hydrolysed fumonisins (HFB_1, HFB_2, HFB_3) were prepared according to Dall'Asta et al. (2012).

All solvents: diethyl ether, methanol, hexane and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA) while KOH was purchased from Carlo Erba (Milan, Italy).

For lipidomic analysis, the following chemicals were used. Methanol (MeOH), ethanol (EtOH), propanol (iPrOH), and hexane (Hex), were of HPLC/MS grade and were purchased from Merck (Darmstadt, Germany), whereas HPLC/MS grade ammonium formate ($HCOONH_4$) was purchased in granular form from Fluka (Buchs SG, Swiss). Authentic Heptadecanoic acid (MW 270.45 g/mol), used as the internal standard (IS), was purchased from Sigma-Aldrich (St Louis, USA). The IS was diluted in EtOH, at the final concentration of 50 µg/mL.

2.2.4 Proximate analyses

Dry matter on milled maize samples was obtained by drying 1 g of sample in a laboratory oven, maintaining the system at constant temperature (105°C) for 24 hours.

Total starch, total nitrogen, and total fat content as well as fatty acids profile were determined according to Dall'Asta et al. (2012).

For the rapid resolution reversed phase HPLC (RR-RP-HPLC) separation was used a Zorbax SB-C8 rapid resolution HT 2.1 x 100 mm 1.8 µm p.s (Agilent Technologies, Santa Clara, CA, USA). Chromatographic conditions for the targeted analysis of free fatty acid and some oxylipins were as reported in Scala et al., (2013). MS product ion scanning were processed using the Mass Hunter software (B.01.03 version). Relative abundance of each oxylipin was calculated and normalized to the internal standard recovery efficiency (heptadecanoic acid - 50 ng). Targeted mining of the HPLC/MS-ESI ToF data were performed with the molecular feature extraction (MFE) algorithm in the Mass Hunter^{TM} software.

2.2.5 Free and total fumonisin determination

Free and total fumonisin were determined according to our previous works (Dall'Asta et al. 2012).

Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed fumonisins B_1, B_2, and B_3. All the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolyzed compounds and referred to as "total fumonisins".

2.2.6 Lipidomic untargeted analysis

Free, conjugated and modified fatty acids were extracted as described by Stumpe et al. (2005) with slight modifications. An amount of maize kernels (100 g) were lyophilized and ground in liquid nitrogen. An aliquot of 20 mg was collected in a clean tube and added of 1 mL of the extraction medium (hexane: 2-propanol 3:2 v/v, containing 0.0025% butylated hydroxytoluene w/v) and 5 µL heptadecanoic acid standard solution 2 mg/ml in EtOH added as the internal standard reference as reported in Scala et al., (2013).

Separation and accurate mass measurements of lipid compounds was performed with a 1200 series rapid resolution HPLC coupled with a G6220A series time of flight mass spectrometer (ToF-MS, Agilent Technologies, CA, USA) equipped with an electrospray (ESI) interface operating in the negative ion mode. LC/MS-ESI ToF data were acquired and deconvoluted into individual chemical peaks using the Mass Hunter™ acquisition software. Untargeted and semi-targeted mining of the HPLC/MS-ESI ToF data were performed with the molecular feature extraction (MFE) algorithm in the Mass Hunter™ software. All the analysis were performed as previously described in Scala et al. (2013).

ESI tandem mass spectra were obtained with a G6410A series triple quadrupole (QqQ, Agilent Technologies, CA, USA). Data were acquired in both the negative and positive ion mode at unit mass resolving power by scanning ions between m/z 100 and m/z 800. MS spectra were averaged and processed with the Mass Hunter™ software. The HPLC-system was the same described above. The collision energy (CE) and the fragmentor voltage (FV) were adjusted to optimize product ion (PI) scan experiments. CE and F values were reported below with the respective spectral data.

The MFE algorithm was used to extract individual molecular species by their accurate mass detected with the ToF MS. Lists of molecular features, which were the detected species characterized by accurate mass, isotopic pattern and absolute abundance, were generated from each analyzed sample and converted into compound exchange files (CEF), which were then processed with Mass Profiler Professional (MPP) 12 (see below). Molecular features detected in the HPLC-MS system were aligned by their retention time (RT) in the chromatographic runs, and accurate mass axis in order to compare their expression across the different maize hybrids in different growth stages. Compounds detected in the different samples and presenting consistent RT (shift below 6 % of the RT) and accurate mass (mass error below 6 ppm) were assigned as the same molecular species. Relative abundance of individual features was obtained by normalizing their peak area by the area of the ISTD.

2.2.7 Statistical analysis

Data on the incidence of all fungi and *Fusarium*, *Aspergillus* and *Penicillium* separately were arcsine transformed before statistical analysis; this is appropriate for observations which are proportions (Fowler and Cohen, 1990). Data on fumonisin production were ln transformed before statistical analysis; this transformation is always required for data that covers a wide range from single-digit numbers to numbers in hundreds or thousands (Clewer and Scarisbrick, 2001).

ANOVA with Tukey's post hoc test was performed on all parameters determined in the maize samples using the statistical package SPSS (PASW ver. 18.0.0, 2009, Chicago, IL, USA).

Maize samples were grouped along the sampling time and according to their content of fumonisins. In detail, high (Hfb) and low (Lfb) tags were assigned for fumonisin content higher and lower than 4000 ppb, respectively. To compare the expression of lipid compounds in Hfb and Lfb, compounds occurring with 100% frequency in the biological replicates in the respective group were selected. Among all the metabolites, only those features consistently detected throughout all the analyzed maize samples were selected for further statistical analysis.

ANOVA with Tukey's post hoc test was performed on the entities detected with 100% frequency in the different samples. Fold changes of filtered entities were compared between Hfb and Lfb maize hybrids and significance was determined by T Student's test. Differential expression was evaluated at each sampling time, including harvest, and visualized by Volcano plots. Changes higher than 2 folds, with $p < 0.05$ after the Benjamini-Hochberg correction, were considered as significant. Principal components analysis (PCA) was then performed on entities filtered following grouping of samples according fumonisins amounts.

The statistical package SPSS (PASW ver. 18.0.0, 2009, Chicago, IL, USA) was used also for these data analysis.

Compound identification and annotation was performed using the METLIN Personal Metabolite Database by means of the ID browser tool, and the Molecular Formula

Generator algorithm. The LIPIDMAPS (http://www.lipidmaps.org/) database was used to infer compound identity. The annotation of free fatty acids (FAs) reported the number of carbon atoms and of double bonds. Other lipids were annotated consistently with the names reported on LIPIDMAPS.

2.3 Results

2.3.1 Chemical composition analysis

The chemical composition of 10 maize hybrids considered for this work was determined at harvest, as reported in Table 2.1. The same parameters were analysed also along the growing season, by sampling 4 different growth stages, and results were compared.

The chemical composition of maize hybrids was found to be constant during the growing season, with the only exception of total fat ($p < 0.005$) and, as a consequence, total fatty acids, even though their relative abundance, expressed as relative percentage on the total fatty acids, remained constant over the observation period (see Table 2.2).

2.3.2 Incidence of infected kernels and fumonisins content at harvest

All the hybrids tested showed a high incidence of fungal infection ranging from a minimum of 46% of H1 to a maximum of 100% of H3, H8 and H15, determined at harvest. The main fungal contamination was due to *Fusarium* spp. in all the hybrids with the only exception of hybrid 9 where *Aspergillus* spp. were dominant (Table 2.3).

Regarding fumonisin, the average contamination found at harvest, in all the samples analysed, was 3996 μg/Kg (range: 33 - 18819 μg/Kg) and 6541 μg/Kg (range: 202 – 22018 μg/Kg) for free FBs and total FBs, respectively. Hidden fumonisins, expressed as the difference between total and free fumonisins, were found in all the considered

samples with the exception of sample H9, ranging from 170 μg/Kg to 9977 μg/Kg with an average free-to-total FBs ratio of 0.48.

Along the growing season, fungal infection did not show significant variation from early dough stages to maize harvest. On the contrary, FB content varied significantly from stage 1 to stage 4. In particular, both for free and total FBs, samples collected at harvest resulted significantly more contaminated than those collected in earlier growth stages, as mean of all hybrids considered (see Table 2.2). Although contamination at harvest was higher than that determined at earlier stages for all the samples, hybrids considered within this study showed different trends of fungal infection and fumonisin accumulation along the growing season. As an example, the trends observed for H11 and H3 are reported in Fig. 2.1. H11 is characterised by a very low fumonisin level (free FBs ≤ LOQ at stage 4) in spite of a strong *Fusarium* infection rate all along the growing season (range: 94 – 100%). On the contrary, H3 showed very high FBs contamination (free FBs: 8839 μg/Kg at stage 4) with an average *Fusarium* incidence of 90% over the observation period.

2.3.3 Correlation between composition, lipid compounds and fumonisin contamination

All the data obtained from the proximate analysis and from the lipid determination performed for the 10 hybrids considered in this study at the 4 growth stages were critically compared by statistical correlation, to highlight their possible relation with fungal incidence and fumonisin accumulation.

In particular, data were compared by Spearman's correlation test ($\alpha < 0.05$). Total fungal incidence was negatively related to total linoleic (Rho = -0.438 , $p = 0.005$), and linolenic acids (Rho = -0.336 , $p = 0.034$).

Fusarium incidence was found to be significantly related neither with FB contamination nor with chemical composition. Very intriguingly, *Aspergillus* incidence was found to be positively related to free FBs content (Rho: 0.444; $p = 0.004$) and to all the considered fatty acids (p values ranging from 0.000 to 0.004).

This correlation, although still unclear, was already observed in previous studies (Dall'Asta et al., 2012).

Concerning mycotoxins, both free and total fumonisins were found to be positively related to all the total fatty acids considered within the study, showing Rho- and p-values in the range 0.349 – 0.999 and 0.000 – 0.027, respectively.

2.3.4 Untargeted lipidomic profile

The lipidomic profile of infected maize at all sampling time tested was studied, grouping hybrids according to their total fumonisin content, with a cut-off level of 2000 µg/Kg: high (Hfb; > 2000 µg/Kg) and low (Lfb; < 2000 µg/Kg) as indicated above (Table 2.3). The cut-off value was chosen basing on the first legal limit proposed in 2006 (European commission), later increased to 4000 µg/Kg due to a motivated stakeholders request (EC, 2007). Notably, H3, H8, H9, H12 and H15 were considered as Hfb whereas the others as Lfb (H1, , H11, H14 and H16). From the analysis, 2776 different entities (for exact mass and/or RT) were found and included in statistics. According to the PCA analysis, the first and the second components (X-Axis component 1: 28,24%; Y-Axis component 2: 9,62%) shared in two almost separated clusters the lipid metabolites formed along the sampling time. Compounds belonging to Hfb and Lfb maize hybrids were plotted in grey and black respectively (Fig. 2.2A), obtaining a significant agreement ($p< 0.05$) between the statistical clustering and the grouping criteria. The 2776 entities were then filtered according to their frequency in the different samples. Among all the metabolites, only those features consistently detected throughout all the analyzed maize samples were selected for further statistical analysis. ANOVA with Tukey's *post hoc* test was performed on the 302 entities detected with 100% frequency and 82 entities resulted to be significantly modified in the different hybrids. To determine the regulation of these entities, the condition Hfb and Lfb were compared with Volcano plotting with Student's T test and Benjamini-Hockberg correction (Fig. 2.2B). Differences in the lipid levels were evaluated in maize hybrids grouped according to the content of

fumonisin, independently on the sampling time. The Student's T test, visualized as a volcano plot, retrieved 5 compounds which were present at a significantly different extent in the two groups (Hfb and Lfb). In particular, these 5 entities were up-regulated in maize hybrids with the high content of fumonisins (Fig. 2.2B).

The same statistical approach was then applied to the samples collected at the harvest time, to better highlight possible differences between Hfb and Lfb. According to the PCA analysis, the first two components (X-Axis component 1: 26,46%; Y-Axis component 2: 15,79%) clustered the lipid metabolites in maize into two highly different subgroups, according to the contamination criteria (Fig. 2.3A). The data were then compared by Volcano Plot analysis (Fig. 2.3B), as previously described, selecting only those metabolites occurring with 100% frequency. The volcano plot, run on 314 lipid metabolites always occurring, retrieved 44 compounds expressed at significantly different extent ($p< 0.05$, Student't T test with Benjamini-Hochberg's correction). In particular 39 and 5 entities, different from previous analysis, were up and down-regulated, respectively, in maize hybrid with a high content of fumonisins (Fig. 2.3).

The 5 and 44 entities resulting from the above statistical analyses (PCA, ANOVA, Student's T test) were searched manually in the single HPLC-ToF/MS chromatograms to confirm their presence and abundance. Sixteen out of the searched 49 compounds were positively confirmed in all the samples and met the fold change threshold of 2. To attempt the identification of the selected lipid compounds, MS/MS fragmentation analysis was carried out and 7 compounds provided a clear fragmentation MS/MS spectrum. Fragmentation parameters, parent ions and daughter ions are reported in Table 2.4A.

Combining the MS/MS spectra and the elemental formula attributed on the basis of the accurate mass determined with the TOF MS analysis it was possible to speculate that the compound eluted at RT and detected as $[M-H]^-$ ion with m/z 295.2275 (match score 95.78%) could be attributed as the 9-hydroxyoctadecenoic acid (9-HODE), an oxylipin involved in maize - *F. verticillioides* interaction (Gao et al.,

2009). The identity of the tentatively assigned 9-HODE was confirmed by comparison of its MS/MS spectrum with that of the authentic compound analyzed in the same conditions (not shown). The entities detected at m/z 654,5974; 682,6408 and 696,6462, possibly corresponding to the [M-H]- ion, upon fragmentation produced MS/MS spectra consistent with sphingolipid species (Zitomer et al., 2009). In order to assign identity to these compounds, experiments in product ion scan (Table 2.4B) were performed in positive ion mode for the $[M+H]^+$ ions. Product ion scan of the $[M+H]^+$ ions with m/z 656, 684, and 698, share the daughter ions at m/z 60, 264, 282, 300 yield from the sphingoid moiety (Merrill Jr. et al., 2005; Zitomer et al., 2009; 2011). Fig. 2.5 reports the obtained MS/MS spectra.

2.4 Discussion

The work presented herein is the first study concerning the interaction between *F. verticillioides* and maize performed in field conditions. Several other studies have been carried out so far under *in vitro* conditions, pointing out the role played by oxylipins in the plant-pathogen cross-talk and in the modulation of mycotoxin accumulation in maize (Gao et al., 2007; McDonald et al., 2004). However, their major limitation is represented by the scarce overlap between the conditions applied and those experimented under natural field infection (Battilani et al., 2011; Jestoi et al., 2008). For this reason, the major aim of this work was the study of the factors affecting fumonisin modulation in several maize hybrids grown in the field and exposed to different/natural ecopathological conditions. In 2010, ten maize hybrids from commercial field located in Northern Italy have been monitored along the growing season, by collecting samples at 4 maturation stages starting from early dough maturity up to the harvest.

As far as the fungal incidence is concerned, all the hybrids considered within this study showed a strong infection rate already at the early dough maturity; this rate generally remained almost constant along the ripening period. On the other hand, fumonisin accumulation seems to follow an increasing trend reaching the maximum

level at harvest. These data are substantially in agreement with previous studies (Bush et al., 2004; Cao et al., 2013). *Aspergillus* spp. infection was found to occur in 5 out of 10 hybrids, but an incidence above 50% was reported only for H9, the hybrid where hidden fumonisins were not detected. This event should be considered in further studies because just observed in other field trials by the authors.

In agreement with previous studies (Dall'Asta et al., 2012), hidden fumonisins were found at significant levels in all the considered samples with exception of H9. As already reported (Dall'Asta et al., 2012; Falavigna et al., 2012), hidden fumonisins can be released as parental compounds under digestive conditions: accordingly, these data are an important confirmation of the necessary monitoring of both free and total fumonisins to get a reliable evaluation of their overall content in maize.

Concerning the trend of mycotoxin accumulation along the growing season, different hybrids showed very different behavior. According to Fig. 2.1, hybrids grown in the same area and showing a similar *Fusarium* incidence differed significantly in fumonisin amount, thus indicating that the choice of the hybrid as well as the ecophysiological conditions played an important role in regulating fumonisin biosynthesis *in planta*. Maize genotypes can be differentiated by their agronomical characteristics, but often also by their chemical composition, that influences not only their technological behavior but also their resistance towards pathogens and/or insects (Battilani et al., 2008; 2011; Blandino and Reyneri, 2008; Loffler et al., 2010). In a previous study, the key role played by fatty acids in the regulation of fumonisin production in maize has been suggested (Dall'Asta et al., 2012). This observation is confirmed by the data reported herein, as total fatty acid amount were found to be positively related to fungal infection and fumonisin accumulation ($p < 0.005$ for all the considered variables). The total fat amount significantly increased during the observation period and showed significant differences between genotypes, while the contribute of each fatty acid remained unvaried during the ripening period considered. Unsaturated fatty acids have been frequently described as modulators of plant resistance pathway upon pathogen attack, even though their profile is strongly

influenced by the environmental conditions experienced by plants during the flowering/growing period (Kachroo et al., 2009). Polyunsaturated FAs, released from membranes by lipases in response to attacks by biotic agents, play a key role in plant-pathogen interaction either directly as free FAs or as precursors of oxylipins (Walley et al., 2013). As an example, linoleic acid levels contribute to the regulation of development, seed colonization, and mycotoxin production in *Aspergillus* spp. (Calvo et al., 1999). FA such as linoleic acid and some of its oxylipin-derivatives [e.g. 9-H(P)ODE] produced by host (e.g. maize) in response to the attack of a mycotoxigenic fungus may trigger the synthesis of mycotoxins such as aflatoxins (Burow et al., 1997) and fumonisins (Gao et al., 2009). In relation to this, some authors suggest 9-oxylipins as mycotoxin-susceptibility factors. In fact, the constitutive and/or induced presence of such fatty acids-derived molecules in the host plant, stimulates the production of mycotoxins (Christensen and Kolomiets, 2011) even if the role of these toxic compounds *pro* infection onset remains to be clarified.

In our study, further analysis for better evidencing lipid markers eventually involved in fungal development and mycotoxin contamination have been performed using an untargeted approach followed by chemometric data analysis. The analysis carried out by considering the lipid compounds detected in hybrid grains along the whole ripening season and at harvest lead us to identify 7 compounds which provided a clear fragmentation MS/MS spectrum. Amongst these, it was possible to recognize an oxylipin, 9-HODE and 3 sphingoid bases. As reported above, 9-HODE is known as mycotoxin-susceptibility factor, since in several plant-fungus interaction systems it was confirmed as crucial for mycotoxin production (Christensen and Kolomiets, 2011); in particular, Gao et al. (2007) reported that in a 9-oxylipin-deficient maize mutant fumonisin B_1 produced by *F. verticillioides* was reduced by 200-fold. Thus, our study confirms that 9-HODE can be produced under field conditions by maize ears during ripening and it is present in an higher amount in those hybrids which better support fumonisin synthesis. This is the first report which describes and pinpoints that naturally occurring *F. verticillioides* contamination of maize leads to

higher fumonisins production in those hybrids in which the synthesis of 9-HODE is triggered.

Fumonisins are potent inhibitors of ceramide synthase(s) (CerS) which biosynthesizes, *de novo* and by recycling pathways, sphingolipids (Pewzner-Jung et al., 2006). In relation to this direct inhibition, sphinganine (Sa) and sphingosine (So) (at a lesser extent) accumulate, whereas *N*-acylsphinganines (dihydroceramides), *N*-acylsphingosines (ceramides, Cer) and more complex sphingolipids decrease into FB_1-challenged cells (Riley et al., 2001; Zitomer and Riley, 2011). Whilst sphingolipid metabolism disruption in mammals has been proposed to be responsible for FB toxicity and possibly carcinogenicity (Merrill et al., 2001), its role in maize and in general in plant-pathogen interaction remains elusive. As a matter of fact, fumonisins significantly alter sphingolipid metabolism even in maize cell cultures (Zitomer and Riley, 2011), thus a role for this metabolites in affecting plant response to pathogen infection can be suggested. In our study, we firstly demonstrate the accumulation of 3 sphingolipids into maize kernels heavily (>2000ppm) and naturally contaminated with fumonisins. These results, suggest that fumonisins may severely interfere with sphingolipid metabolism even *in vivo.* If sphingolipid synthesis alteration is causal or a mere effect of *F. verticillioides* and fumonisins contamination remains to be ascertained. From the studies available, the plant ceramides are involved in signaling pathways that promote hypersensitive response and associated programmed cell death in plants (Buré et al., 2013; Markham et al., 2013). In relation to this, fumonisins, which have not a clear cut role in pathogenicity (Proctor et al., 2013), may help the pathogen in its competition with other maize-feeder pests and/or pathogens.

We may suggest that the specific lipidomic profile in Hfb maize hybrids, with particular regard to oxylipins and sphingolipids, may affect the production of fumonisins by *F. verticillioides*.

Table 2.1 Characterization of maize hybrids from Northern Italy, analysed at harvest: chemical composition (mean ± standard deviation, n = 3 technical replicates).

Code	Harvest Area	FAO	Proximate composition (%)				Fatty acids (g/100 g d.m.)				
			Moisture	Starch	Proteins	Fat	C16:0	C18:0	C18:1	C18:2	C18:3
H1	MO	300	34.1±0.1	68.5±1.2	6.3±0.5	3.4±0.2	11.9±0.1	2.1±0.1	31.4±0.1	54.0±1.3	0.5±0.0
H3	CR	600	22.3±0.2	77.9±1.4	6.4±0.2	3.2±0.1	9.8±0.2	7.3±0.3	22.6±0.2	59.6±1.5	0.7±0.1
H8	CR	600	21.1±0.2	72.1±0.9	6.4±0.3	3.1±0.3	15.7±0.2	1.9±0.2	26.4±0.3	55.5±2.1	0.5±0.0
H9	PR	500	18.4±0.1	76.0±1.2	6.1±0.8	3.0±0.3	14.3±0.6	1.8±0.1	34.9±0.2	48.7±1.8	0.4±0.0
H11	MO	500	30.0±0.1	74.0±1.8	6.3±0.3	2.9±0.2	11.9±0.3	2.2±0.5	29.4±0.2	56.0±1.7	0.4±0.0
H12	PR	500	21.4±0.3	67.5±1.6	6.4±0.3	2.6±0.2	12.2±0.7	1.6±0.5	31.9±0.3	53.4±2.3	0.9±0.1
H13	CR	700	20.6±0.2	77.2±1.2	6.6±0.5	2.1±0.1	13.5±0.4	1.2±0.4	26.9±0.3	57.6±2.2	0.8±0.1
H14	CR	700	24.1±0.2	74.8±1.3	6.7±0.4	3.7±0.3	11.2±0.6	1.6±0.4	25.4±0.2	61.1±1.5	0.7±0.1
H15	CR	700	24.1±0.1	72.3±0.9	6.5±0.2	2.9±0.2	11.7±0.4	1.6±0.2	28.0±0.2	58.1±1.3	0.5±0.2
H16	CR	700	25.4±0.3	77.2±1.1	6.7±0.3	3.0±0.2	10.8±0.3	1.7±0.2	26.2±0.1	60.7±1.3	0.6±0.1

Table 2.2 Results of ANOVA for all the parameters considered. Only significant differences were reported and each value is a mean value of the 10 maize hybrids considered (n = 10 biological replicates). Different letters indicate significant differences.

Stage	Free FBs (μg/Kg)		Total FBs (μg/Kg)		Total Fat (g/100g d.m.)		Total C18:0 (g/100g d.m.)		Total C18:1 (g/100g d.m.)		Total C18:2 (g/100g d.m.)		Total C18:3 (g/100g d.m.)	
1	323	b	1058	b	0.94	b	0.01	b	0.27	b	0.52	b	0.00	b
2	1766	ab	2428	b	1.63	ab	0.03	b	0.43	ab	0.90	ab	0.01	b
3	2136	ab	3502	ab	2.49	ab	0.04	ab	0.70	ab	1.42	ab	0.01	ab
4	3996	a	6541	a	2.99	a	0.07	a	0.85	a	1.68	a	0.02	a
significance[ł]	**		**		**		**		**		**		**	

[ł] * significant with p<0.05; **: significant with p<0.01

Table 2.3 Infection and fumonisin accumulation in maize hybrids from Northern Italy, determined at the harvest. Data are expressed as mean ± standard deviation (n = 3 technical replicates).

Code	**Total fungi**	***Fusarium***	***Aspergillus***	**Free FBs**	**Total FBs**
	Infection rate (%)			µg/Kg	
H1	46	40	4	495 ± 25	934 ± 47
H3	100	90	0	8839 ± 442	18815 ± 941
H8	100	58	0	2388 ± 120	5580 ± 279
H9	82	4	50	3623 ± 182	3636 ± 181
H11	78	78	0	< LOQ	709 ± 35
H12	72	38	2	18819 ± 941	22019 ± 1101
H13	80	64	0	1333 ± 67	3140 ± 157
H14	88	56	2	< LOQ	202 ± 10
H15	100	92	0	3523 ± 176	8678 ± 434
H16	74	58	6	859 ± 43	1714 ± 86

Table 2.4 A) HPLC-MS/MS data of lipid compounds resulted differentially expressed in Hfb, notably up-regulated, compared to Lfb maize hybrids along the growing season and at harvest. A threshold of 2.0 fold was set as minimum difference in lipid compound expression in the two groups. Spectra were acquired in negative ion mode. (CE: collision energy; FV: fragmentor voltage Q1 – Q3: typical daughter ions obtained from parent ion fragmentation). B) Product ion scan of the $[M+H]^+$ ions with m/z 656, 684, and 698 by LC-MS/MS.

A)

Retention time (min)	CE (mV)	FV	m/z	Q1	Q2	Q3	Identification
1.56	16	140	295.2	194.9	277.0	-	9-hydroxyoctadecenoic acid
18.09	34	140	654.6	410.5	355.5	309.6	Cer (t18:0/22:0(2OH))
18.69	38	140	682.0	337.2	383	438.3	Cer (t18:0/24:0(2OH))
18.63	42	140	527.4	257.3	274.9	231.0	n.i.*
18.37	10	50	587.5	493.6	113.0	215.4	n.i. *
8.90	20	140	561.4	270.1	-	-	n.i. *
17.82	20	140	696.5	372.4	457.0	-	Cer (t18:0/26:0)

* Not yet identified

B)

Parent ion $[M+H]^+$	CE	Product ions	Neutral Loss ($RCONH_2$)
656,4	28	60.2; 264,3; 282,4; 300,4	356
684,2	34	60.2;252,3; 264,3; 282,2; 300,4	384
698,2	28	60.2; 264,3; 282,3; 300,4	398

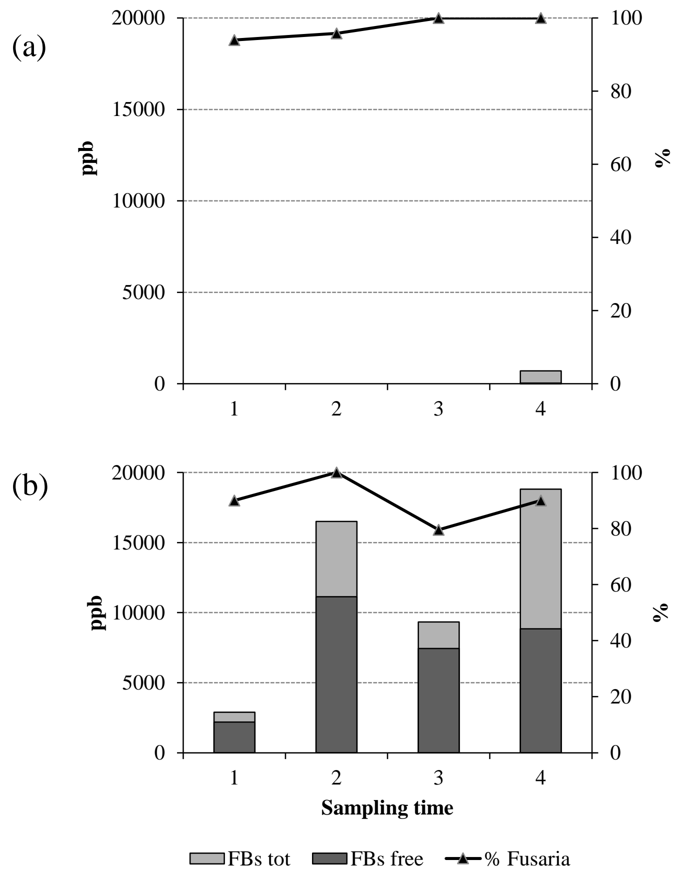

Fig. 2.1 Example of different trends in *Fusarium* incidence and fumonisin accumulation obtained along the growing season: (a) Hybrid 11; (b) Hybrid 3.

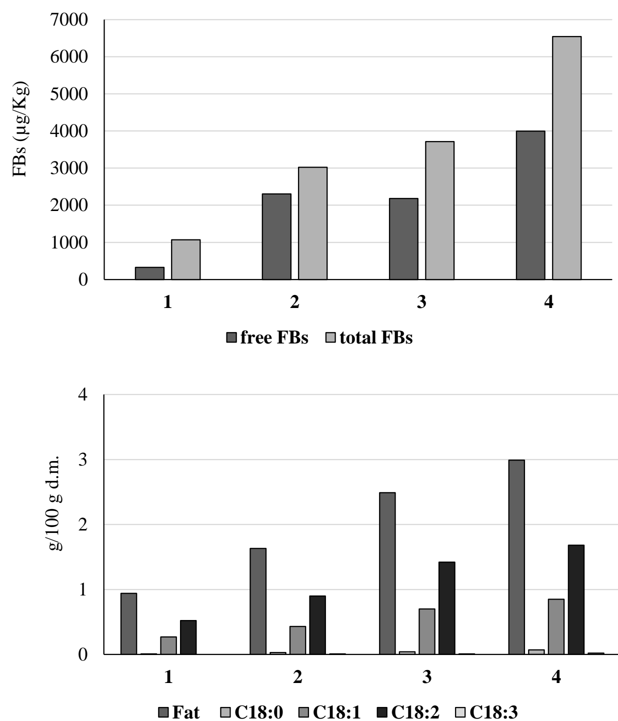

Fig. 2.2 Trends observed in fumonisin contamination, target oxylipins and total fatty acids levels during the growing season. Data are expressed as mean of 10 different samples.

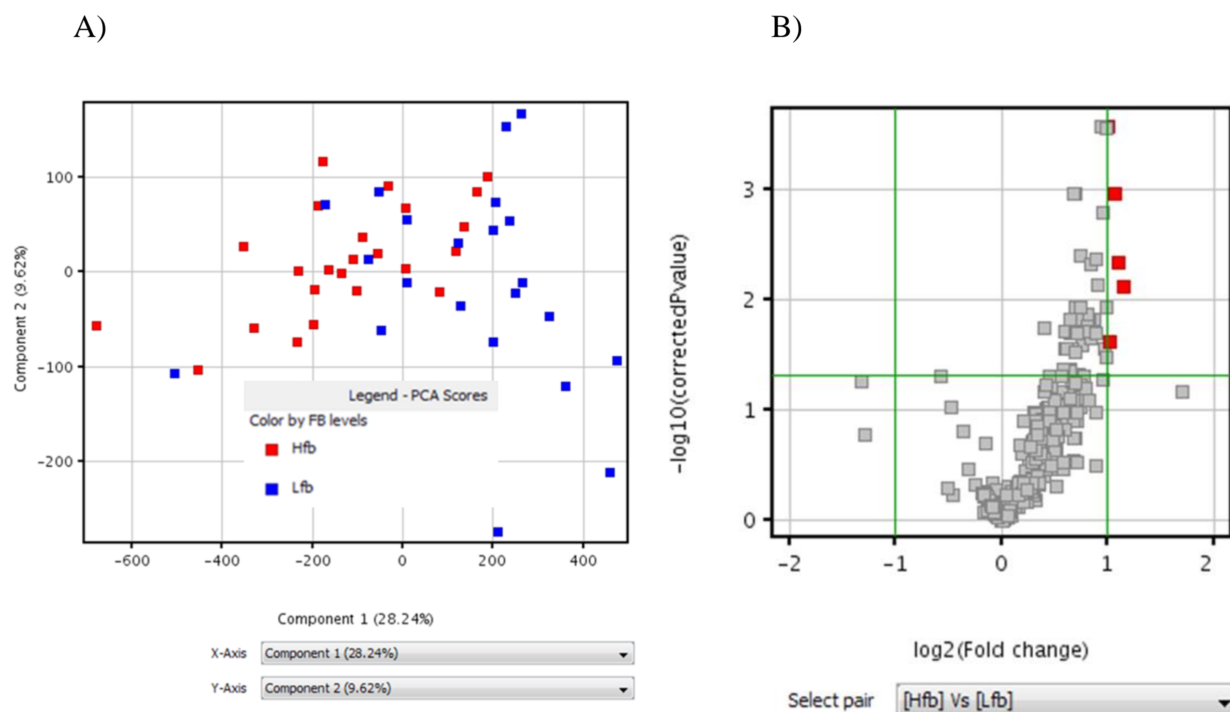

Fig. 2.3 A) PCA score plot of data generated by HPLC-ESI/ TOF-MS analysis of maize hybrids along the sampling time and grouped according to the fumonisins content. Blue square represent the samples with low content of fumonisins (Lfb), the red square the samples with high content of fumonisins (Hfb). B) Volcano plot analysis of 82 entities present in maize hybrids at all the sampling time. On X axis is reported the fold change to indicate the abundance variation of compounds produced by maize hybrids at all the sampling time with an high content of fumonisins (Hfb) compared to maize hybrids with low content of fumonisins (Lfb). The species on the right side were more abundant, whereas those on left side were less abundant in Hfb.

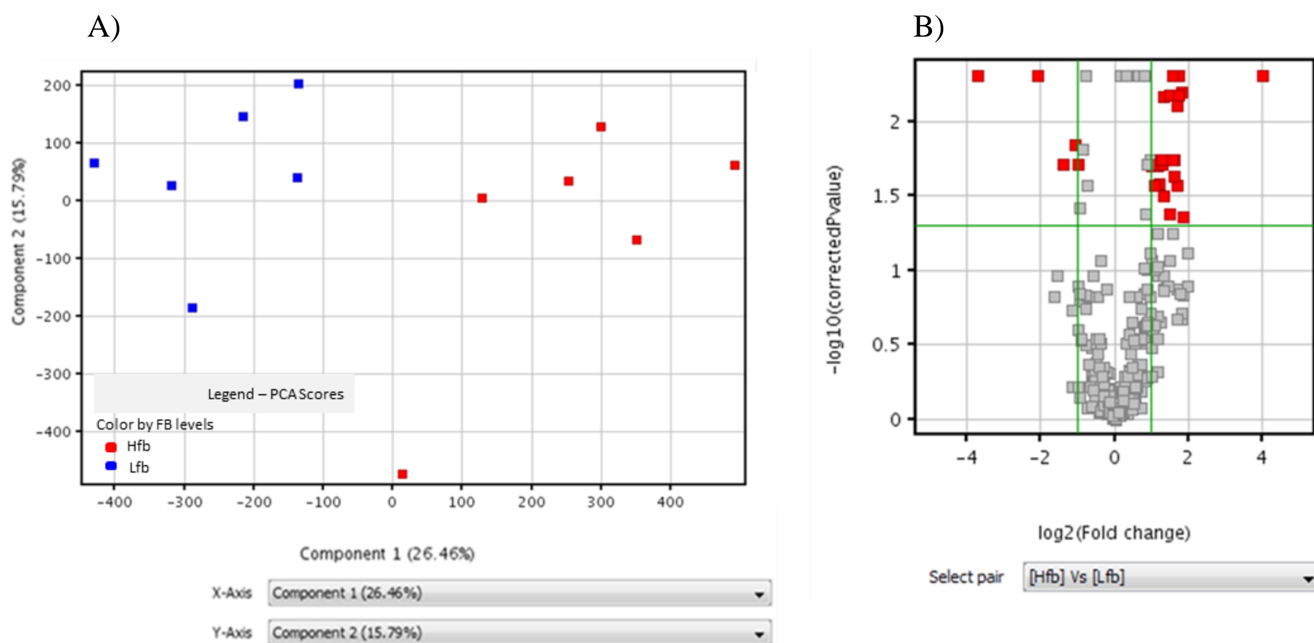

Fig. 2.4 A) PCA score plot of data generated by HPLC-ESI/ TOF-MS analysis of maize hybrids at harvest time and grouped according to the fumonisins content. Blue square represent the samples with the low content of fumonisins (Lfb), the red square the samples with the high content of fumonisins (Hfb). B) Volcano plot analysis of 314 entities present in maize hybrid at harvest time and grouped according to the content of fumonisin. On X axis is reported the fold change to indicate the abundance variation of compounds produced by maize hybrids at harvest time with an high content of fumonisin compared to maize samples at harvest time with low content of fumonisin. The species on the right side were more abundant, whereas those on left side were less abundant in maize samples at harvest time with an high content of fumonisin.

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CHAPTER 3

Fusarium verticillioides-maize kernel ecosystem: conidiation, colonization, fumonisin B_1 production and gene expression by kernel assay

In preparation, Molecular Plant Pathology

Fusarium verticillioides-maize kernel ecosystem: conidiation, colonization, fumonisin B_1 production and gene expression by kernel assay

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Abstract

Maize is the third cultivated cereals worldwide, and is known to be susceptible to the attack of different pathogens and insects in field during the growing season that can cause yield losses, both in term of quality and quantity.

Many laboratories have conducted *in vitro* experiments devoted to the comprehension of mechanisms involved in plant response to fungal infection and mycotoxin contamination, but results are often not comparable. In this study, a proposed standardized method for quantifying different biological parameters (fungal growth, biomass and mycotoxin contamination) has been used in the ecosystem *Fusarium verticillioides*-maize kernel.

Superficially sterilized maize kernels have been inoculated in glass vials with a fungal spore suspension (10^6 spores/mL), incubated for 15 days and analysed every three days. Results showed that conidia and fumonisin B_1 production increased till day12, while the maximum in ergosterol synthesis was reached on day 9. Gene activation showed that all the tested plant genes for oxylipin production were overexpressed respect to the chosen reference gene during pathogen infection. Also fungal genes for oxylipin production are active during incubation, even if these genes were under expressed referring to the chosen reference gene.

3.1 Introduction

Fusarium verticillioides is one of the most important maize pathogens worldwide. Besides macerating the seed upon colonization and thus reducing yield, this pathogen produces one of the most harmful mycotoxins, fumonisins, secondary metabolites that cause a range of species-specific toxic effects on humans or animals after the ingestion of contaminated food or feed. Many environmental factors can affect fungal ability to colonize the host, reproduce via production of conidia, secondary metabolism, and fumonisin synthesis. Many environmental factors play a key role in inhibiting or promoting fungal metabolisms. In particular, temperature, water status of the substrate and its pH, nutrients availability and competition with other pathogens are major driving forces regulating fungal metabolism.

Recent studies have demonstrated the role of some oxidized lipid molecules, produced by the plant and the pathogen, that may act as signals in plant-pathogen ecosystems (Blée, 2002; Christensen and Kolomiets, 2011; Gao and Kolomiets, 2009; Tsitsigiannis and Keller, 2007). This molecules are produced via oxidation of polyunsaturated fatty acids (PUFA) through the action of dioxygenases like lipoxygenases or linoleate diol synthases present in the plant and in the fungus (Andreou et al., 2009; Camera et al., 2004; Feussner and Wasternack, 2002; Mosblech et al., 2009). Once produced by the plant, these molecules can mimic the physiological roles of fungal endogenous oxylipins that have been shown in numerous studies to regulate fungal growth, development, sporogenesis and mycotoxin biosynthesis, favoring or inhibiting these processes. In particular, studies showed that plant oxylipins have an either negative or positive effect on conidia production (Brodhagen and Keller, 2006; Tsitsigiannis and Keller, 2006) or regulate the ratio of sexual/asexual spores produced by the fungus (Tsitsigiannis et al., 2004a; Tsitsigiannis et al., 2004b), as well as biosynthesis of mycotoxins (Burow et al., 1997; Gao et al., 2009; Roze et al., 2007) and *quorum sensing* (Affeldt et al., 2012).

In this study, we applied the kernel assay developed by Christensen et al. (2012) primarily for *Aspergillus flavus*, another mycotoxin producing fungus that infects

corn seeds, to extend our understanding on the interaction between living kernels and *F. verticillioides* in terms of timing and kinetics of pathogenic and developmental processes such as vegetative growth and colonization of kernels, production of conidia and fumonisins while simultaneously monitoring the expression of both fungal and plant dioxygenases. Since the majority of published studies focused on the fungal development and secondary metabolism using dead kernels, this study was designed to shed light at temporal changes in the physiological processes of both the host and the pathogen, and even more importantly to identify the timing of when the pathogen kills the host cell and switches to the saprophytic stage in the disease cycle. Fungal colonization was monitored by measuring accumulation of fungal specific complex membrane lipid ergosterol, enumeration of conidia, , and fumonisin B1 production. Expression of several selected lipoxygenase genes of maize and fungal fatty acid dioxygenases including a lipoxygenase and linoleate diol synthase during 15 days of incubation time.

3.2 Materials and Methods

3.2.1 Chemicals

All the chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.) with exception for phenol:chloroform=1:1 (pH 4.3) purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

3.2.2 Spores suspension preparation

Fusarium verticillioides strain 7600 (also known as M3125, Fungal Genetics Stock Center, Kansas City, KS, USA) was grown in Petri plates containing Potato Dextrose Agar (PDA; Difco, Sparks, Maryland) at 28°C for 1 week. Five mL of autoclaved 0.1% Tween-20 solution was added, the surface was scraped and the inoculum suspension was diluted with autoclaved 0.1% Tween-20 and filtered through 4 layers of autoclaved cheesecloth. Spore concentration was adjusted to 10^6 spores/mL.

3.2.3 Kernels selection and preparation

Kernels of maize B73 inbred line, susceptible to *F. verticillioides* ear rotting and fumonisin contamination, were used in this study. An effort was made to select the kernels of similar shape and size, preferably flat, produced in the same geographic area during the single growing season. Kernel assays were essentially carried out as described by Christensen et al. (2012). Shortly, kernels were placed into a 200 mL flask, surface sterilized with 70% ethanol by shacking at room temperature for 5 min, 1 min with sterile water and 10 min with 6% sodium hypochlorite. Finally, they were rinsed three times with distilled sterile water by shaking for 5 min each time, and dried at room temperature on autoclaved towels in sterile conditions. In order to facilitate fungal infection, the embryo side was wounded at a depth of 0.5 mm using a sterilized razor blade.

3.2.4 Sample inoculation

Groups of 4 kernels were placed in autoclaved 20 mL glass scintillation vials and their weight were recorded. The kernels were inoculated with 200 µL of a spore suspension containing 10^4 spores/mL, capped and mixed by Vortex® for 10 sec.

A humidity chamber was prepared by placing 5 autoclaved sheets of paper towel in a plastic container, to which 100 mL of distilled sterile water was added. All kernels were placed in the vials with the embryo side up to avoid difference in growth caused by the differential colonization of diverse kernel tissues. The caps were loosened to allow air exchange and the vials were placed in the humidity chamber.

The chamber was not air- or water-tight and additional 50 µL of sterile distilled water were daily added to each vial throughout the experiment to make sure that a high moisture level was maintained during the entire time course.

The samples were incubated under 12h light/12h dark photoperiod at 28ºC for 3, 6, 9, 12 and 15 days. At every time point, samples were frozen at -80ºC until used in the analysis.

3.2.5 Conidia counting

Four vials with kernels were used as replicates for conidia counting. Two and a half mL of methanol were added to each vial and mixed thoroughly using a Vortex®. One hundred µL of spore suspension were extracted two times as technical replicates and diluted in a tube (Eppendorf, Hamburg, Germany) with 100 µL of sterile distilled water. Each 200 µL aliquot was counted twice with a hemocytometer.

3.2.6 Ergosterol analysis

Four vials with kernels were used as replicates for ergosterol analysis. To extract ergosterol, 2.5 mL of methanol and 5 mL of chloroform (1:2 v/v) were added to each vial and mixed for 10 sec followed by incubation in the dark for 24 hours at room temperature.

Then, the extracts were filtered through a 0.45 µm nylon membrane and placed in analytical amber vials. The samples were injected directly into a HPLC LC-20AT system (Shimadzu, Kyoto, Japan) equipped with a 4.6 U ODS-C18 column (200 Å, 250 ± 4.6 mm) and a Shimadzu SPD-20A UV/VIS detector set to monitor at 282 ηm. Methanol (100%) at flow rate of 1.5 mL/min was used as mobile phase. A comparison between peak areas of samples to a standard curve generated from HPLC-grade ergosterol was used to quantify ergosterol in the samples.

3.2.7 Fumonisin B_1 analysis

Four vials with kernels were used as biological replicates for fumonisin B1 analysis. Fumonisin B1 was extracted in 10 mL of acetonitrile (ACN)/deionized water (DW) (50/50, v/v) overnight, at room temperature without agitation. Subsequently, 2 mL of the extract were mixed with 6 mL of DW and applied directly to the C-18 solid phase extraction column.

Before loading the samples, the column was preconditioned by rising with 2 mL of ACN (100%) followed by 2 mL of DW.

After loading the samples, the column was washed with 2 mL of DW followed by 2 mL of ACN/DW (15/85, v/v). Samples for HPLC analysis (containing FB_1) were then eluted with 2 mL of ACN/DW (70/30, v/v).

FB_1 was derivatized with o-phthaldehyde (OPA) by transferring 0.1 mL of the column eluate to a vial with 0.1 mL borate buffer (0.05 M boric acid/0.05 M sodium borate [50/50, v/v], pH8.5) and 0.1 mL OPA (0.1mg/mL in ACN with 0.5% mercaptoethanol). After 10 min the reaction was stopped by adding 0.5 mL of ACN/0.01 M boric acid (40/60, v/v).

FB_1 was then analyzed on a HPLC LC-20AT (Shimadzu, Kyoto, Japan) system equipped with an analytical Zorbax ODS column (4.6 150 mm) and a variable wavelength Shimadzu RF-10Axl fluorescence detector (excitation 335ηm/emission 440ηm). A linear gradient was used (solvent A: ACN/0.1 M sodium phosphate (40/60), pH 3.3; solvent B: ACN/0.1 M sodium phosphate (60/40) , pH 3.3) as follows: 100% A to 100% B in 10 min, 100% B for 5 min.

Standards of FB_1 were analyzed by HPLC and peak area measurements were used to generate a standard curve and, subsequently, to quantify the FB_1 level in a sample by comparing peak area with the standard curve.

3.2.8 RNA extraction and gene expression analysis

Samples were ground in liquid nitrogen to obtain a very fine powder and 100 mg of powder were placed into a 2 mL tube (Eppendorf, Hamburg, Germany), preventively frozen in liquid nitrogen. Two hundred µL of kernel extraction buffer (50 mM TRIS pH 8.0, 150 mM LiCl, 5mM EDTA pH 8.0, 1% SDS – all stocks made on DEPC treated water) and 200 µL of phenol:chloroform=1:1 (pH 4.3) were added to each tube (Eppendorf, Hamburg, Germany), immediately mixed with Vortex® until thawed, and incubated on ice for 5 min. The mixture were decanted into 1.5 mL PHASE LOCK tubes (Eppendorf, Hamburg, Germany) and centrifuged at 10,000 RPM for 10 min at 4°C. Two hundred µL of phenol: chloroform=1:1 (pH 4.3) were added a second time to each PHASE LOCK tube, mixed with Vortex®, incubated on

ice for 5 min and centrifuged at 10,000 RPM for 10 min at 4°C. Two hundred µL of chloroform (pH 4.3) were added to each tube, mixed with Vortex®, incubated on ice for 5 min and centrifuged at 10,000 RPM for 10 min at 4°C. One hunfred µL of supernatant were removed and placed into new 1.5 mL tubes and 350 µL of Qiagen RTL Buffer (Qiagen, Hilden, Germany) were added and thoroughly mixed. Then, 250 µL of 100% ethanol were added to each tube and thoroughly mixed. The entire content of each tube (700 µL) was transferred into Qiagen RNA spin-column (Qiagen, Hilden, Germany), and centrifuged at 10,000 RPM for 15 sec at room temperature and the filtered solution discarded. Five hundred µL of Qiagen Buffer RPE (Qiagen, Hilden, Germany) were added and spun at 10,000 RPM for 15 sec at room temperature, the filtered solution was discarded. Five hundred µL of Qiagen Buffer RPE (Qiagen, Hilden, Germany) were added and spun at 10,000 RPM for 2 min at room temperature and the filtered solution discarded. The spin columns were then placed into clean collection tubes and spun at 10,000 for 1 min at room temperature. Finally, the spin columns were placed into new 1.5 mL microcentrifuge tubes, 40 µl of Qiagen RNase-free water (Qiagen, Hilden, Germany) were added and spun at 10,000 RPM for 1 min at room temperature. The microcentrifuge tubes were then heated at 65°C for 5 min to stop the enzyme. The amount and quantity of extracted RNA was estimated with Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

The extracted RNA was purified following the DNASE I RNASE-FREE kit protocol (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).For quantitative reverse transcription-PCR (qPCR) assays, a one-step qPCR procedure was performed using Thermo Scientific Verso One-Step RT-qPCR Kits (Thermo Scientific, Waltha, MA, U.S.A). Reactions were optimized for RNA and primer concentrations with each 10 µl reaction consisting of 40 ng of DNase-free RNA and 200 nM primers. qPCRanalysis was performed with an Applied Biosystem StepOnePlus Real-Time PCR instrument. Primers used in this study are described in Supplementary Table 4.1. The PCR program consisted of a 15 min cDNA synthesis step at 50°C, followed by

polymerase activation step of 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30s at 54°C (*FLOX1* and *MLOX1*) or 30s at 60°C (*LDS1*, *LDS2*, *LOX2*, *LOX3*, *LOX4*, *LOX5* and *LOX12*), and 30s at 72°C followed by a melt curve analysis (60 or 54°C to 95 °C). Primers were designed using Primer3Plussoftware in accordance with the criteria required for quantitative PCR primer design (Udvardi et al., 2008) or taken from the literature (Scala et al., 2013). The specificity of primers, lack of primer-dimer formation, and the absence of contaminating genomic DNA was verified, respectively, using amplicon dissociation curves, PCR in the absence of cDNA, and by PCR analysis of RNA samples before reverse transcription.

3.2.9 Data analysis

Analysis of variance was applied to conidiation, colonization and fumonisin production data; Tuckey's post hoc test was used to determine statistically significant differences. Pearson's correlation coefficient between all parameters considered was calculated.

Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin #2 ABI PRISM 7700 Sequence Detection System). All data were normalized to β-tubulin and Cullin as housekeeping gene, respectively, for *F. verticillioides* and maize (Real-Time PCR Application Guide, Bio-Rad, Hercules CA). The expression of the target gene corresponds to the fold increase (or decrease) of the gene in the sample compared to the calibrator sample (day 0), or to the previous time point.

All data analysis was managed using SPSS (PASW ver. 18.0.0, 2009, Chicago, IL, USA).

3.3 Results

3.3.1 Kinetics of kernel colonization, sporulation and fumonisin production

The vegetative growth of *F. verticillioides* on maize kernels was visible since day 3 and it became more abundant along the incubation period until 15 days after inoculation (Fig. 3.1).

Conidia production showed a significant increase during the incubation period, from 15 to 2275 x 10^6 spores/g kernel, with the maximum value reached at day 12, comparable to sporulation at day 15 (Fig. 4.2 A). Pearson's coefficient showed a strong correlation between incubation time and conidia production (0.92) (Table 3.3).

Kernel colonization by *F. verticillioides*, reported as μg of ergosterol per g of kernels, significantly increased until day 9 then significantly decreased at 12 dpi and remained at a similar level at 15 dpi (Fig. 3.2 B). However, when conidia production was calculated based on fungal biomass as measured by ergosterol levels, both referred to g of kernels, a continuous increase was observed until the end of the incubation period, with significant differences observed until 12 dpi (Fig. 3.2 C). Time affected both colonization and fungal biomass-dependent conidiation.

Fumonisin B1 was detected at each time point but a significant increase was noticed between day 6 and 9 and between day 9 and 12 (Fig. 3.2 D). The biomass-dependent fumonisin B1 production showed a persistent increase throughout the entire incubation time, with a significant difference between day 6 and 9 and day 9 and 12 (Fig. 3.2 E). Pearson's coefficient highlighted a strong correlation between FB_1 synthesis and time (0.903) but it was also strongly correlated with conidia production (0.912) (Table 3.3).

When conidiation, fungal biomass and fumonisin B1 production along the incubation period were rated on the respective maximum values, it clearly showed that the ability to produce spores and fumonisin B1 had a very similar pattern and both spore number and mycotoxin content reached maximum levels at those time points coinciding with the optimal fungal growth (Fig. 3.2 F).

3.3.2 Plant and fungal gene expression during seed colonization

Plant: the molecular analysis showed that the maize housekeeping gene cullin is not expressed after 9 dpi providing for the first time an important molecular evidence that seeds were completely colonized by the fungus between day 9 and 12 dpi. Therefore, gene expression was not measured at day 15 when seeds were evidently dead.

Gene expression analysis showed that maize *LOX1*, *LOX2* and *LOX4*, *LOX5* and *LOX12* were all highly expressed in response to infection with the most rapid and strongest suppression observed for *LOX1* starting at 3 dpi (over 10 fold induction), followed by a similar pattern observed for *LOX2* transcripts, while *LOX5* and *LOX3* displayed a significant expression reduction compared to the control at 6 dpi. The *LOX12* gene was significantly repressed at 9 dpi (Fig. 3.3). These results suggest that most of the LOX genes tested are required for resistance to *F. verticillioides*. In fact, these findings strongly supported the results of functional analyses of the transposon-insertional knock-out mutants in several of the LOX genes tested here; *LOX4* and LOX5 were dramatically more responsive to *F. verticillioides* colonization (Park, Kolomiets et al., unpublished data). Likewise, a *LOX12* mutant was expressed following *F. verticillioides* infection not only in the kernels but in all the below- and above-ground organs tested, leading to increased susceptibility and increased conidiation and fumonisin B1 production in the kernels (Christensen et al., 2013, Molecular Plant Microbe Interactions: accepted).

Fusarium verticillioides. Supporting our hypothesis that some fungal oxylipins are required for normal pathogenicity processes, all selected fungal oxylipin biosynthesis genes were induced during the infection of kernels, even if they were down-regulated with respect to the β-tubulin gene. *FLOX1* and *LDS1* expression were significantly affected by time (Table 3.2B).

Correlation. Some interesting correlations are highlighted regarding gene expression (Table 3.3). Calculation for correlation expressed with the Pearson's coefficient showed that *MLOX1*, *LOX4* and *LOX12* were correlated with fungal metabolisms (Table 3.3). In particular, *MLOX1* was strongly and negatively correlated to *FLOX1*

and *LDS2*, but also negatively related to the ergosterol content; *LOX4* was positively correlated to *LDS1* and FB_1 synthesis; *LOX12* was positively correlated to *LDS1*.
The expressions of *FLOX1*, *LDS1* and *LDS2* were all correlated to tissue colonization expressed as ergosterol content. *LDS1* and *LDS2* expression was also correlated to conidia production. A strong correlation (coefficient 0.95, $p \leq 0.01$) was found between *LDS1* expression and FB_1 synthesis.

3.4 Discussion

Kernel bioassays are generally accepted in many laboratories as relatively easy tools to study mycotoxin producing fungal pathogens and their interactions with the host seed (Christensen et al., 2012). Despite the increasing number of publications about the *F. verticillioides*-maize ecosystem, several key aspects of this pathosystem remain unclear. Specifically, following inoculation of the seed, the critical times of the highly dynamic processes of fungal development, pathogenicity and disease progression including the time points at which the pathogen undergoes vegetative growth and seed colonization, asexual reproduction and production of mycotoxins. Therefore, this study aimed at answering some of these questions using a well-defined model system, maize B73 inbred line and *F. verticillioides* strain 7600, the genomes of which has been sequenced.
This study showed that, with the inoculation method used, the pathogen completed tissues colonization at day 9, as evidenced by the finding that no more ergosterol was produced after 9 h of infection. Conidial production continued until day 12 and no further significant changes in spore production were observed, similarly to the findings of an *in vitro* experiment by Rossi et al. (2009). However, conidia production rate, as calculated per unit of fungal biomass, showed a strong increase on day 12 and 15 and it could be considered a reaction to nutritional source depletion; exhausting itself and the growth substrate, the fungus produced conidia to enhance its survival and dissemination.

The dynamics of conidia production and FB_1 synthesis followed a similar pattern during the incubation period and the two processes were highly and positively correlated. Many studies supported the idea of a correlation between these two pathways (Brodhagen and Keller, 2006; Calvo et al., 2002). Shim and Woloshuk (2001) discovered that the disruption of *FCC1*, a *Fusarium* cyclin C_1-like gene, resulted in the inhibition of conidia production and the suppression of FB_1 synthesis, caused by the inactivation of *FUM5*, the polyketide synthase gene involved in fumonisin synthesis. Furthermore, the disruption of maize 9-lipoxygenase, *ZmLOX3* gene, also resulted in a severe reduction of both conidia production and FB_1 synthesis (Gao et al., 2007).

No correlation between *Fusarium* biomass, as measured by ergosterol content, and FB_1 synthesis was found, in a study on more than 20 strains of *Fusarium* by Melcion et al. (1997). Bluhm et al. (2008), tried to correlate the growth and toxin production by the deletion of a gene (*ZFR1*) but even if the grow of mutant (*Δzfr1*) was reduced on certain substrate and fumonisin production was decreased, no correlation was found.

Recently, a relationship between plant- and fungus-produced oxylipins and fungal metabolism, including sporulation, tissue colonization and mycotoxin synthesis, was demonstrated (Brodhagen and Keller, 2006; Christensen and Kolomiets, 2011; Tsitsigiannis and Keller, 2007). All these fungal developmental and pathogenicity processes can either be enhanced or inhibited by the presence of oxidized metabolites. It has been hypothesized that the potent regulatory functions of both plant and fungal oxylipins is likely due their extreme structural similarities and thus, plant oxylipins can mimic fungal oxylipins in their endogenous signaling roles. Oxylipins are produced by the action of enzymes including lipoxygenases, in plants, or linoleate diol synthases, in fungi, encoded by gene families. The genes required for oxylipin synthesis are differently expressed in a space and time-dependent manner, depending on the genotypes, tissue and on the environmental stimuli. This differential expression of specific branches of the oxylipin pathway leads to the production of a

mixture of diverse oxylipins, the so called “oxylipin signature” profile, of the considered tissue and time after a specific stimulus is applied.

An important outcome of this study is the finding of the time at which the host seeds completely succumb to fungal infections and die. The vitality of seeds has been tested by measuring the expression of the constitutively expressed plant housekeeping gene, cullin, at every time point tested in this study. Starting at day 12, the transcripts of the cullin gene were hardly detectable in the infected kernels, suggesting that between days 9 and 12 seeds are no longer viable. Therefore, we conclude that starting at day 12 (or earlier), once the seed is no longer responsive to infection, the fungus switches to a saprofitic mode of nutrition. This finding is important for any study involving plant-pathogen interactions, i.e. when the host is no longer alive to be able to organize any type of defensive reactions. Interestingly after day 12, no significant changes in conidia production, ergosterol and FB_1 synthesis were noticed.

In this study, several selected maize lipoxygenase (LOX) genes were tested in their response to *F. verticillioides* colonization of the seed. The host LOX genes selected were either those whose function in the interactions with this pathogen was reported previously such as *LOX3*, *LOX4*, *LOX5* and *LOX12* or the genes closely related to the 9-LOX gene subfamily, *LOX1* and *ZmLOX2*. *LOX1* and *LOX2* were induced to the highest levels compared to other LOX genes tested. Despite the high sequence identity between these two genes (more than 90% nucleotide and amino acid sequence identity), *LOX1* and *LOX2* were differentially induced by infection. While *LOX1* was induced 10-fold as early as at day 3 and then declined to about a 4-fold induction level at day 9, *LOX2* was induced up to 4 to 5 fold and remained induced at the same level for the rest of seed life (9 days). The level of expression of these two genes followed an opposite trend with respect to the others; they were strongly expressed at 3 dpi with an expression decrease in the following time points, while other genes were expressed later. Maize *LOX1* gene encodes for a dual positional specific lipoxygenase (Huon et al., 2009) and its expression is induced in plant leaf in response to wounding. The expression of this gene is also stimulated by methyl

jasmonate treatments of leaves (Kim et al., 2003). Despite the possibility that methyl jasmonate accumulation was avoided by vial cap loosening , that permitted gases exchange with the external environment, the sudden and strong expression of this gene versus the others is not surprising because, during sample preparation for the inoculation, a wound in the kernel embryo was made. To date, the function of *LOX1* and *LOX2* genes in the interactions with *F. verticillioides* is unknown, precluding any speculation about the relevance of highly induced expression of these two genes in response to *F. verticillioides* infection.

Published studies by Gao et al. (2007, 2009) showed that *ZmLOX3*-mediated signaling is involved in the regulation of fungal secondary metabolism including biosynthesis of fumonisins and aflatoxins. In particular, Gao et al. (2007) demonstrated that the maize knock-out *lox3-4* mutant, in which this gene is not expressed, significantly accumulated lower levels of fumonisins as compared to the wild type seed after the inoculation with the wild type *F. verticillioides* strain. We have observed that this gene began to be significantly expressed from day 6, which coincided with a significant increase of fumonisin contamination, further underlining the relationship between these two pathways.

LOX4 and *LOX5* are also close paralogs, sharing over 95% identity at both aminoacid and nucleotide levels (Park et al., 2010). *LOX4* and*LOX5* are expressed at relatively low levels in uninfected maize organs tested. *LOX4* is preferentially expressed in the below-ground organs, while transcripts of *LOX5* were only strongly induced in wounded leaves as well as in response to insect infestation, suggesting the role of *ZmLOX5* in plant resistance response against herbivore insects (Park, 2013). Furthermore, the *LOX4* and *LOX5* mutants were similar to each other in their enhanced response to kernel colonization and conidia production by *F. verticillioides* (Park, 2013), suggesting that the two genes are required for the defense reaction against this pathogen. This study showed that both genes were induced in response to pathogen inoculation, although their induction was not as strong as that of *LOX1* and

LOX2. It is possible that relatively low and late response of these two genes is the reason why B73 seeds were susceptible to *F. verticillioides* infections.

In the most recent study, *ZmLOX12* was demonstrated to be implicated in maize defense against *F. verticillioides* as the knock-out disruption of this gene resulted in a dramatic increase in susceptibility to *F. verticillioides* not only in the kernels but in all the below- and above-ground organs tested, including increased susceptibility and increased conidiation and fumonisin B_1 production in the kernels (Christensen et al., 2013). Here, we observed a very low and late induction of this gene, prompting our hypothesis that the low expression of this gene in B73 is yet another potential reason behind the well documented susceptibility of B73 to fumonisin contamination and *Fusarium* ear rot.

Regarding the expression study of the fungal oxylipin biosynthetic genes, transcript accumulation of one lipoxygenase and two linoleate diol synthase (*LDS*) genes were tested. In *Aspergillus* spp. and other fungal genera, the *LDS* genes are named Ppo (psi producing oxygenase) as they are known to produce a mixture of oxidized molecules called Psi factors. The change in the ratio between these oxidized lipids affect the production of spores, increasing the production of sexual or asexual spores, depending on specific oxylipin composition or secondary metabolism such as mycotoxin synthesis. The silencing of specific Ppo genes resulted in the reduced ability to colonize tissues by the fungus and an altered ratio between sexual/asexual sporulation (Tsitsigiannis and Keller, 2006; Tsitsigiannis and Keller, 2007). In this study we observed a positive correlation between the pattern of expression of these three genes and ergosterol synthesis. Also a positive correlation was observed between the expression of *LDS1* and *LDS2* and conidia production (Table 3.3). Interestingly, a positive strong correlation was also observed between the expression of *LDS1* and FB_1 synthesis, supporting the notion that the production of this mycotoxin may be regulated by the oxylipin products of the *LDS1* isoform.

Table 3.1 Sequences of the primers used for molecular analyses.

Primers	Sequence	Amplicon size (bp)
Plant Primers		
MLOX1	F5' TTCCGTGAAGTGTGGTTCTC 3' R5' GAGCCTTATTACAACAGTCCTCA 3'	
LOX2	F5' GCTGGCGGTAACCACTTATTA 3' R5' ACACCATGCATGTGACCAATA 3'	
LOX3	F5' TACCACTACCACCCCAGGAGT 3' R5' AGCACTGCGAAACGACTAGAA 3'	
LOX4	F5' TGAGCGGATGGTTTGTAGAT 3' R5'ATTATCCAGACGTGGCTCCT 3'	
LOX5	F5' GGGCAGATTGTGTCTCGTAGTA 3' R5' ATATTCAAGCGTGGACTCCTCT 3'	
LOX12	F5' AATTGACAAGCTGCGTCCTT 3' R5' TCCAAACCAATCATCGCAA 3'	
Cullin	F5' GCGTTTGCTCCATTCACTTT 3' R5' CCATAACTTTGCGGCTCTTC 3'	
Fungal Primers		
FLOX1	F5’ACGATTCCCAAAGACGAGCAAGTG3’ R5’AGGCCGATGTTGTGTCCTTGTTCA3’	215
LDS1	F5’GGACTCGCTGCGATCGTGTGG3’ R5’TCGCCCTTCTGGGCAATGGC3’	210
LDS2	F5’AGACCCCCACCGAGGCCAAG3’ R5’CCACTGCCCAGCCTCCCAGA3’	191
B Tubulin	F5’ACATTCGTCGGAAACTCCAC3’ R5’CAGCATCCTGGTACTGCTGA3’	190

Table 3.2 A) Average values (mean of biological replicates) of conidia counting (number of conidia/g kernel), ergosterol (µg ergosterol/g kernel) and fumonisin B_1 (µg fumonisin B_1/g kernel) calculated for each time point: different letters are referred to significantly different values; **B)** Average values of the fold change of gene expression in response to *F. verticillioides* infection compared to day 0: different letters are referred to significantly different values.

Table 3.2 A

Day	**Conidia** ($x10^6$/g kernel)		**Ergosterol** (µg/g kernel)		**Fumonisin B_1** (µg/g kernel)		**Conidia/Ergost.** ($x10^6$/ µg)		**FB_1/Ergost.** (µg/ µg)	
3	15	c	3	c	267	c	5	c	98	bc
6	536	c	21	b	1032	c	27	b	53	c
9	1546	b	38	a	8755	b	41	b	245	b
12	2471	a	22	b	12721	a	114	a	590	a
15	2275	a	18	b	12049	a	126	a	672	a

Table 3.2 B

	***Fusarium verticillioides* primers**			**Maize primers**					
	FLOX1	**LDS1**	**LDS2**	**MLOX1**	**LOX2**	**LOX3**	**LOX4**	**LOX5**	**LOX12**
Time	**	*	ns	*	ns	*	ns	ns	**
day3	-9.7 b	-2.1ab	-4.3	10.5 a	3.9	-0.8 b	0.1	-1.4	-3.1 b
day6	-5.9 a	-4.5 a	-4.2	5.7ab	4.3	2.2 a	-0.9	-1.4	4.8 a
day9	-5.4 a	-0.9 b	-3.6	4.2 b	6.1	1.5 a	1.3	-5.6	5.8 a

** $p \leq 0.01$; * $p \leq 0.05$; - not significant

Table 3.3 Significant correlation expressed as Pearson's coefficients (2-tails test), the lack of value means no correlation. LOX2, and LOX5 were not included because no significant correlations were found.

	ergosterol	**FB_1**	**FLox1**	**Lds1**	**Lds2**	**MLox1**	**Lox4**	**Lox12**
conidia	0.49*	0.91**	-	0.85**	0.78*	-	-	-
ergosterol		-	0.68*	0.71*	0.88**	-0.70*	-	-
FB_1			-	0.95**	-	-	0.67*	-
FLox1				-	0.93**	-0.98**	-	0.77*
Lds1					-	-	0.80**	-
Lds2						-0.93**	-	-
MLox1							-	-0.74*
Lox3							-	0.96**

** p≤0.01, * p≤0.05

Day 3

Day 6

Day 9

Day 12

Day 15

Fig. 3.1 Visual observation of the inoculated kernels at each time point.

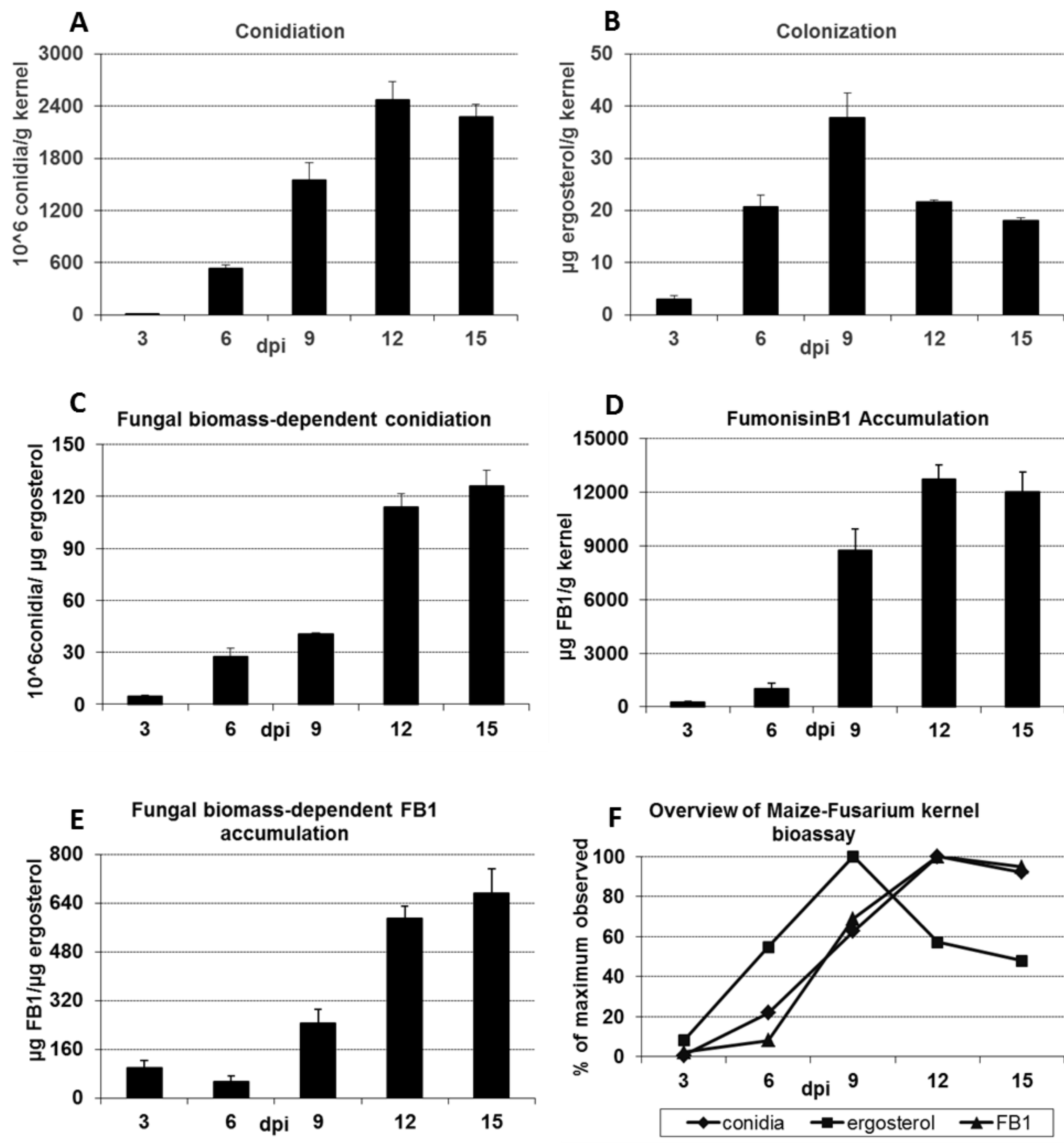

Fig. 3.2 Dynamics of conidia production (A), colonization expressed as ergosterol content (fungal biomass) (B), production of conidia expressed on ergosterol production (C), fumonisin B_1 synthesis (D), fumonisin B_1 expressed on ergosterol production (E) and relative percentages of conidia production, ergosterol and fumonisin B_1 calculated on the maximum value observed (F) during the incubation period (dpi=days post inoculation).

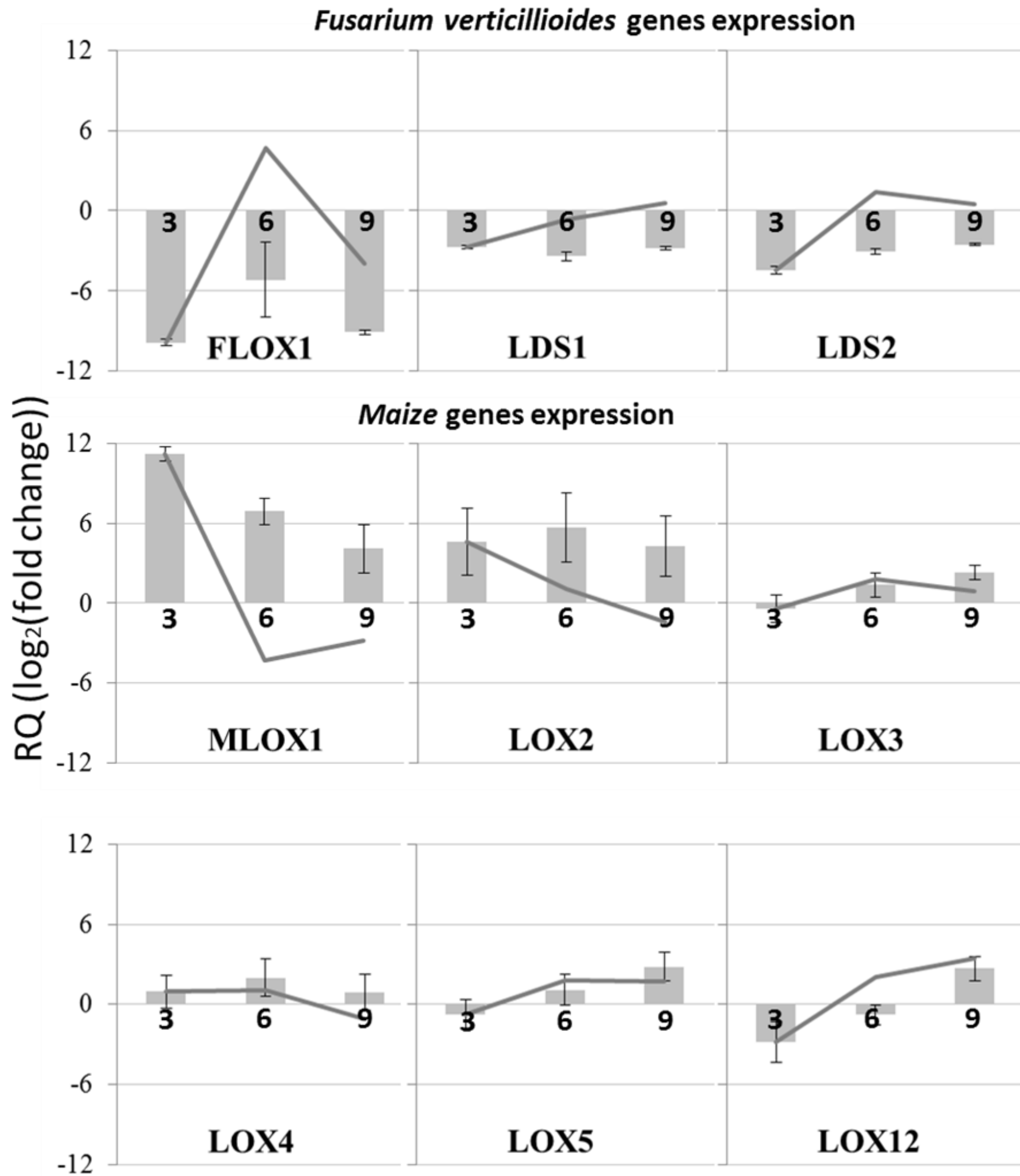

Fig. 3.3 Relative expressions (RQ=relative quantification) of the selected genes normalized with the comparison to the reference gene expression (Cullin for maize and β-tubulin for *Fusariumverticillioides*), as compared to the expression level at day 0 (bars) or evaluating the expression increment (lines): standard errors were calculated between biological replicates.

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CHAPTER 4

Effects of drying treatments on the presence on fungal species and fumonisin contamination in maize kernels

Effects of drying treatments on fungal species and fumonisin contamination in maize kernels

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Abstract

This study aimed to verify the effect of drying thermal treatments on fumonisin content in maize, both in free and hidden forms. Two drying temperatures x time conditions (70°Cx24h; 95°Cx9h) where applied and fungal incidence and fumonisins contamination were determined before and after the treatment. Drying treatments showed to decrease the fungal population in kernels. Referring to fumonisin, the contamination increased after the drying treatment. An explanation for this seems to be the release of some fumonisin from maize matrix, due to the changes in matrix components caused by drying temperature.

4.1 Introduction

Maize is an important crop worldwide, used as food, feed and source for processed products or energy. Independently from its use, post-harvest maize is stored for variable periods before its final utilization. Many ecological parameters are known to affect storage safety, but among them, water activity (a_w) is one of the most important to be controlled. Usually, maize harvesting is carried out when kernel humidity is still too high for a safe storage (Trucksess et al., 1988; Battilani et al., 2011); early harvest is therefore suggested to limit mycotoxin contamination (Battilani et al., 2011; Cotty and Cardwell, 1999). A moisture content <14% (correspondent to < 0.70 a_w) is considered necessary for a safe storage (Magan and Aldred, 2007; Yilmaz and Tuncel, 2010). Fungal pathogens associated with maize, mainly the toxigenic species *Aspergillus flavus* and *Fusarium verticillioides,* (Battilani et al., 2008; Giorni et al., 2009) can continue to develop and produce mycotoxins, aflatoxins and fumonisins respectively, during storage in a conducive environment. Therefore, a prompt maize drying is commonly carried out, before grain transfer in storehouses, to avoid the post-harvestincrease of mycotoxins contamination (Silva et al., 2008).

Many different technologies can be used for cereal drying, from solar radiation (Folaranmi, 2008), natural and unheated air (Kaaya and Kyamuhangire, 2010) to more sophisticated and expensive dryers using heated air (Costa et al., 2010; Jittanit et al., 201; Tuncel et al., 2010). Drying costs increase moving from natural to artificial drying, but the latter is commonly applied being more rapid and safer for the grain.

Several authors studied the effect of drying temperature on fungi and related mycotoxins. Hawkins et al. (2005) found that the incidence of *F. verticillioides* was significantly reduced by the use of drying temperatures above 60°C, while 70°C were requested to reduce *A. flavus* incidence, however these results were obtained with quite long treatments (4 days). Drying at temperatures higher than 70°C could result in the complete inhibition of all the spoilage agents present in the grains (moulds, insects, bacteria and yeasts), but it could also produce the loss of grain quality,

causing kernel cracking (Brown et al., 1979; Davidson et al., 2000; Haros et al., 2003; Navratil and Burris, 1984) and structural damage of maize components, first of all starch (Bhuiyan et al., 2010; Izli and Isik, 2013).

Referring to mycotoxins, controversial data are reported in the literature. In particular, it was reported that the use of very high temperatures (≥190°C), like those reached during roasting and frying, seem to reduce the concentration of mycotoxins (Humpf and Voss, 2004). However, the reduction could be only apparent; in fact, it was recently demonstrated that the presence of reducing sugars, amino acids or proteins could mask fumonisins through the conjugation with these matrix components (Seefelder et al., 2003; Berthiller et al., 2009). Consequently, common analyses do not reveal the presence of these mycotoxins, but they can be released in the intestinal tract and produce the same toxic effect of the natural compounds (Dall'Asta et al., 2010; Motta and Scott, 2009).

In a recent study on fumonisin fate along the maize chain, hidden forms of fumonisins had an increasing trend during drying (Falavigna et al., submitted). This was previously found also by Ono et al. (2002) and frequently reported by farmers (Battilani, personal communication).

Therefore, the present study was developed with the aim of investigating the possible effects of drying temperatures on fumonisin contamination in the grain, including the presence of hidden forms and taking into account the possible role of maize hybrids.

4.2 Materials and Methods

4.2.1 Sample collection

In 2010 and 2012, commercial maize fields grown in Cremona and Piacenza (Northern Italy) were sampled at harvest. Each field was divided in 3 sectors, each one sampled by collecting ten ears following a X-shape design; a total of thirty ears were sampled from each field and considered as a representative sample for the field.

In the first year, 6 maize hybrids (H3, H8, H13, H44, H15, H16) were collected in Cremona (Northern Italy). In 2012, only 2 maize hybrids were sampled in triplicate,

one in Piacenza (H3) and one in Cremona (H8) (Northern Italy). After husk elimination, ears were shelled, the kernels of each sample were mixed and 3 sub-samples of about 500g were collected; one sub-sample was used as a control (untreated) and the other 2 sub-samples were used for the drying treatments.

4.2.2 Drying test

Maize kernel sub-samples were placed in a stove (NSC9180, So.Ge.Sa. Srl, Potenza, Italy) and treated with 2 different combinations of temperature and time: (i) control (no treatment), (ii) 70°C for 24 hours; (iii) 95°C for 9 hours. Measurement of a_w level was performed both before and after the drying process using AquaLab LITE (version 1.3 © Decagon devices Inc., WA, USA) equipment.

4.2.3 Incidence of infected kernels

Fifty kernels were randomly selected from each sample, surface disinfected in a 1% sodium hypochlorite solution for 2 min, then in a 90% ethanol solution for 2 min, rinsed with sterile distilled water and dried under a sterile flowhood. Then, kernels were placed into Petri dishes containing Potato Dextrose Agar (Oxoid LTD., Basingstoke, Hampshire, England) and incubated at 25°C for 7 days.

After incubation, fungal strains which developed from the kernels were counted and identified at genus level. In particular, *Fusarium*, *Aspergillus* and *Penicillium* spp. were taken into account. The incidence of infected kernels was reported as the number of kernels with growing moulds related to the total number of plated kernels.

4.2.4 Starch components determination

Maize flour samples were analyzed using a Megazyme amylose/amylopectin assay kit (Megazyme International Ireland, Bray Business Park, Bray, Ireland). Amylose determination was obtained after the selective quantitative precipitation reaction of amylopectin with concanavalin A (ConA) (Gibson et al., 1997). The percentage of

starch components was obtained through the determination of amylose and total starch concentration using a spectrophotometer UV 1601 (Shimadzu Italia, Milano, Italia).

4.2.5 Fat content determination and fatty acid analysis

Fat content was determined utilizing a Soxhlet extractor (Velp Scientifica, Monza-Brianza, Italy). In particular, 5 g of ground sample were extracted using diethyl ether as a solvent. Then, in order to determine the fatty acid profile, 50 mg of extracted fat fraction was placed into a tube and dissolved in 3 ml of hexane; 2 ml of KOH/CH_3OH 5% solution were added and the solution was vigorously shaken in order to derivatize the fatty acids, for their transformation in the corresponding methyl esters; 1 µl of the obtained sample was analyzed in a GC-MS apparatus (Agilent Technologies 6890N gas-chromatograph coupled to an Agilent Technologies 5973, Agilent technologies St. Clara, CA, USA) on a SUPELCOWAX 10 capillary column (Supelco, Bellefonte, Pennsylvania, USA). Oven temperature started from 80°C for 3 min and increased of 20°C min^{-1}up to 260°C, maintained for 5 min. Total run time was 21 min. The carrier gas was helium with a total flow of 18 ml min^{-1}. The injector and interface temperatures were 280°C. Split modality was chosen as injection mode. Mass spectra were recorded in full scan modality by electronic impact at 70 eV, in the mass range of 40-500 m/z.

4.2.6 Fumonisin determination and analysis

Free fumonisin extraction. In order to extract free fumonisins, 5 g of milled corn kernels was extracted with 40 ml of water/methanol mixture (3/7 v/v) by shaking the sample in a high-speed blender Ultraturrax T 25 (v=1; 4000 rpm, IKA, Stauffen, Germany) for 3 min. Then the sample was filtered on filter paper and 4 ml of the extract were evaporated to dryness under nitrogen flow and the residue was redissolved in 1 ml of water/methanol mixture (3:7 v/v) before LC-MS/MS analysis.

Total fumonisin extraction. For total fumonisin extraction, the corn sample (2.5 g) was previously treated with 50 ml of aqueous KOH 2N in order to obtain the hydrolysis of hidden fumonisins. The samples were blended in a high-speed blender Ultraturrax T 25 (v = 1, 4000 rpm) and then stirred at constant speed at room temperatures for 1 hour. After this step, hydrolyzed fumonisins were extracted adding 50 ml of acetonitrile, shaking the solution for 5 min utilizing the high-speed blender Ultraturrax T 25 (v = 1, 4000 rpm). In order to separate the two formed layers, samples were centrifuged for 10 min at 3500 r.p.m. at 25°C. After this step, 4 ml of extract containing hydrolyzed fumonisins were placed into a vial and dried under nitrogen flow. Then, the dried residue was redissolved in 0.4 ml of water/methanol mixture (3:7 v/v) and analyzed by LC-MS/MS.

Sample analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.) on a Synergy Fusion RP 50 x 2 mm Column (Phenomenex). H2O (A) and CH3OH (B) both acidified with 0.2% of HCOOH were used as eluents. Gradient started at 70% of eluent A, going to 35% in 3 min and then to 10% in other 12 min and here remained for 3 min. Then column was riequilibrated in 7 min.

MS detection was performed using an ESI + source: capillary voltage, 4.0 kV; cone voltage, 50 V; extractor voltage, 2 V; source block temperature, 120°C; desolvation temperature, 350°C; cone gas flow and desolvation gas flow (nitrogen), 50 L/h and 700 L/h, respectively. Detection was carried out using a multiple reaction monitoring (MRM) mode by monitoring two transitions for each analyte, as follows: 722.4→334.4 (CE 40 eV), 722.4→352.3 (CE 35 eV) for FB_1, 706.4→336.4 and 706.4→318.4 (CE 35 eV) for FB_2 and FB_3, 406.5→370.5 and 406.5→388.5 (CE 20 eV) for HFB_1, 390.5→336.4 and 390.5→372.5 (CE 20 eV) for HFB_2 and HFB_3. The first transition reported was used for quantification, while the second transition was chosen as qualifier. For each sample, the entire procedure was performed in duplicate (n = 2). Matrix-matched calibration curves (calibration range 10-1000 µg/kg) were

used for parent fumonisins (FB_1, FB_2 and FB_3) and hydrolysed fumonisins (HFB_1, HFB_2, HFB_3) quantification.
Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolysed fumonisins B_1, B_2, and B_3. All the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of parent and hydrolysed compounds and referred to as "total fumonisins after hydrolysis".
Fumonisins B_1, B_2 and B_3 standard solutions (a mixture of FB_1, FB_2 and FB_3, 50 μg/ml each, in acetonitrile/water, 1:1 v/v) were purchased from Romerlabs (Tulln, Austria). Hydrolysed fumonisins (HFB_1, HFB_2, HFB_3) were prepared in laboratory.
All solvents: diethyl ether, methanol, hexane and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA) while KOH was purchased from Carlo Erba (Milan, Italy).

4.2.7 Statistical analysis

Data on the incidence of total fungi and the genera considered separately, *Fusarium* and *Aspergillus*, were arcsine transformed and data on fumonisin production were logarithmically transformed before statistical analysis (Fowler and Cohen, 1990; Clewer and Scarisbrick, 2001). Fumonisin B free and FBs total after drying treatment were rated on the value of untreated samples.
Pearson's correlation coefficients among all the main factors considered in the study were calculated for the data collected in both years (2010 and 2012); therefore, analysis of variance for 2012 data was run and Tukey's post-hoc comparison test was used to highlight differences between means.
All data were analysed using the statistical package SPSS (PASW ver. 19.0.0, 2009, Chicago, IL, USA).

4.3 Results

4.3.1 Maize hybrids characteristics at harvest

Year 2010

In 2010, kernel a_w at harvest in the 6 maize hybrids considered in the study ranged between 0.89 and 0.96. Fungal incidence in maize kernels varied from 74% to 100% (total infection), with *Fusarium* as the dominant genus, contributing between 56% and 92% to kernel colonisation and *Aspergillus* spp. isolated only in hybrids H14 and H16, with 2 and 6% incidence, respectively (Table 4.1). *Penicillium* spp. were isolated occasionally, in particular only in 2% of the kernels of hybrid H16, therefore, they were excluded from further analysis.
Regarding FBs, maize hybrids showed very different contaminations levels, ranging from 33 to 8839 µg/kg of free FBs and 202-18815 µg/kg of total FBs in H14 and H3, respectively (Table 4.1).
Regarding the chemical composition, the protein, starch, amylose and amylopectin content was found to be similar in the different hybrids, with around 10% as maximum variation observed. The lowest protein content was reported in H13 and H14, hybrids with the highest amount of amylose. The lowest starch and amylopectin content was detected in H8 and H15.
The variability was more relevant regarding fat content, with H13 having around half the amount of fat per 100 g of kernels compared to H14 (2.68 *vs* 4.88 g/100g). The contribution of fatty acids to the total fat content varied between hybrids, with H8 richer in C16:0, H3 in C18:0 and H14 in C18:1, C18:2 and C18:3 compared to the other hybrids sampled.

Year 2012

In 2012, when only H3 and H8 were considered, kernel a_w=0.86 was measured at harvest in both hybrids. *Fusarium* spp. and *Aspergillus* spp. were alternatively dominant in H8 and H3, respectively, and together contributed to around 50% of fungal incidence, quantified around 96% of the infected kernels (Table 4.1). Also in

2012 production, *Penicillium* spp. were found only in one hybrid (H3) and with a low incidence (7%) and then excluded from further analysis.

The highest FB contamination was found in H8, with 8630 µg/kg of free FBs and 10162 µg/kg of total FBs (Table 4.1).

The chemical composition was similar in the 2 hybrids, excepted for the fat content, with H3 richer than H8 (+30%). Regarding the fatty acids, more relevant differences were noticed for C18:1 and C18:2, with an higher content for both of them in H3.

4.3.2 Maize hybrids characteristics after the drying treatment

Year 2010

After the drying treatment, maize kernels showed an average reduction of a_w around 50%, 40% in H15. However, the drying effect of the two temperatures-time combinations applied (70°C for 24h and 95°C for 9 h) was very similar in all the maize hybrids included in the experiment (Table 4.2).

The thermal treatment influenced the total fungal incidence , with mean reductions of 21% and 77% at 70°C and 95°C, respectively. In particular, *Fusarium* spp. resulted more susceptible, with a reduction of around 90% in both treatments applied (data not shown).

Considering the variation of free and total FBs after thermal treatments, H13 and H16 showed a FB reduction in comparison to the untreated samples. Comparable values in free and total FBs were found in H3 after drying at 95°C and in H8 after drying at 70°C, both free and total FBs increased, more markedly total FBs, in the other drying conditions (Fig 4.1). Increases in free and total FBs were noticed in H15, after both drying treatments, with 70°C x 24 h being more effective. The increase was dramatically more relevant in H14, especially for the free forms, with 1600% and 4600% increase when dried at 95°C and 70°C, respectively (Fig. 4.1).

Regarding the chemical composition of maize kernels, both protein and starch content resulted unvaried after drying while an average increment of around 35% was

observed in the amylose content in both 70°C x 24h and 95°C x 9h treatments, with amylopectin almost unvaried (Table 4.2).

An average reduction of 26% and 14% in the fat content was detected at 70°C and 95°C drying treatments, respectively; differences between hybrids were limited, with the exception of H14 that showed a 50% and 40% reduction, respectively, with the 2 thermal treatments. A very similar average trend was observed for all the fatty acids analyzed with the only exception of stearic acid that presented higher average reductions of around 40% and 30% at 70°C and 95°C, respectively. With regard to thestearic acid content in the different hybrids, an 80% reduction in H3 in both drying treatments and of 60% and 50% in H14, were observed with the exposure at 70°C and 95°C (Table 4.2), respectively.

Year 2012

Drying treatments resulted able to reduce the a_w level of around 75% in both the maize hybrids considered, ranging from 0.86 to 0.20 a_w. Total fungal incidence resulted only slightly influenced by the treatment at 75°C (10% reduction) while the 95°C x 9 h treatment caused a fungal reduction of about 50% (Table 4.2). Regarding FB content after drying, a reduction was noticed both in H3 and H8 after the exposure at 95°C x 9 h, mainly in the free forms; almost no variation happened in H8 after 24h at 70°C, while an increase in both forms, more relevant in the free forms, was observed in H3. (Fig 4.1).

After drying, both H3 and H8 did not show any variation in protein, starch and amylose content. Fat was mainly influenced by the treatment at 70°C x 24h, showing a reduction of 10%, while no variations were found at 95°C x 9h. Also differences in the fatty acid composition resulted very low, with reductions from 10 to 20% for the treatment at 70°C and values ranging from +5% to -10% for the treatment at 95°C for palmitic, oleic, linoleic and linolenic acid. The only fatty acid that resulted highly modified by the thermal treatment was stearic acid that was increased by the drying treatments from 50% to 125% at 70°C and 95°C (Table 4.2), respectively.

4.3.3 Statistical analysis of data

Year 2010

The Pearson's correlation analysis run on data collected in 2010 showed an important influence of thermal treatments on fungal infection; the negative significant correlation confirmed that the exposure to high temperatures can reduce the incidence of fungal incidence in kernels (P≤0.01). Moreover, both amylose and amylopectin resulted influenced by the treatment, being the first increased (P≤0.01) and the latter decreased (P≤0.05) (Table 4.3).

The a_w level resulted negatively correlated with starch (P≤0.05) and amylose contents (P≤0.01) but it was positively correlated with fungal incidence (P≤0.05); fungal incidence was negatively correlated with the amylose content (P≤0.05) (Table 4.3).

Regarding FBs, both free and total FBs were correlated with the hybrid, as well as hidden forms, and also correlated to protein (positively) and fat (negatively) content; in particular, oleic acid seems to play the most important role (P≤0.01). Furthermore, oleic acid was also positively correlated with linoleic acid (P≤0.01) (Table 4.3).

Year 2012

Regarding maize hybrids collected in 2012, the ANOVA highlighted a relevant role of hybrids on fungal incidence and FB content (both free and total FBs) (Table 4.4). In particular, H8 resulted to be mainly infected by *Fusarium* species and to have an higher FB content in both forms.

Significant differences between H3 and H8 were found also for fat content and fatty acids composition, in particular with more palmitic, oleic and linoleic acid per 100g of kernels in H3 (Table 4.4).

Thermal treatments significantly reduced fungal incidence; in particular the treatment at 95°C caused 47% of reduction of total fungal incidence, but it significantly increased the stearic acid content in maize kernels. However, no significant effects were found on FB contamination (Table 4.4).

4.4 Discussion

Drying treatments confirmed their importance in reducing fungal incidence in maize. In particular, long time treatments (24 hours) and lower temperatures (70°) resulted more effective in reducing the incidence of fungal populations with respect to the exposure to higher temperatures (95°C) for a shorter time (9 hours). In previous studies, drying temperatures higher than 70°C resulted able to completely inhibit spoilage fungi present in maize (Davidson et al., 2000), but in our study they were only reduced. However, our treatments were, at maximum, only for 24 hours while in the same previous experiment longer exposure times were used (4 days). Anyway, using lower drying temperature could result in a higher fungal presence but in a better maize grain quality since less subjected to cracking (Davidson et al., 2000, Haros et al., 2003).

Another important parameter was the a_w level; in fact, in 2010, when a_w at harvest was between 0.89 and 0.96, thermal treatments induced a lower reduction in a_w but an higher effect on fungal reduction and chemical composition of maize (amylose and fat in particular). In 2012, the a_w level at harvest was lower (0.86 a_w) and the effect of drying treatments was the highest on the a_w level but produced very small modifications in maize chemical composition. This underlines once again the relevant role played by harvest time on maize quality (Battilani et al., 2011).

Interestingly, H14 resulted the maize hybrid with the highest reduction in fat content after drying treatments (-51% and -45% respectively at 70°Cx24h and 95°Cx9h) and it was the hybrid showing the highest average increase of free (+3079%), total (+659%) and hidden (+195%) FBs after treatments.

Even if the fat content after drying treatments was never investigated before, mycotoxin fate was previously documented. In previous studies, fumonisins resulted increased after artificial drying and it was probably due to modifications in matrix components that favored mycotoxin release (Ono et al., 2002; Falavigna et al., submitted).

Surprisingly, in 2010, at harvest, H3 presented the maximum content in stearic acid with respect to other maize hybrids which was probably due to hybrid characteristics but that was strongly reduced by the drying treatments (82% reduction both at 70°Cx24h and 95°Cx9h treatments) to values present in all the other maize hybrids.
In 2010, H3 and H16 gave the highest average increase of the amylose content with both thermal treatments (47% and 51%, respectively) even if changes of the starch content were similar to those of other maize hybrids (+5% at 70°Cx24h and +2% at 95°Cx9h). These kind of modifications can be explained by the fact that starch granules are melted by treatments with temperatures higher than 60°C (Izli and Isic, 2013) and, in general, drying treatments with temperatures higher than 80°C increase the amylose content (Bhuiyan et al., 2010).

Table 4.1 Incidence of infected kernels (%), free and total fumonisins (μg/kg) and chemical composition of kernels(g/100g) of maize samples collected in 2010 and 2012.

Year Hybrids	Water activity (a_w)	Fungal incidence (%)	Fusarium spp. (%)	Aspergillus spp. (%)	Free FBs (μg/kg)	Total FBs (μg/kg)	Proteins (g/100g)	Starch (g/100g)	Amylose (g/100g)	Amylop (g/100g)	Fat (g/100g)	C16:0 (g/100g)	C18:0 (g/100g)	C18:1 (g/100g)	C18:2 (g/100g)	C18:3 (g/100g)
2010																
H3	0.94	100	90	0	8839	18815	8.17	77.91	13.53	64.38	4.14	0.407	0.300	0.936	2.467	0.027
H8	0.96	100	58	0	2388	5580	8.00	72.05	14.24	57.81	3.91	0.615	0.075	1.030	2.167	0.020
H13	0.89	80	64	0	1333	3140	7.59	77.22	15.59	61.63	2.68	0.361	0.033	0.721	1.545	0.020
H14	0.95	88	56	2	33	202	7.29	74.82	15.57	59.25	4.88	0.548	0.078	1.238	2.983	0.035
H15	0.94	100	92	0	3523	8678	8.22	72.26	14.88	57.38	3.76	0.440	0.061	1.055	2.189	0.020
H16	0.94	74	58	6	859	1714	8.26	77.18	14.11	63.07	3.99	0.429	0.070	1.045	2.419	0.023
2012*																
H3	0.86	97	10	54	1106	4968	7.65	77.30	18.68	58.62	2.86	0.330	0.039	0.926	1.521	0.037
H8	0.86	95	51	21	8630	10162	7.25	73.27	18.21	55.06	2.01	0.247	0.037	0.593	1.097	0.030

*** Data reported for 2012 are mean values of 3 replicates.**

Table 4.2 Variation per cent of the main maize hybrids parameters after drying treatments at 70°C for 24 hours and 95°C for 9 hours with respect to the untreated maize samples. For 2012 mean values of three replicates are given.

2010	Water activity (a_w)		Fungal incidence (%)		Proteins		Starch		Amylose		Amylopectin		Fat	
Hybrid	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**
H3	-50	-50	-33	-96	5	2	5	2	51	43	-5	-7	-20	-15
H8	-52	-50	-41	-62	5	2	5	2	32	29	-2	-5	-25	-14
H13	-51	-45	6	-45	5	2	5	2	27	29	-1	-5	-21	6
H14	-49	-46	-57	-89	5	2	5	2	31	25	-2	-4	-51	-45
H15	-40	-38	-4	-84	5	2	5	2	28	28	-1	-5	-12	-5
H16	-47	-50	3	-87	5	2	5	2	45	56	-4	-10	-26	-14
Mean variation	***-48***	***-47***	***-21***	***-77***	***5***	***2***	***5***	***2***	***36***	***35***	***-3***	***-6***	***-26***	***-15***
2012														
Hybrid	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**	**70°C**	**95°C**
H3	-79	-76	-11	-57	-5	-5	-1	2	-12	+12	3	-1	-14	-23
H8	-80	-72	-7	-37	-5	-4	6	5	5	-7	6	9	-11	21
Mean variation	***-80***	***-74***	***-9***	***-47***	***-5***	***-5***	***3***	***4***	***-4***	***3***	***5***	***4***	***-13***	***-1***

Table 4.3 Significant correlations expressed as Pearson's coefficient (2-tails test) of relevant parameters examined in 2010.

	Water activity (a_w)	Fungal Incidence	Proteins	Starch	Amylose	Amylopectin	Fat	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Free FBs	Total FBs	Hidden FBs
Hybrid	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	-0.61**	-0.69**	-0.72**
Drying treatment	-0.84**	-0.87**	nc	nc	0.81**	-0.55*	nc	nc	nc	nc	nc	nc	nc	nc	nc
Water activity (a_w)		0.60*	nc	-0.50*	-0.94**	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Fungal Incidence			nc	nc	-0.60**	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Proteins				nc	nc	nc	-0.50*	-0.48*	nc	-0.49*	nc	-0.52*	0.52*	0.51*	nc
Starch					0.54*	0.57*	nc	nc	nc	nc	nc	nc	nc	nc	nc
Amylose						nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Amylopectin							nc	nc	nc	nc	nc	nc	nc	nc	nc
Fat								0.81**	0.50*	0.96**	0.99**	0.78**	nc	-0.48*	-0.51*
Palmitic acid									nc	0.83**	0.75**	0.56*	nc	nc	nc
Stearic acid										nc	0.49*	0.48*	nc	nc	nc
Oleic acid											0.93**	0.66**	-0.47*	-0.53*	-0.54*
Linoleic acid												0.81**	nc	nc	-0.47*
Linolenic acid													nc	nc	nc
Free FBs														0.97**	0.72**
Total FBs															0.86**
Hidden FBs															

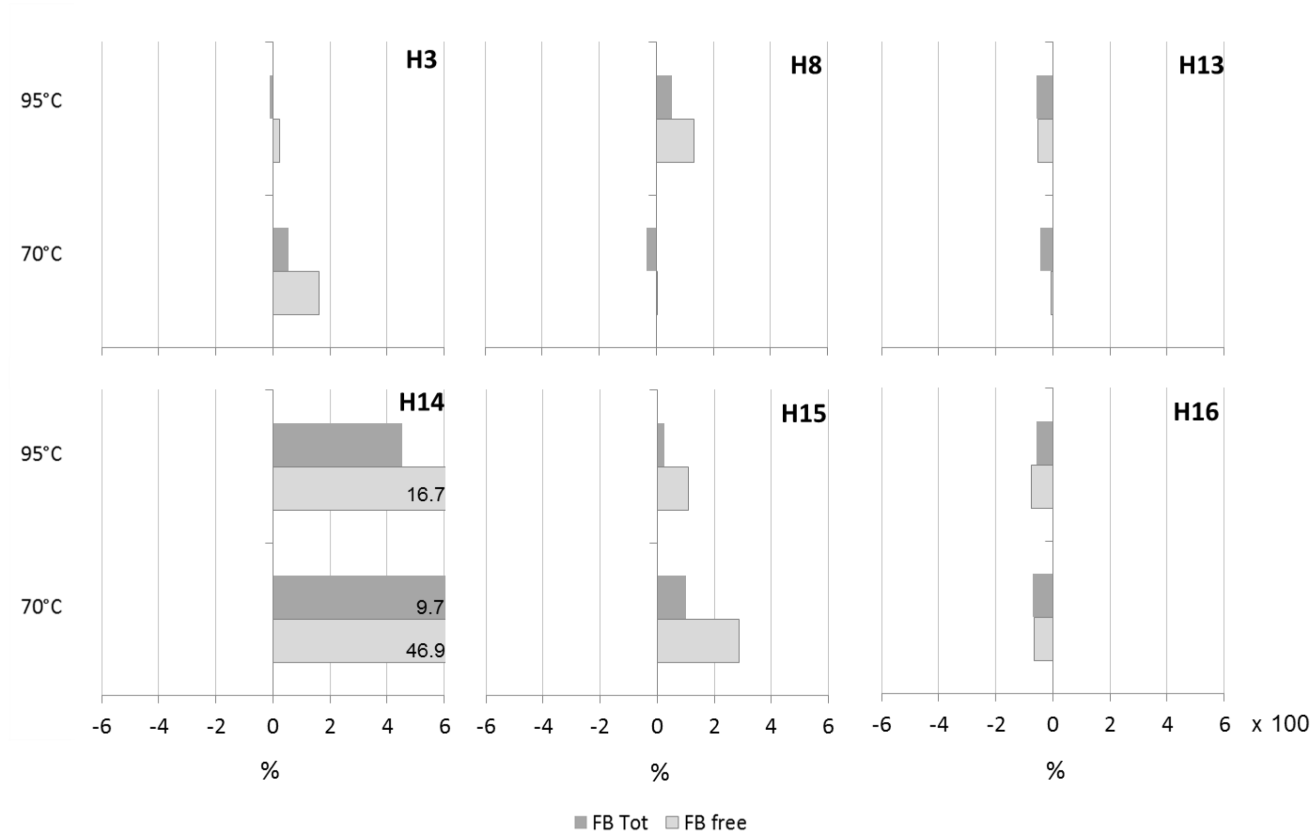
nc: not correlated; *: P≤0.05; **: P≤0.01

Table 4.4 Analysis of variance for fungal infection, fumonisin content and chemical composition of kernels in maize hybrids collected in 2012.

	Fungal incidence (%)		Free FBs (µg/kg)		Total FBs (µg/kg)		Free FBs /Total FBs	
Significance	n.s.		**		**		**	
H3	75		1325	b	4825	b	0.26	b
H8	81		7058	a	10420	a	0.69	a
Significance	**		ns		ns		ns	
Test	96	a	4868		7565		0.54	
70°C	87	a	5061		7937		0.59	
95°C	51	b	2646		7366		0.29	

	Protein (g/100g)		Starch (g/100g)	Amylose (g/100g)	Amylop. (g/100g)	Fat (g/100g)		C16:0 (g/100g)		C18:0 (g/100g)		C18:1 (g/100g)		C18:2 (g/100g)		C18:3 (g/100g)
Significance	*ns*		*ns*	*ns*	*ns*	**		**		*ns*		**		**		*ns*
H3	7.3		77.5	18.6	58.9	2.5	a	0.31	a	0.05		0.82	a	1.29	a	0.03
H8	7.3		75.9	18.1	57.8	2.1	b	0.25	b	0.07		0.65	b	1.08	b	0.03
Significance	*ns*		*ns*	*ns*	*ns*	*ns*		*ns*		**		*ns*		*ns*		*ns*
Test	7.5	a	75.2	18.4	56.8	2.4		0.29		0.04	b	0.76		1.31		0.03
70°C	7.4	b	77.0	17.7	59.3	2.1		0.26		0.06	b	0.68		1.10		0.03
95°C	7.2	b	77.8	18.9	58.9	2.3		0.29		0.09	a	0.77		1.15		0.03

a)



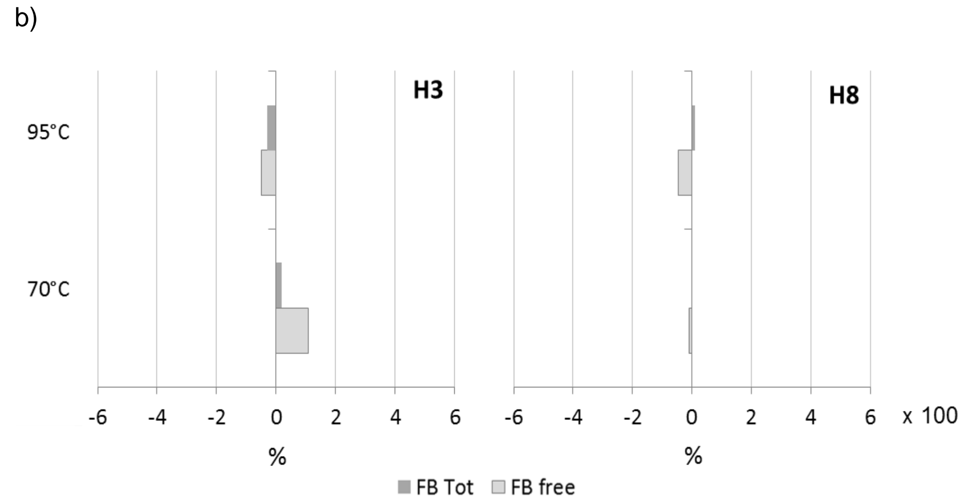


Fig. 4.1 Variation per cent of total and free fumonisin (FBs) production in maize hybrids after drying treatments respect to the untreated samples collected in 2010 (a) and in 2012 (b). Because of the great variation of percentage values, data are reported as percentage variation/100.

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CHAPTER 5

Dynamics of fungi and related mycotoxins during cereal storage in silo bags

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Dynamics of fungi and related mycotoxins during cereal storage in silo bags

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Abstract

The aim of this work was to investigate the dynamics of fungi and related mycotoxins during cereal storage in silo bags. A 2-year trial was organised (2009-2011); in each year, two silo bags, filled with maize and durum wheat respectively, were prepared. During storage, meteorological data were collected from a close station and temperature, CO_2 and O_2 were measured inside the silos. Grain was sampled from silo filling (September) every 40 days until June (7/8 samples per silo per year). Water activity of grain, colony forming units (CFU) and mycotoxin content (fumonisins, aflatoxins and ochratoxin in maize, deoxynivalenol and ochratoxin in wheat) were determined.
Temperature inside the silo bags followed the trend of external temperature, with a more limited variation. A decrease of O_2 was observed from 16.4% to 2.0% in maize and from 15.7% to 15.1% in wheat; the decrease was balanced by CO_2 increase. Minor variations were registered in grain water activity and CFU and mycotoxin content did not change significant throughout the storage period; the results were consistent in the two years. On the basis of these results, silo bags prove to be suitable for safe cereal storage.

5.1 Introduction

Long storage of cereals is a common practice worldwide and permanent structures, such as horizontal or vertical silos, sometimes equipped with temperature or air control, are used to safeguard product quality and safety (Lacey, 1989). Cereals must be protected in field against fungal attacks that could become the initial inoculum for further infection during storage. Post-harvest development of fungi, in fact, can cause severe product losses and furthermore mycotoxin contamination of grain could take place when toxigenic strains are present (Magan and Aldred, 2007).

Fusarium, *Penicillium* and *Aspergillus* spp. are fungi frequently isolated from stored maize and wheat worldwide, and many species of these genera are able to produce mycotoxins (Magan et al., 2003). These fungi live in different, partially overlapping ecological niches (Giorni et al., 2009), and the environmental parameters determine the dominant species.

Fusarium spp. is commonly dominant in the field, where water activity (a_w) is not a limiting factor for most of the crop growing period; it can continue its development during storage only when grain is not properly dried (Magan and Lacey, 1984a; Magan and Lacey, 1984b; Magan and Lacey, 1984c). *Aspergillus*, on the contrary, is more xerophilic and could get the upper hand during storage, because of the low moisture content of grains, even if *Fusarium* was demonstrated to be more competitive than *Aspergillus* and *Penicillium* (Marin et al., 1998).

The nature of substrate, a_w, temperature and air gas composition are the most important parameters able to affect fungal growth and mycotoxin production post-harvest (Magan et al., 2003; Magan and Lacey, 1984a; Magan and Lacey, 1984b; Magan and Lacey, 1984c) and their management is considered fundamental to prevent further contamination during storage.

Traditional storage systems, such as silos and warehouses, are often very expensive and not flexible in terms of volume. They are rigid systems, not always the correct size for

the quantity of cereal to be stored; the presence of headspaces, where the environmental conditions are possibly appropriate, can favour fungal development and mycotoxin production (Magan and Aldred, 2007).

The silo bag is an alternative storage system, recently considered in Argentina (Bartosik et al., 2008; Bartosik et al., 2003) and Australia (Darby and Caddick, 2007), that is less expensive and more adaptable to storage requirements (Darby and Caddick, 2007; Muck and Holmes, 2006; Rodriguez et al., 2002). These bags are versatile, as they can be sealed up at different volumes, depending on the cereal mass to be stored. The silo bag is a hermetic storage technique; the respiration of the stored biomass produces a slightly anaerobic internal environment that results unfavourable for pathogens and parasites activity (Muck and Holmes, 2006). It was confirmed by Locatelli et al. (2010) who observed an inhibition of insect growth and a rise of their mortality along with CO_2 increase in durum wheat stored in silo bags.

The aim of this study was to investigate the dynamics of fungi, of related mycotoxins and of environmental parameters in maize and wheat stored in silo bags as useful parameters to evaluate cereals safety.

5.2 Materials and Methods

5.2.1 Silo bag preparation and sampling

In a grain warehouse located in San Giovanni di Ostellato (Ferrara province), in northern Italy, both in 2009 and 2010, two large bags, of a triple polyethylene layer of 240 μm thickness, were used for the silage trial. The silo bags were 75 m long and 2.70 m in diameter; they were filled with about 240 tons of cereals and then hermetically sealed. The storage period started after the cereal harvest (July and September, respectively for wheat and maize), with the filling of the silo bags, and lasted until late May-early June of the following year. The differences between the life cycle of the two cereals selected

for the study explain the different dates in which the two silo bags were filled; so the maize silo bags were prepared on 24th August and 8th September, and the durum wheat silo bags on 2nd July and 17th August, in 2009 and 2010, respectively. Grain sampling was planned during the storage period every 40-45 days (Table 5.1). Due to the delay in maize silo bag preparation, only six samples were withdrawn in 2010. A code number (1-7) was attributed to each sampling date; the same code identified very close sampling dates in the 2 years.

Three holes were made in order to sample the cereals, 120 cm above ground level, at the start, in the middle and at the end of each silo bag; they were closed with a circular foldout window.

The internal gas concentration was measured using a CheckPoint (Dansensor, Glenn Rock, NJ, USA), a portable gas analyser for modified atmosphere packaging, able to determine both oxygen (O_2) and carbon dioxide (CO_2) concentration. It was equipped with a syringe that could be inserted through the bag. Gas composition measurement was done close to the holes before sampling. A gas sample was automatically drawn into the analyser, where electrochemical and infrared analysers determined the O_2 and CO_2 concentrations, respectively. The hole produced by the syringe needle was then closed with sticky tape in order to inhibit any gas exchange with the open air.

A thermal probe (Thermometer HD 9010 Delta OHM S.r.l., Padova, Italy) was used for temperature measurement immediately before each sampling.

Grains were sampled using a probe, a cylindrical rod composed of two long hollow tubes, (150 x 5 cm). Five sub-samples were collected inserting the probe with different slopes, from each sampling point and at each date, to obtain 6-7 kg of grains. Samples were stored at -4°C until shipping to the laboratory, managed in less than 24 hours.

Water activity (a_w) of grain, fungal colony forming units (CFU) and mycotoxin content (fumonisins, FBs; aflatoxins, AFs; and ochratoxin A, OTA in maize; deoxynivalenol, DON and OTA in wheat) were determined in all the samples collected.

Meteorological data, intended as air temperature, were recorded by a meteorological station placed close to the silo bags.

5.2.2 Determination of water activity

AquaLab LITE (version 1.3© Decagon devices Inc., WA, USA) equipment was used to determine a_w in kernels. This equipment uses a dielectric humidity sensor to measure sample a_w (accuracy ±0.015 a_w). Approximately 6 g of grains, 20 kernels for wheat and 10 for maize, were randomly selected from each sample and a_w was measured immediately after sample delivery.

5.2.3 Quantification of fungi

Samples were milled using a Universal Cutting Mill PULVERISETTE 19 (Fritsch Gmbh, Idar-Oberstein, Germany) with a 1 mm sieve. In the grinding chamber a rotor fitted with knives comminutes the sample in combination with 3 fixed knives. A FRITSCH Cyclone (Fritsch Gmbh, Idar-Oberstein, Germany) connected to the mill helps in keeping the sample temperature stable and in recovering the flour produced.

Sub-samples (10 g) of maize and wheat flour were blended with 90 mL of sterile 0.1% peptone:water (w/v) using a homogenizer (Bagmixer® 400, Interscience, Paris, France) and serial dilutions from 10^{-2} until 10^{-7} were plated in Petri dishes with PDA (Oxoid LTD., Basingstoke, Hampshire, England) added with 50 mg of chloramphenicol and incubated at 25°C for 6 days.

The total number of fungal colonies was counted using a colony counter (Suntex Colony Counter 570, Suntex Instruments Company Ltd, Taipei, Taiwan) and the identification at genus level was carried out based on colony morphology (Leslie and Summerell, 2006). The result was expressed as CFU/g of flour.

5.2.4 Quantification of mycotoxins

5.2.4.1 Fumonisin standards

FB_1 and FB_2 standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). FB_1 and FB_2 (1 mg) were separately dissolved in 10 mL acetonitrile/water (1:1 v/v); the concentration was calculated using the weight indicated by the manufacturer. These solutions were diluted to obtain HPLC calibrant solutions in acetonitrile/water (3:7 v/v) at individual concentrations of FB_1 and FB_2 between 2.5 and 50 μg/L.

5.2.4.2 Fumonisin analysis

Fumonisins were extracted from 20 g of sample with 200 mL of 0.4 M phosphate buffer [840 mL Na_2HPO_4 (56.8 g/L) + 160 mL $NaH_2PO_4xH_2O$ (55.2 g/L), pH 7.5] (Pietri and Bertuzzi, 2012). After extraction for 45 min using a rotary-shaking stirrer and filtration through a folded filter-paper, 5 mL of the extract were purified through an immunoaffinity column (Fumonitest WB, Vicam, Watertown, MA, USA). After washing the column with PBS (2 mL), FBs were slowly eluted (0.5 mL/min) with methanol (6 mL) into a graduated glass vial; subsequently, the eluate was concentrated to 2 mL under a gentle stream of nitrogen. Analysis was carried out using a HPLC-MS/MS system, consisting of a LC 1.4 Surveyor pump, a Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo-Fisher Scientific, San Jose, CA, USA) and a PAL 1.3.1 sampling system (CTC Analytics AG, Zwingen, Switzerland); the system was controlled by an Excalibur 1.4 software (Thermo-Fisher). After dilution of the extract (0.1 mL brought to 1 mL) with acetonitrile/water (3:7 v/v), FBs were separated on a Betasil RP-18 column (5 μm particle size, 150x2.1 mm, Thermo-Fisher) with a mobile-phase gradient acetonitrile/water (both acidified with 0.2 % formic acid) from 25:75 to 55:45 in 9 min, then isocratic for 3 min; the flow rate was 0.2 mL/min. The 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. 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 a LC-Quan 2.0 software. The performances of the analytical method are reported in Table 5.2.

5.2.4.3 Trichothecenes standards

Deoxynivalenol, 3-Ac-DON, 15-Ac-DON, NIV, HT-2, T-2 and diacetoxyscirpenol (DAS) were purchased from Sigma (St. Louis, MO, USA). Single trichothecene stock solutions of 500 µg/mL were prepared in acetonitrile and, only for DON, the concentrations were measured by UV spectrophotometry at 218 nm using the value 6400 as the molar absorption coefficient (Widestrand and Pettersson, 2001). The concentrations of the other trichothecenes were calculated from the weight of the toxin in the vial supplied by the manufacturer. DAS was used as the internal standard. Trichothecene working solutions of 25-500 µg/L were prepared by diluting with methanol.

5.2.4.4 Trichothecenes analysis

Trichothecenes were extracted from samples (25 g) with 100 ml of acetonitrile-water (86+14 v+v); an aliquot (6 mL) of the filtrate was slowly pressed trough a MycoSep 227 column (Romer Labs, Washington, DC, USA). An aliquot (200 µL) of the internal standard (DAS 10 mg/L) was added to 2 mL of the eluate. The solution was evaporated to dryness and derivatised with 200 µL trimethilsilylimidazole:trimethilclorosilane (1+0.2 v+v) (Sigma, St. Louis, MO, USA) for 15 min in subdued light. Then 0.8 mL

hexane was added, the solution was washed with 1 mL 0.2 M phosphate buffer pH 7.5, and the hexane phase was used for GC-MS. The GC-MS system consisted of Trace GC Ultra (Thermo Scientific, San Jose, CA, USA) equipped with a ISQ single-quadrupole mass spectrometer (Thermo Scientific); the system was controlled by Excalibur 2.1 software (Thermo Scientific).The analysis was carried out using a capillary column Rtx-5MS, 30m x 0.25mm i.d., 0.25 μm film thickness (Restek Corporation, Bellefonte, PA, USA). Helium was the carrier gas with a column head pressure of 55 kpa. The sample was injected (2 μL, split ratio 1:30) into the GC-MS by a programmed temperature vaporisation (PTV) injector. The PTV temperature was raised from 70°C to 250°C (held for 2 min) at 10°C/sec. The oven temperature programming was from 70°C (held for 1 min), to 245°C at 10°C/min and then to 300°C (held for 1 min) at 30°C/min. MS transfer-line and ion source temperature were 300°C and 200°C, respectively. Electron ionization at 70 eV and selected ion monitoring (SIM) were used for detection. Fragment ion peaks monitored were 393, 407, 422, 512 for DON, 377, 392, 467 for 3-Ac-DON, 350, 377, 392, 407, 467 for 15-Ac-DON, 377, 392, 407, 467, 510, 585 for NIV, 350, 377, 392 for DAS, 287, 347, 350, 377, 466 for HT-2, 290, 347, 377, 436 for T-2; ion peaks used for the quantification were 393 for DON, 377 for 3-Ac-DON, 392 for 15-Ac-DON, 510 for NIV, 350 for DAS, 466 for HT-2 and 290 for T-2. The performances of the analytical method are reported in Table 5.2.

5.2.4.5 Aflatoxin standards

Aflatoxin standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). For each AF (B_1, B_2, G_1, G_2), a stock solution of 5-8 μg/mL was prepared in benzene:acetonitrile (98:2, v/v, 2 mL) and stored at –20°C. The solutions were calibrated spectrophotometrically at 350 nm (A.O.A.C., 2005). The working standard solution was prepared after evaporation under nitrogen of an aliquot (100 μL for AFB_1 and AFG_1, 50 μL for AFB_2 and AFG_2) of each stock solution and re-dissolution in chloroform (10 mL)

by ultrasonication. An aliquot (100 μL) of this solution was evaporated under nitrogen and re-dissolved in the HPLC mobile phase (0.5-5 mL), to obtain calibrant solutions at individual concentrations of AFB_1, AFB_2, AFG_1, AFG_2 between 0.3 and 6 μg/L.

5.2.4.6 Aflatoxin analysis

Aflatoxins were extracted from a 25 g portion of sample with 250 mL acetone-water (70 + 30 v/v) (Bertuzzi et al., 2012), using a rotary-shaking stirrer for 45 min. After filtration through a folded filter-paper, an aliquot of the filtrate (5 mL) was diluted with distilled water (45 mL) and the solution was purified through an immunoaffinity column (Easi-Extract Aflatoxin, R-Biopharm Rhône LTD). After washing the column with 5 mL distilled water, AFs were eluted into a graduated glass vial with methanol (2.5 mL). The eluate, concentrated under a gentle stream of nitrogen, was brought to 1 mL with acetonitrile:water (25 + 75 v/v) and vortex-mixed for few sec; then, the extract was filtered (HV 0.45 μm, Millipore), before HPLC analysis. Analysis was performed using an HPLC instrument, consisting of two PU-1580 chromatographic pumps, an AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco Corporation, Tokyo, Japan); the instrument was controlled by a Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 μm 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 0.5 mL/min. AFs were detected after post-column photochemical derivatisation (UVE, LCTech GmbH, Dorfen, Germany); the fluorimeter was set at 365 nm excitation and 440 nm emission wavelengths. The performances of the analytical method are reported in Table 5.2.

5.2.4.7 Ochratoxin A standard

Ochratoxin A standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of 40 μg/mL was prepared in benzene:acetic acid (99+1 v/v) and stored at -20°C. The solution was calibrated spectrophotometrically at 333 nm using the value 5550 for the absorption coefficient (A.O.A.C , 1995). The working standard solution was prepared after evaporation under nitrogen of an aliquot (100 μL) of the stock solution and re-dissolution in the HPLC mobile phase by ultrasonication. This solution was diluted to obtain eight HPLC calibrant solutions at concentrations of OTA between 0.04 and 1.2 μg/L.

5.2.4.8 Ochratoxin A analysis

Ochratoxin A was extracted from a 10 g portion of sample, with 100 mL of a mixture of sodium bicarbonate 0.13M-methanol (50 + 50 v/v) for 45 min using a rotary-shaking stirrer. After filtration through a folded filter paper, an aliquot of the filtrate (5 mL) was diluted with PBS (50 mL) and purified through an immunoaffinity column (Ochraprep, R-Biopharm Rhône LTD). The column was washed with PBS (2 mL) and OTA was slowly eluted (0.5 mL/min) with methanol acidified with acetic acid (98 + 2 v/v, 2.5 mL) into a graduated glass vial: the eluate was concentrated under a gentle stream of nitrogen, brought to 1 mL with acetonitrile:2% acetic acid (41 + 59 v/v) and vortex-mixed for a few sec. The extract was filtered (Millex HV 0.45 μm, Millipore Corporation, Bedford, Massachusetts, USA) before HPLC analysis. The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut, USA), equipped with a Jasco AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco Corporation, Tokyo, Japan) set at 333 nm excitation and 470 nm emission wavelength. The system was governed by a Borwin 1.5 software (Jasco). A RP-18 column (4 μm particle size, 125 × 4 mm i.d., Merck) was employed at ambient temperature, with a

mobile phase of acetonitrile:2% acetic acid (41 + 59 v/v) at 1.0 mL/min. The performances of the analytical method are reported in Table 5.2.

5.2.5 Statistical analysis

Data concerning CFU/g and mycotoxins were logarithmically transformed before the statistical analysis to reduce the variance (Clewer and Scarisbrick, 2001). The effect of year, sampling date and their interaction were considered in the analysis of variance run with all the data collected using the statistical package SPSS (PASW ver. 18.0.0, 2009, Chicago, IL, USA). Tukey HSD (Honestly Significant Difference) post-hoc comparison test was used to highlight differences between means.

5.3 Results and Discussion

5.3.1 Interstitial gas composition

All the factors investigated significantly influenced both CO_2 and O_2 concentration in both matrices considered, with few exceptions (Table 5.3). The level of CO_2, detected in silo bags filled with maize, was significantly different in the two years of the study (Table 5.4); the mean CO_2 content was 13.0% and 8.5%, in the storage season 2009-2010 and 2010-2011, respectively. This concentration varied significantly between the storage seasons considered also in wheat silo bags, where 5.5% and 3.2% were detected, respectively.

During the storage period, an increase of CO_2 concentration in silo bags filled with maize, was observed (Fig. 5.1) and the final concentration resulted significantly higher than the initial one. The content increased from 6.1% to 19.5% and from 5.0% to 14.9% in the first and in the second year, respectively, following a very similar trend.

The CO_2 concentration in the wheat silos showed a fluctuating trend in both years; the average content varied between 2.7% (5th sampling) and 6.3% (2nd sampling) (Fig. 5.1);

however, a significant increase of the CO_2 level was detected in wheat silos during the last sampling (7th) in both years with respect to the previous samplings (5th, 6th).
Referring to O_2, the average content in the two storage seasons was significantly different only for maize, with 5.4% in the first and 11.5% in the second year; on the contrary, in wheat silos, these values were very close, i.e. 15.5% and 16.2%, in 2009-2010 and 2010-2011 respectively, and not statistically different (Tables 5.3 and 5.4).
In maize silos, the O_2 concentration decreased significantly during both storage seasons, but more markedly in the first year. The O_2 concentration started, in fact, from comparable values, 16.4% in 2009 and 17.4% in 2010, but reached very different final values, 0.4% and 3.7% respectively. In wheat, the O_2 content showed limited fluctuations, ranging between 18.2% (5th sampling) and 13.4% (2nd sampling). The trends of the two years resulted very similar, both in maize and wheat silo bags (Fig. 5.1).
Due to the hermetic closure and to the gas and water insulation guaranteed by the silo bag, the composition of the interstitial atmosphere changed during storage, as expected. The trend of CO_2 and O_2 was complementary with an increase of CO_2 and a decrease of O_2 concentration, attributable to the respiration of the grain mass enclosed. The interstitial air resulted more anaerobic in maize; this could be primarily ascribed to the higher respiration rate of this matrix (Bailey, 1940), but kernel size could also play a role; in fact, because better packaging is possible with the smaller wheat kernels (Khatchatourian and Binelo, 2008), the limited interstitial space presumably further reduced the respiration rate.
The changes in the interstitial atmosphere found in this study confirm the results obtained by (Rodriguez et al., 2008) in Argentina in a trial where the hermetic conditions were preserved. On the contrary, in experiments carried out in Australia, where the hermetic conditions were not well-kept (Darby and Caddick, 2007), grain spoilage occurred. A CO_2 concentration similar to the first year of our study was

reported by Bartosik et al. (2008) for dry and wet maize that reached 18.2% and 18.5%, respectively. On the contrary, relevant differences in the CO_2 concentration in the interstitial air of stored wheat were reported by Rodriguez et al. (2008); they showed a crucial role of grain moisture content (MC) during storage. The CO_2 concentration increased with increasing MC of grain; i.e. 30% CO_2 was reached with 19% MC, while 5% of CO_2 was measured in grain with MC<13%. They explained differences in the CO_2 content with a greater biological activity of grains with higher MC, but also with mould respiration that became active in MC higher than 13.5%. The influence of spoilage respiration was confirmed by comparison, for the same MC, of good and poor quality stored wheat (Rodriguez et al., 2008); this influence was ascertained also for soybean grains (Bartosik et al., 2008).

5.3.2 Temperature

Temperature was significantly influenced by the year, the sampling date and their interaction (Table 5.3) and, according to ANOVA, for both cereals the variance was mainly explained by the sampling date.

The average temperature measured inside the silo in each year was very similar for both cereals, 14.0°C and 14.1°C in maize, 14.3°C and 15.7°C in wheat, during 2009-2010 and 2010-2011 seasons, respectively.

The internal temperature of maize silo bags initially decreased from 24.3°C to the minimum value of 8.5°C during the 4th sampling, and then increased up to 19.7°C. In the wheat silo bags, a very similar trend was observed; starting from the initial value of 24.9°C, the minimum value of 7.2°C was measured at the 4th sampling and 20.3°C at the end of storage. The dynamics of the internal temperature (Fig. 5.2) followed the external pattern in both cereals and years considered, but the range of variation of internal temperature was tighter.

Changes in the inside-silo temperature, measured at the centre of the mass, were limited, compared with the air temperature measured by the sensors placed outside the silos. The silo bags demonstrated a capacity to dissipate the accumulated heat, presumably released by biotic mass respiration to ambient and ground. These results are in agreement with previous studies conducted both in Argentina (Bartosik et al., 2008; Rodriguez et al., 2002) and in Australia (Darby and Caddick, 2007). Bartosik et al. (2008) also underlined that this ability to disperse the heat produced could be explained by the favourable volume/area ratio of the silo bag.

Temperature is a parameter found to influence CO_2 concentration only in the case of grain MC above 14%, according to Rodriguez et al. (2008), but in our study the MC values were lower than 14%, which is considered a pre-requisite for good storage (Weinberg et al., 2008). The initial decreasing trend of temperature, together with the low water content of kernels and the low O_2 content of interstitial air, presumably concurred in creating an unfavourable environment for mould development.

5.3.3 Water activity

The ANOVA highlighted a significant influence of year and sampling date on a_w level for both grains and, in wheat, the a_w was also influenced by their interaction (Table 5.3). The a_w changed slightly in the two years of the study (Table 5.4) in both silo bags; the content varied from the initial value of 0.64 a_w to the final value of 0.52 a_w in maize, and from 0.57 a_w to 0.48 a_w in wheat. A significant decrease in a_w content occurred after the 4th sampling in both cereals. Water activity followed an opposite trend compared with temperature (Fig. 5.2); it decreased after the 4th sampling, when temperature increased quickly in both cereals.

The range of a_w variation during storage was limited, in agreement with the observations of Rodriguez et al. (2008); these results confirmed the air tightness of the system and its

good insulation. In fact, statistically significant a_w increases were reported by Pacin et al. (2009) when the handling was not adequate, or localised spoilage formation, as reported by Darby and Caddick (2007), when the hermetic closure was not guaranteed.

In our experience, no moisture stratification was noticed by visual controls in the peripheral layer, in agreement with the Argentinean data (Bartosik et al., 2008) while an increase of MC of seeds in the external layer was observed by Darby and Caddik (2007); they calculated higher yield losses in silo bags than in the traditional bunkers due to the higher percentage of seeds exposed to the peripheral layer.

The higher a_w, measured in the 2009-2010 storage period compared to 2010-2011, irrespective of a lower temperature, probably justifies the higher respiration rate of maize and the higher CO_2% measured. In our case the initial MC of seeds corresponded to the commercial MC level, which is below 14%; then we measured the dynamics of a_w, whereas in both the Argentinean and Australian studies the parameter evaluated was MC. Despite the different parameters measured, the data are almost comparable, being related by the sorption isotherm (Maiorano et al., 2009), even if a hybrid related difference has recently been underlined (Battilani et al., 2011).

5.3.4 Fungal contamination

Fusarium resulted the dominant genus isolated from both cereals during the two seasons of the study, while other genera, such as *Aspergillus* and *Penicillium,* have been occasionally isolated. The predominance of the *Fusarium* genus was found also by (Pacin et al., 2009), whereas Gonzalez Pereyra et al. (2011) found that the dominant genus in silo bag was *Aspergillus.* These differences are probably due to the different geographical areas and years in which the experiments were carried out; samples collected by Gonzalez Pereyra et al. (2011) came from the province of San Luis, while Pacin et al. (2009) collected samples from provinces of Buenos Aires, Córdoba, and Santa Fé.

The ANOVA showed that the sampling date affected the fungal contamination both in maize and in wheat, while *Fusarium* contamination was very similar in the two sampling years (Table 5.4); furthermore, the interaction year x date was also significant in both cereals stored. The CFUs/g in both cereals showed a decreasing trend, more marked in durum wheat in both years, and the final contamination resulted to be significantly lower compared to the initial values (Fig. 5.3). Based on these results, the contribution of mould respiration to O_2 decrease is probably very limited throughout the storage period. These results are in agreement with Pacin et al. (2009); they also reported no significant variation in mould propagules/g of maize stored in silo bags for 120-226 days.

5.3.5 Mycotoxin contamination

Two different fungal metabolites were constantly found as cereal contaminants, FBs in maize and DON in wheat. Among the other mycotoxins, only aflatoxins were rarely found and only in traces.

The ANOVA showed a significant influence of the year on fumonisin contamination in maize and DON in wheat and the former was also influenced by the interaction year x date.

Fumonisins in maize and DON in wheat were nearly double in the second year compared with the first year (Table 5.4). This is in agreement with a previous study conducted in Argentina by Hennigen et al. (2000); they found a marked difference in terms of fumonisin contamination for the same maize varieties during two consecutive growing seasons, due to different environmental conditions.

A slight, not significant increase in FB content was observed in maize during storage in 2009-2010; on the contrary, a slight decrease was registered in 2010-2011. Deoxynivalenol showed a limited fluctuation during the first year, a bit wider in the second year, but significant differences in kernel contamination were never observed (Fig. 5.3).

Mycotoxin contamination is in agreement with fungal behaviour; even if the level detected in the silo bags at the beginning of the storage period differed between the two seasons, no significant variation occurred during cereal storage. These results are apparently not in agreement with Pacin et al. (2009); they found a significant increase in FB concentration at the silo bags opening after storage lasting from 4 to 8 months, but this result is based on mean data, with some silos filled with grain at improper (high) humidity for storage. Pacin et al. (2009) also studied the role of silo bag closure and opening times on FB contamination, with a 3 and 4 months interval, respectively. The highest level of FB contamination was found in silo bags closed later or opened later and, in these silo bags, the level of contamination also showed a greater variability with higher values with respect to the general mean contamination. The initial grain quality, in terms of fungal contamination and MC, showed a crucial influence on the contamination level at the end of the storage period, while no correlation was found between storage duration and fumonisin contamination.

Levels of FB contamination both higher and lower with respect to those determined at the closing time were reported by Pacin et al. (2009) at the silo bags opening; this was observed also in this study, with an increasing and a decreasing trend respectively in the first and second year. Nevertheless, the ability to preserve the initial hygienic quality is confirmed, these variations being not significant, and presumably related to the modified internal conditions of the silo bags. The effectiveness of silo bags in maintaining the good quality of stored grains was recently also demonstrated by Gonzalez Pereyra et al. (2011). They reported that, when handling is adequate, the fungal and mycotoxin contamination is lower in maize stored in silo bags than in trench silos.

5.4 Conclusions

Aside from the cheapness and the ease of use of silo bags, this storage method seems to be an effective tool to store cereals; neither the fungal contamination nor the content of

mycotoxins changed significantly during storage in the two years of the study, both in maize and in durum wheat.

Few studies on the use of silo bags for grain storage are available and information regarding fungi and mycotoxin dynamics during storage is very poor. Good management of silo bags, intended as attainment of an hermetically sealed ambient, without any gas and water exchange with the external environment throughout the storage period, is confirmed to be crucial, as is the MC of kernels at storage time. Our results confirmed that, when these good practices are followed, the internal environment of the silo bag is able to preserve the initial hygienic conditions of the grain mass during storage, both for maize and durum wheat, with no significant variation in mycotoxin contamination or in CFU counts. Therefore, the use of this storage tool could contribute to the optimisation of cereal storage.

Table 5.1 Dates (and codes) of maize and wheat sampling during the 2009-2010 and 2010-2011 storage.

	SAMPLING DATES			
	2009-2010		**2010-2011**	
Sampling code	**maize**	**wheat**	**maize**	**wheat**
1	21 Sept	21 Sept		16 Sept
2	04 Nov	04 Nov	03 Nov	03 Nov
3	17 Dec	17 Dec	13 Dec	13 Dec
4	04 Feb	04 Feb	28 Jan	28 Jan
5	24 Mar	24 Mar	15 Mar	15 Mar
6	29 Apr	29 Apr	02 May	02 May
7	31 May	31 May	08 June	08 June

Table 5.2 Mean recovery (%, mean of 3 replicates), limit of detection (LOD; μg/kg) and limit of quantification (LOQ; μg/kg) of fumonisins (FBs), aflatoxins (AFs) and ochratoxin A (OTA) in maize flour and deoxynivalenol (DON), nivalenol (NIV), toxins T-2 and HT-2 and OTA in wheat flour.

	Recovery	LOD	LOQ
Maize			
FBs	95.5	10	30
AFs	96.8	0.04	0.1
OTA	94.4	0.1	0.3
Durum Wheat			
DON	92.4	3	10
3-Ac-DON	90.1	5	20
15-Ac-DON	88.8	5	20
NIV	85.7	10	30
T-2	90.1	10	30
HT-2	91.0	10	30
OTA	94.8	0.1	0.3

Table 5.3 Results of the analysis of variance of parameters measured during maize and durum wheat storage in silo bags in 2009 and 2010.

	CO_2	O_2	T	a_w	CFU *Fusarium*	toxin
MAIZE						
year	**	**	**	*	n.s.	**
date	**	**	**	**	**	n.s.
Year x date	*	n.s.	**	n.s.	**	*
DURUM WHEAT						
year	**	n.s.	**	**	n.s	**
date	**	**	**	**	**	n.s.
Year x date	**	*	**	*	*	n.s.

** significant (P≤0.01), * significant (P≤0.05), n.s. not significant

Table 5.4 Mean value of parameters quantified during maize and durum wheat storage in silo bags in 2009 and 2010.

	MAIZE		DURUM WHEAT	
	2009	2010	2009	2010
CO_2 (%)	13.03	8.51	5.52	3.18
O_2 (%)	5.41	11.49	15.50	16.21
T (°C)	13.97	14.09	14.26	15.65
a_w	0.62	0.59	0.57	0.52
DON (µg/kg)			1001	2266
FB_1+FB_2 (µg/kg)	3089	5682		

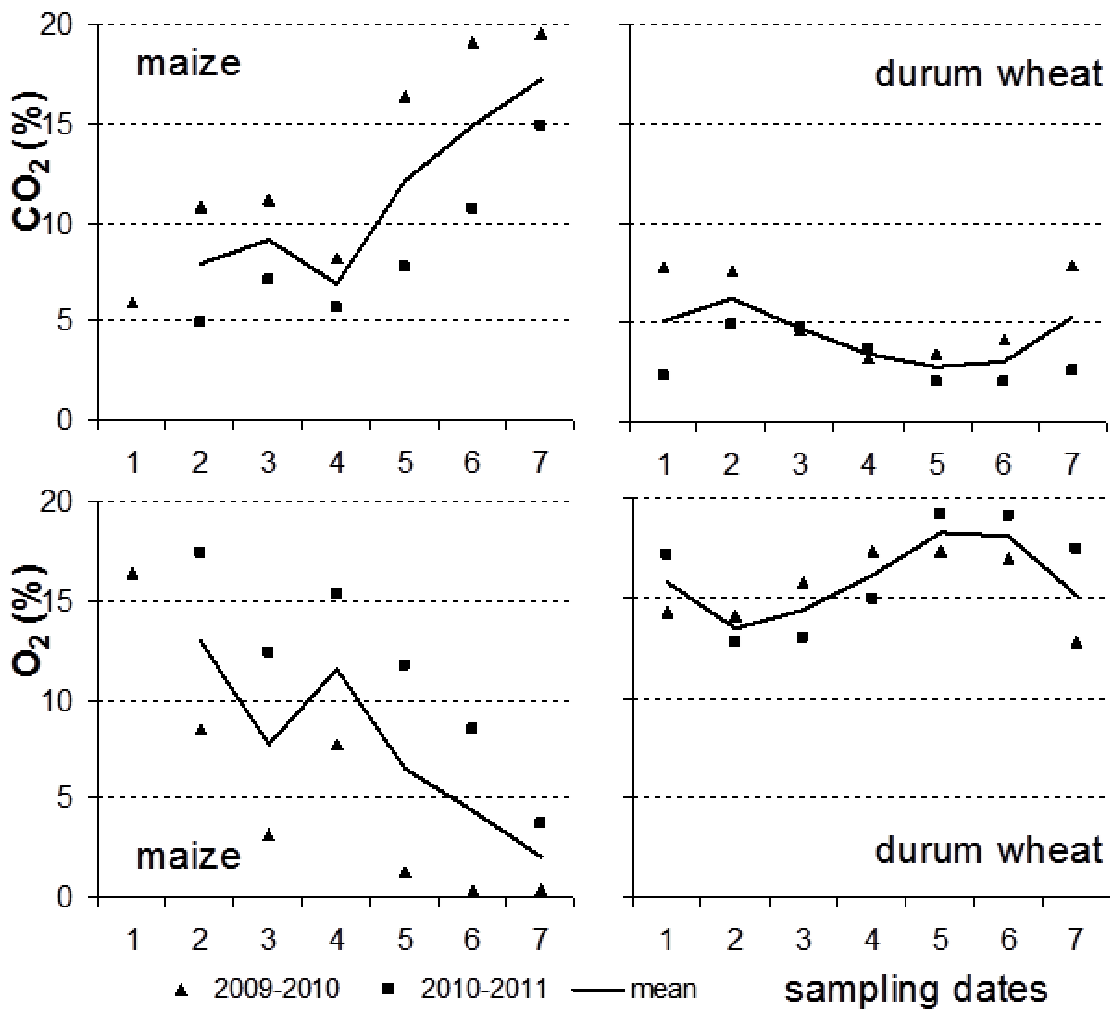

Fig. 5.1 Dynamics of carbon dioxide (CO_2) and oxygen (O_2) concentration in the atmosphere of silo bags filled with maize and durum wheat during storage; the line represents the mean of the two years (2009-2010 and 2010-2011).

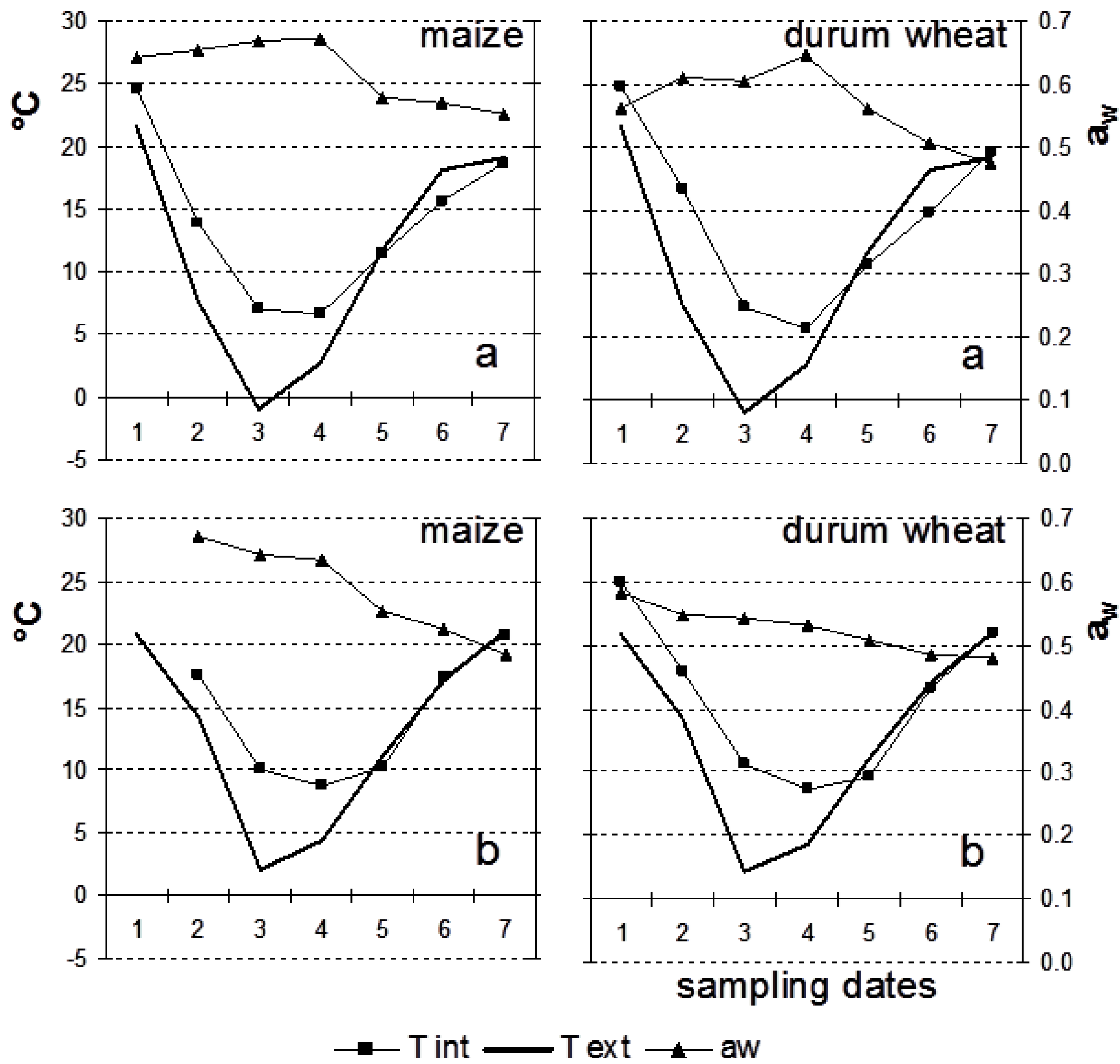

Fig. 5.2 Dynamics of water activity and temperature (external and internal) in maize and durum wheat stored in silo bags in 2009-2010 (a) and 2010-2011 (b).

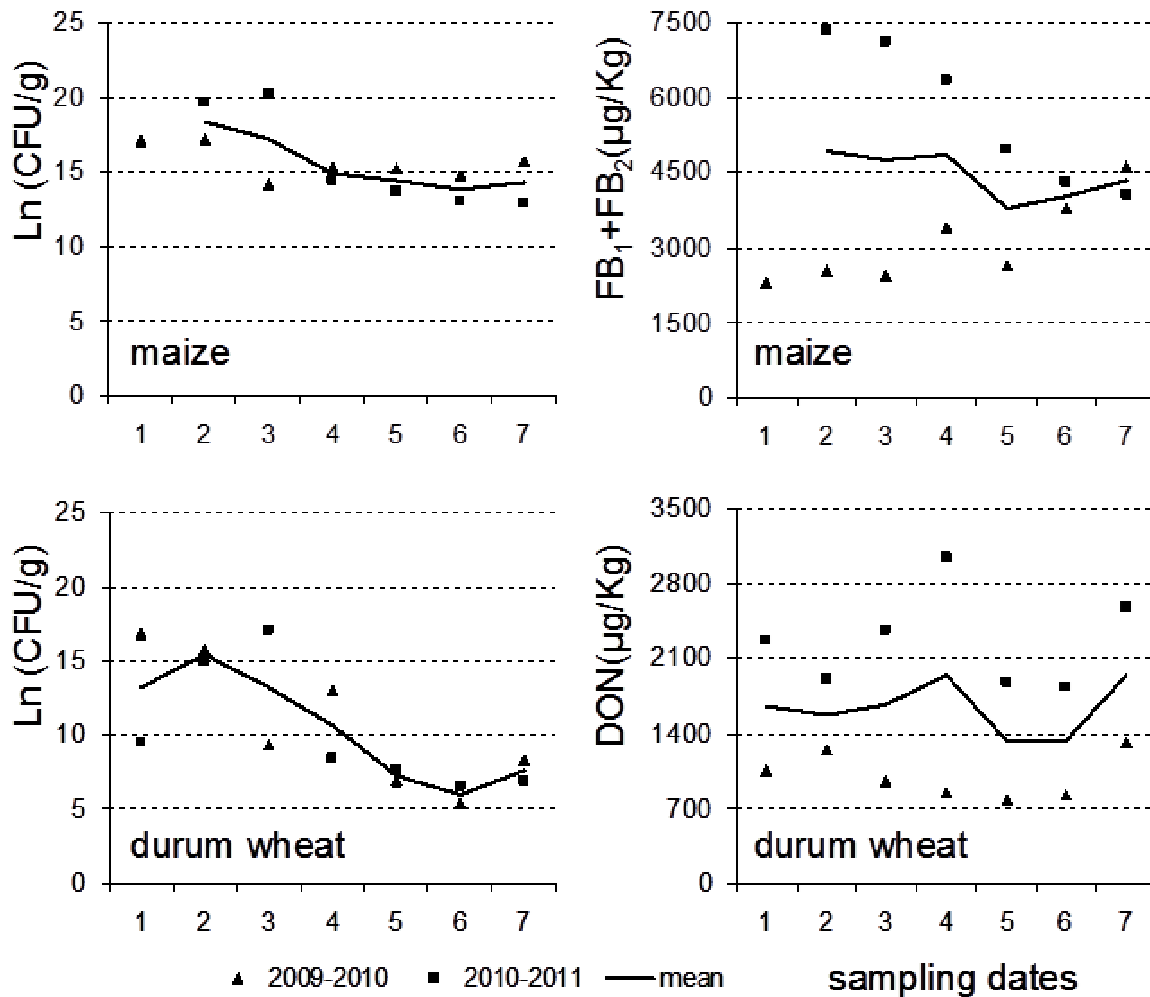

Fig. 5.3 Dynamics of *Fusarium verticillioides* and fumonisin in maize and *F. graminearum* and deoxynivalenol in durum wheat stored in silo bags during the two years of the trial: 2009 (▲) and 2010 (■) and their mean (─).

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CHAPTER 6

Conclusions and Perspectives

The aim of this work was the study of the pathosystem *F. verticillioides*-maize to better understand the role of environmental and ecological conditions on fungal development and on fumonisin production, both in the free and hidden forms.

Since the dough maturity stage, a strong infection incidence was just present on maize plant in field, remained almost constant till to the harvest time. On the other side, fumonisin accumulation, both in the free and hidden forms, continued to increase, reaching the maximum level at harvest, underlining that kernels conditions are not inhibitory for toxin synthesis till to crop ripening. The level of contamination varied among hybrids, and even if it is mainly dependent on the environmental condition of the growing area, different hybrids from the same area showed comparable *Fusarium* incidence but very different levels of fumonisins contamination. Being genetic resistance not known in commercial hybrids, data strengthened the idea that differences in chemical composition of maize hybrid kernels could affect fumonisins synthesis.

The analysis of chemical composition and its changes during maize growing season, in relation to *Fusarium* development and fumonisin production, confirmed the key role of fatty acids on fungal metabolisms. Furthermore, the analysis of lipid profile showed the presence of some molecules derived from fatty acid (oxylipins) clearly present during the interaction *F. verticillioides*-maize. This molecule, together with other oxidized fatty acid derived molecules, have been recently recognize to be involved in the cross talk plant-pathogen. The ecosystem *F. verticillioides*-maize was investigated also *in vitro* to better understand the gene expression of both the plant and the pathogen in relation to the colonization (ergosterol synthesis), the reproduction (conidia production) and fumonisin synthesis. The pathogen is able to colonize the seeds in 9 days post inoculum, where the maximum of ergosterol content was found and no more ergosterol was produced in longer incubation. The conidia production and fumonisins synthesis reached the maximum 12 days post infection, confirming the relationship between these two pathways. The analysis of gene expression showed that the seeds are dead from day12,

because no amplification of the housekeeping gene was found. The expression of plant genes, involved in oxilipins synthesis, were or began to be overexpressed during incubation. On the other side, fungal genes involved in oxylipin synthesis showed activation during the incubation period, but they resulted under expressed compared to β-tubulin, the *Fusarium* housekeeping gene we considered.

Another critical point for pathogens and toxins contamination control in cereals is the management of the grain mass after harvest. Drying is a common practice for maize growers and it give the possibility to modulate harvest time, taking also into account mycotoxin contamination.. Two different drying treatments were tested, 70°Cx24h and 95°Cx9h, and they were both able to reduce fungal contamination, but fumonisin detected varied. The chemical changes of the matrix during drying treatment, like for example starch granules gelatinization, seemed to cause the release a quote of the hidden fumonisin.

Storage is another critical point to be managed to safeguard grain quality. If good storage conditions are not guaranteed, the grain mass could face spoilage and quality reduction. The storage system represents a cost, that could vary based on the technology, so growers are always interested in new low cost methods. In the last ten years a new technology, silo bags, became of great interest among growers because of the reduced investment, the versatility and the effectiveness of the methods. The micro aerobic atmosphere, created inside the silo bag by the grain mass respiration, is able to inhibit spoilage, both caused by fungi and insects, and it has also an inhibitory effect on toxin production.

Many interesting results have been obtained on the ecosystem *F. verticillioides*-maize, the dynamic of infection and of fumonisin production and masking during the cultural season, the environmental conditions that can favor or inhibit the fungal development

and the production of fumonisin have been investigated. However, it could be interesting to improve the knowledge of other aspects of this ecosystem.

In particular, it would be interesting to define:

- The complete set of signal molecules, produced by the plant, that can act a role in affecting fungal metabolism, to define a signature profile and how this profile changes during pathogen infection;
- The signature profile for the fungal pathogen *Fusarium verticillioides*;
- The chemical characteristics that give resistance or susceptibility to different maize hybrids with respect to *Fusarium* infection and fumonisin production and masking;
- The prediction on the proportion of hidden fumonisins to better take into account their contribute to total ingestion.