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S.S.D: AGR/07

Genome Wide Association Studies to identify genes for resistance to *Fusarium* Ear Rot in maize

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Academic Year 2015/2016

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Abstract

Fusarium verticillioides is the causal agent of *Fusarium* ear rot (FER) in maize and contaminates grains with fumonisin, a family of mycotoxins involved in several human and animal diseases. Quantitative genetic variation exists for resistance to FER and fumonisin contamination among genotypes, however, resistant maize hybrids are currently not available.

The aim of this work was the identification of genetic markers associated to resistance against *F. verticillioides*.

A bioassay was used to screen inbred lines of the maize association population for FER resistance, GWAS and candidate gene approaches were applied to identify markers.

GWAS was performed using a 227K SNP matrix and resulting in 206 significant markers.

Genes involved in *F. verticillioides* response in developing maize kernels were retrieved from a previous RNASequencing study while maize *R* genes were retrieved from scientific literature. Resistant (CO433 and CO441) and susceptible genotypes (CO389 and CO354) were selected to amplify and sequence candidate genes. Polymorphisms detected were used to find association with phenotypes scored using the bioassay. Four significant markers were found.

Finally, the correlation between FER phenotypes scored in field experiments and bioassay phenotypes was investigated. A population of 172 RILs (CO441 x CO354), was tested. No correlation was found.

KEYWORDS: Maize, *Fusarium verticillioides*, Genome Wide Association Studies, Candidate genes, SNPs, Rolled Towel Assay.

Abstract

Fusarium verticillioides è l'agente responsabile della Fusariosi della Spiga del mais, contamina la granella con fumonisine, micotossine responsabili di diverse patologie umane e animali.

Per la resistenza alla fusariosi e all'accumulo di fumonisine esiste variabilità tra genotipi diversi ma non sono ancora disponibili ibridi immuni.

L'obiettivo di questo lavoro è stato quello di individuare marcatori associati alla resistenza a *F. verticillioides.*

Mediante un bioassay è stato testato un association panel per la resistenza a *F. verticillioides.*

Al fine di identificare i marcatori di resistenza sono stati applicati un approccio GWAS e uno per geni candidati.

L'analisi GWAS è stata eseguita con 227K SNPs restituendo 206 marcatori significativi.

Da un lavoro di RNASequencing sono stati individuati i geni coinvolti nella risposta a *F. verticillioides* mentre i geni *R* sono stati recuperati della letteratura scientifica.

Genotipi resistenti (CO433 e CO441) e suscettibili (CO354 e CO389) sono stati scelti per individuare polimorfismi nei geni candidati da associare ai fenotipi rilevati mediante il bioassay. Quattro marcatori sono risultati significativi.

Infine, la correlazione tra l'incidenza della fusariosi rilevata in campo e mediante bioassay è stata analizzata in una popolazione di 172 RIL derivanti da CO441 x CO354, tuttavia, non è stata individuata alcuna corrispondenza.

PAROLE CHIAVE: Mais, *Fusarium verticillioides*, Genome Wide Association Studies, Geni candidati, SNPs, Rolled Towel Assay.

Introduction

1 Maize

1.1. Origins and systematics

Maize, corn or Indian corn, (*Zea mays*) is a monocot belonging to the *Maydeae* subfamily in the *Poaceae* family. Among all the cultivated species no one has an enigmatic evolutionary origin as maize (Iltis, 2000). The genus *Zea* is native to Mexico and Central America. The wild relatives of cultivated corn are known as “teosinte” or “teocintli” both annual or perennial. This term derives from the Nahuátl indian language; in Mexico teosinte in called “madre de maiz” (Iltis, 2000). Modern corn is derived from domestication of *Zea parviglumis* started about 10,000 years ago.

1.2. Morphology

The stem or stalk is generally 2-3 m tall, however short cycle varieties can be less than 1 m tall and tropical corn can reach 6 meters in high. Some varieties tend to produce tillers, this depends also on environmental factors such as climate, soil and growing conditions.
The number of leaves, mainly between 12 and 18, depends on the cycle of the variety and on tillers numbers; a leaf is usually between 30-150 cm in length and 15 cm in width.
Corn is a monoecious plant that develops two distinct inflorescences named tassel and ear.
The tassel is the male inflorescence, it is located at the upper end of the stalk. It is a panicle of different shape and flowers are grouped by two in spikelets. Each flower contains three stamens.
The ear is the female inflorescence and it is produced by an axillary bud along the stalk. The axis of the ramification bears some leaves called bracts that envelop the ear. The female spikelets are made of two flowers, only one is fertile. From each flower a long stile or silk develop quickly to come out from the top of the ear. The cob is the axis of the ear where female spikelets are fixed.
The kernel is the fruit-seed of the *Poaceae* family.
Corn kernels are attached to the cob by a short pedicel. From outside the kernel is formed by the pericarp (the fruit part), the aleuronic layer and near the pedicel is located the embryo which account for about 10% of the kernel; the remaining 85% of the kernel volume is filled by the endosperm which is the starchy reserve tissue of the kernel.
Several types of kernels exist and they are used to describe different kinds of corn:

- *Zea mays indentata* (dent corn): the kernel have a depression on the top formed during the drying of the soft starch at the kernel top while the lateral starch is more compact. This kind of corn is the most grown nowadays.
- *Zea mays indurata* (flint corn): the kernel hearth is made of soft starch surrounded by compact starch.
- *Zea mays saccharata* (sweet corn): this corn is not able to polymerize sugars into starch. When fully mature the kernel appears wrinkly.
- *Zea mays everta* (popcorn): kernels are very small, the starch structure is similar to flint corn but in this case the kernel reacts to the heat producing flakes.
- *Zea mays amylacea*: this corn is characterized by the presence of the waxy gene and the starch composition of the kernel is mainly due to amylopectin. Amylose extender hybrids present 70% of amylose.
- *Zea mays tunicata*: it is not cultivated as a crop. This maize was one of the first cultivated corn.

1.3. Economic importance of corn

Corn is the most cultivated cereal in the world (Poloni and Schirawski, 2014). It is grown on 183 million of hectares accounting for a production of 1 billion tons in 2014. In Europe nearly 19 million of hectares are cultivated with corn for a global production of 129 million tons. In Italy the land dedicated to this crop accounts for 869,947 hectares with a mean production of 10,6 tons per hectare and a global production of 9,239,545 tons (faostat.fao.org, 2016).
The plant is utilized mainly for human consumption and animal livestock feeding; it is also widely used for non-food application as bioethanol and biogas production, oil extraction and in the starch industry (Poloni and Schirawski, 2014).

1.4. Corn diseases

Maize is affected by an average of 100 pathogens, but only few are present in a given location depending on various factors and only a few of the diseases present tend to become severe. The extent of the damage caused by corn diseases, in a particular season, depends on several factors including the susceptibility of the hybrid to the specific disease, the level of pathogen inoculum present and the environmental conditions of that season. Most diseases are caused by microscopic fungi that affect the plant to gain nourishment. Symptoms vary depending on the fungus: color changing, leaf death, rotting of stalks and ear, abnormal development of tassel and ear and even

plant death. The most important and destructive diseases are leaf blight, stalk rots, ear and kernel rots, seedling diseases and sometimes bacterial and viral diseases. Diseases can occur at any growing stage from germination to maturity resulting in different yield losses depending on the growing stage. Disease losses are both quantitative, caused by plant death or reduced vigor of the plant, and qualitative, if there are changes in the quality of the harvested grains. An additional problem related to ear and kernel rot is the contamination of grains with mycotoxins that may be dangerous for human and animal consumption (Sweets and Wright, 2008; Ali and Yan, 2012).

1.4.1. Ear rots

A large number of fungi can attack and invade developing ears and kernels causing a various number of diseases classified as ear rots. Several fungi that are naturally present in the field can attack ears before harvesting affecting the quality and appearance of corn.

Field inspections are able to detect ear rots; fungal growth and contamination usually don't continue during storage if corn is dried and conserved properly after harvesting.

Ear rots usually occur when rainfall is above normal values from silking to harvesting. Exceptions to these requirements are represented by *Aspergillus flavus* and *Aspergillus parasiticus*: these fungi are favored by hot and dry conditions and drought stress during pollination enhances the infection by *A. flavus*.

Fungi associated with ear rots belonging to the genus *Alternaria* and *Cladosporium* are the causal agents of the "black corn"; *Diplodia maydis* and *D. macrospora* causes *Diplodia* era rot; *Fusarium verticillioides* is the agent of *Fusarium* ear rot (FER) while *F. graminearum* causes *Gibberella* ear rot; *Penicillium* is responsible of *Penicillum* ear rot or blue-eye; *Aspergillus* are the agent of *Aspergillus* ear rot; *Nigrospora* causes *Nigrospora* ear rot and *Trichoderma* is the agent of *Trichoderma* ear rot.

Ear rot severity is more intense in ears damaged by other agents, such as hail, birds and insects that allow a more easy fungal penetration.

Ears well covered by the husk and maturing in a downward position are less damaged by ear rots than ears maturing upright or with open husks. Ears with husk tight to kernels are more prone to ear rots (Sweets and Wright, 2008).

2. The genus *Fusarium*

The genus *Fusarium* was described and named for the first time by Link in the 1809 based on the fusiform shape of the macroconidia. The taxonomy inside the genus *Fusarium* changed several times during centuries, nowadays three different concepts of species, morphological, biological and phylogenetical, exist for the classification of these fungi. In 2006 the information of 70 species were summarized (Refai et al., 2015). Refai et al., (2015) reported 80 species of *Fusarium* that are able to produce toxic compounds known as mycotoxins. *Fusarium* species are generally found in fertile cultivated soil and are able to persist as hyphae or chlamydospores in plant residues or organic matter.

2.1. Ear rots caused by *Fusarium* spp.

Fusarium is a genus of filamentous fungi belonging to the family *Nectriaceae* of the *Ascomycota* phylum. Several *Fusarium* species are widespread pathogens of maize plants both in temperate and semi-tropical areas.

Fusarium affects the root system, the stem and ear causing yield loss thought to be around 10-30%. Maize fusariosis can be grouped in red and pink fusariosis.

F. graminearum, the asexual stage of *G. zeae*, is the main agent of red fusariosis or red ear rot. Pink ear rot is mainly caused by *F. verticillioides* associated with other *Fusarium* species (Logrieco et al., 2002).

2.1.1. *Fusarium* ear rot caused by *Fusarium verticillioides*

F. verticillioides (Saccardo) Nirenberg (synonym *Fusarium moniliforme*; teleomorph *Gibberella fujikuroi* MP-A or *Gibberella moniliformis*) (Bömke et al., 2008) is a hemibiotrophic fungus that is the causal agent of FER and other diseases that affect corn plants.

F. verticillioides belongs to the *G. fujikuroi* species complex that includes pathogens of important crops such as maize, rice, barley, sugarcane, sorghum, pineapple, mango and pine. *G. fujikuroi* species complex is composed by 11 sexually fertile biological species called mating populations (MP-A to MP-K) and anamorphic species in the sections *Liseola, Elegans* and *Dlaminia* (Bömke et al., 2008). All the members of this species complex are known to produce several mycotoxins and other secondary metabolites.

F. verticillioides is not host specific and can affect several species as sorghum, wheat, rice, oats, beans, cotton, asparagus, bananas, sugar beets, figs, stone fruits, several forages, and sugar cane (Bacon and Hinton, 1996).

F. verticillioides is the causal agent of ear and kernel rot of corn. This disruptive disease occurs, theoretically, in all places where corn is grown; the yield is significantly reduced in years with drought, hot climate and insect attack (Refai et al., 2015; Zila et al., 2013; Lanubile et al., 2010). The temperature range useful to *F. verticillioides* is between 10 and 35°C with 30°C as the optimal temperature.

F. proliferatum, *F. subglutinans* and other members of the *Liseola* section of *Fusaria* are minor causal agent of FER (Mesterházy et al., 2012).

2.1.2. *Gibberella* ear rot caused by *Fusarium graminearum*

F. graminearum (*Gibberella zeae*) is the principal causal agent of *Gibberella* ear rot (GER); this rot is typical of northern region with wet and less hot climate, the optimal growing temperature for *F. graminearum* is 25°C.

The distribution of *Fusarium* species changes each year according to the climate: during hot years, in northern climate areas, ears are infected by *Fusarium* species typical of southern region while in cold and wet years *Fusarium* species responsible of GER are found in southern areas.

From a single ear, or kernel, different *Fusarium* species can be recovered depending on weather condition of the growing area (Mesterházy et al., 2012). *F. graminearum* is the most relevant species of the genus *Fusarium* in central Europe, North America and Asia and it is spreading in northern Europe (Refai et al., 2015).

2.2. Symptoms of *Fusarium* diseases

Fusarium can affect the entire corn plant. It is associated with seed rot and seedling blight, stalk rot and ear rot (King and Scott, 1981).

Seed rot and seedling blight: *Fusarium* and other fungi such as *Pythium, Rhizoctonia solani,* and *Penicillium oxalicum* are responsible of these diseases.

Seed rot occurs before germination, the seed is overgrown by fungi and tends to decompose rapidly.

Seedling blight occurs after germination and can be pre-emergence or post emergence.

In pre-emergence the seedling is killed before its emergence from the soil, the seedling turns brown, the coleoptile and root system tend to rot. In post-emergence the seedling emerges from the soil

before turning from green to yellow and die. Brown lesion and mold can appear on decaying tissues. The seedling root system is poorly developed (Sweets and Wright, 2008).

Stalk rot: it is one of the most destructive diseases of corn worldwide. Yield losses due to stalk lodging can be consistent if harvest is delayed or if windstorm happens. Several pathogens are responsible of stalk rot. *Fusarium* stalk rot causes decaying of root, crown and stalk. Disease symptoms start after pollination with death of the bottom leaves of the plant. Rotting is more severe as the plant mature. The stalk tends to decompose and the pit assumes a pink-salmon color. *Fusarium* stalk rot tend to prevail during warm and hot conditions (Sweets and Wright, 2008).

Gibberella stalk rot is caused by *F. graminearum*, basal leaves turn to a gray-green color, internodes are softened and pink-reddish discoloration appears in the diseased stalks (Sweets and Wright, 2008). This disease is favored by rainy weather and high values of relative humidity. Early attacks result in stunted ears since the translocation of photosynthesis products is more difficult.

Ear rot: on the ear *F. verticillioides* is associated with two different symptoms.

Affected kernels can be found alone or in groups scattered through the ear, in some cases the entire ear can be compromised. Usually a whitish-pink or lavender fungal growth on kernels or silk can be found.

The other symptom is referred as starburst: white streaks appear on the kernel. Fungal hyphae develops in the kernel pericarp starting from the silk attach point to the kernel pedicel.

Rotted kernels can be removed during harvesting with proper adjustment of the combine (Munkvold and Desjardins, 1997; Lanubile et al., 2010).

In some cases *Fusarium* can be present inside the entire plant as a symptomless endophyte. The plant is apparently healthy, but kernels can be contaminated by the fungus (King and Scott, 1981; Munkvold and Desjardins, 1997). Symptomless infected kernels are difficult to remove at harvesting.

Gibberella ear rot can be readily identified peeling back the husk and looking the ear. The diseased ear is characterized by a pink-reddish mold that starts at the tip of the ear and develops toward the base. If the ear is severely affected the mold is widespread, the silks and husk are adherent to the ear and mold is present between kernels and husk. With the exception of very susceptible hybrids or when the infection occurs early during ear development, the disease affects only a part of the ear. Although the mold has usually a reddish-pinkish color it can appear yellow to yellow-orange or yellow-red and it may develop also around any kind of ear injuries. Cool and wet weather conditions immediately after silking are favorable for GER development (Sweets and Wright, 2008; Woloshuk and Wise, 2010).

2.3 Ways of infection

Corn plant can be infected by *Fusarium verticillioides* in several ways as shown in **Figure 1**.

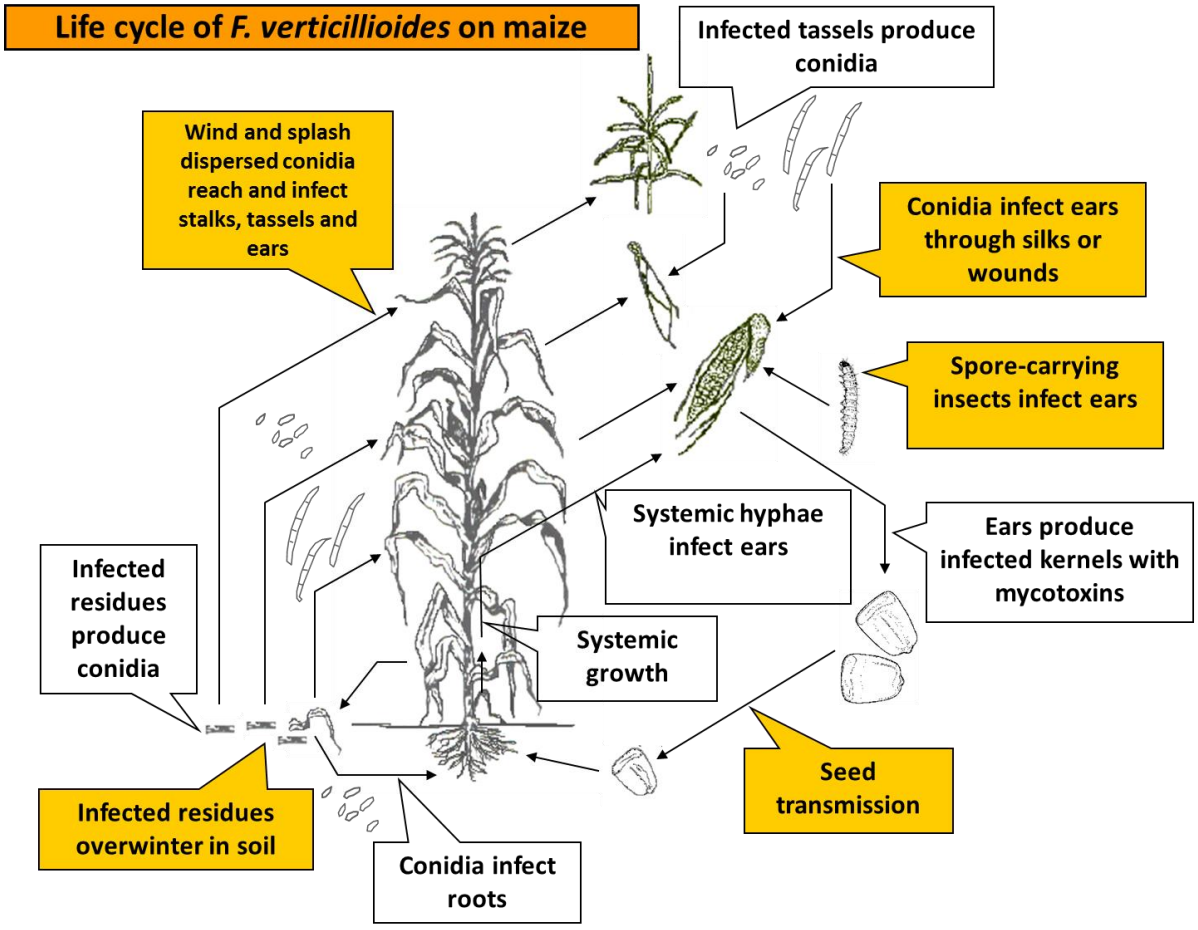

Figure 1. The multiple pathways of infection available to *F. verticillioides* to penetrate in maize plants (Battilani et al., 2003).

Fusarium can survive on infected plant residual and then spread to new plants.

- Seed: this kind of transmission is associated mainly with seedling disease, strains originated from the seed can be found throughout the entire plant. The presence of *Fusarium* infection of the seeds can result in seedlings with no apparently detrimental effect.
- Soil: fungal inoculum can be conserved on infected residues in the soil. When the climatic conditions are favorable the fungus can start growing and came in touch with young corn roots causing infection.
- Silk: silk infection can happen by air-born or water-splashed conidia developed on other plants or crop residues. The moisture of the silks and rainfall at the end of the growing season can help silk infection.

- Insects: insects have an important role in *Fusarium* infections creating wounds where infection conditions are optimal, in this case infection is caused by airborne or water-splashed inoculum after insects injuries. All the plant is subjected to insect damages: the stalk is weakened by insect galleries and fungal growth, while the ear is compromised by galleries and *Fusarium* development on the kernels (Munkvold and Desjardins, 1997).

  These insects are usually referred as corn borers, they feed on the pith, leading weakness, and ear causing yield losses and promoting fungal infection. Different insect species are associated with these symptoms depending on maize growing area (Samayoa et al., 2015).

  One of the most important species is *Ostrinia nubilalis* of the Crambidae family. This insect, native of Europe, has been introduced in North America, it is now spread in all the Boreal Emisphere from tropical to temperate environments. This insect can feed upon the entire plant, the worst damages are caused on the stalk, ear peduncle and kernels. Damaged ears are susceptible of secondary infection by *F. verticillioides* and other fungi.
- Other injuries: birds and hail damages offer to fungi a possible way of penetration (Mesterházy et al., 2012).

2.4. *Fusarium verticillioides* as endophyte

F. verticillioides can act both as dangerous corn pathogen, creating yield loss and threatening human and animal health, or as endophyte. Endophytic fungi represent diverse taxa that inhabits plant host without disease symptoms. One of the most famous case is that of tall fescue (*Festuca arundinacea*) infected by *Epichloë cenophiala* (*Neotypodium coenophialum*) (Mulkvold and Desjardins, 1997). Infected plants have beneficial effect increasing root development gaining drought tolerance and mineral uptake, moreover the endophyte produces toxic compounds that protect the grass from herbivorous animals.

It is not clear if the relationship between corn and *Fusarium* is beneficial. It results in animal toxicoses and several times the infection is highly detrimental for corn plant. However, it is reported that *F. verticillioides* is able to protect corn plant against other fungal infections. If corn seedlings are simultaneously infected by *F. verticillioides* and *Ustilago maydis*, common smut growth is negatively affected by *Fusarium* (Lee et al., 2009). Co-inoculation on the ears with *F. verticillioides* and *A. flavus* results in less *Aspergillus* growth than in ears infected only with *A. flavus* (Zummo and Scott, 1992); furthermore, *F. verticillioides* can protect corn seedlings from *Fusarium graminearum* infection (Van Wyk et al., 1988).

The role of endophytic infection is still unclear because *Fusarium* can be present in ubiquitous ways and it is difficult to establish *Fusarium*-free plants (Munkvold and Desjardins, 1997).

3. Mycotoxins

Mycotoxins are secondary metabolites produced by several species of fungi, and a species can produce several mycotoxins. These molecules can be dangerous to humans and animals by ingestion, inhalation or skin contact. Mycotoxigenic fungi can affect several plants and row materials and fungi affecting cereals are a main concern. It is reported that 25% of the world cereals amount is contaminated by mycotoxins; nuts, spices, fruits and related by-products can be contaminated too. In the U.S. it is estimated that $932 million are lost as mycotoxins contaminated crop products (Smith et al., 2016).

Several fungi are able to produce mycotoxins: Aflatoxins (AFs) is produced by *Aspergillus* spp., Ochratoxin A (OTA) is produced by *Aspergillus* and *Penicillium*, ergot alkaloids are produced by *Claviceps* spp. and altenuene, alternariol, alternariol methyl ether, altertoxin, and tenuazonic acid are produced by *Alternaria* species.

Fungi of the genus *Fusarium* are responsible of several mycotoxins production, the most common are fumonisins (FUM), trichothecenes (TCTs) especially deoxynivalenol (DON), zearalenone (ZEN) and some emerging mycotoxins, known as enniatin, fusaproliferin, moniliformin, beauvericin.

Mycotoxins can be found alone or in combination with others produced by different species (Marin et al., 2013).

The main concern about mycotoxins production and accumulation is the ears, but mycotoxins formation in rotted stalks, infected leaves and the whole plant can represent a risk for forage and corn-silage (Logrieco et al., 2002).

3.1. Fumonisins

Fumonisins were isolated for the first time in 1988 by W.C.A. Gelderblom from cultures of *F. moniliforme* (sin *F. verticillioides*) (Munkvold and Desjardin, 1997).

The structure is similar to that of sphinganine which is the precursor of sphingolipids.

Fumonisins are produced by species in the *Liseola* section as *F. verticillioides* and *F. proliferatum*; *F. napiforme, F. dlamini* and *F. nygamai* are also fumonisin producers.

At least 12 different fumonisins are known, the most important are those of the B series (B1, B2 B3). From a toxicological point of view FB1 is the most relevant; the other three series of fumonisins are A, C and P (Logrieco et al., 2002; Marin et al., 2013).

Fumonisins, particularly the B1, strongly inhibit the enzyme ceramide synthase (CER) that catalyzes the acylation of sphinganine and the recycling of sphingosine. This increases the level of intracellular sphinganine and other sphingoids molecules with cytotoxic effect (Marin et al., 2013).

Fumonisins are the most common mycotoxins in maize grown in temperate condition, they are produced before harvesting and during the earliest stage of storage, with proper drying and storage the water activity in the grain is too low to allow *Fusarium* growth. Fumonisins are heat stable until 150°C, and partially degraded during fermentation.

FBs are known for their acute and chronic toxicity and FB1 has been classified by the IARC as possibly carcinogenic for humans (Marin et al., 2013).

FBs don't have a high acute toxicity even though some animals can show symptoms of acute intoxication. Liver and kidney are the target organs, but differences can occur according to the species, sex and race. In horses, and related species, FB-contaminated feed ingestion is recognized as the cause of leukoencephalomalacia: a brain fatal disease that presents liquefactive necrotic lesion in the white and gray matter of the brain. It is lethal just few days after consumption. In pigs FBs are associated with cardiotoxic effects and pulmonary oedema (Desjardins and Proctor, 2007; Marin et al., 2013).

In humans it is associated with oesophageal cancer in South Africa and liver cancer in China. Closed to the Mexico-Texas border FBs are associated with the neural tube defects that can result in fetal death. In this case the administration of folate may reverse the toxic effect of FBs. Therefore, the problem of FBs contaminated food during pregnancy remains for populations which rely on a corn based diet and with low folate intake (Marin et al., 2013).

In their experiment Williams et al. (2007) studied the effect of fumonisin on maize seedlings of the variety Silver Queen. They found that after 13 days of watering the plants with a solution of fumonisin, at different concentrations, leaf lesions and stunting were visible on seedlings. Plants treated at higher fumonisin levels showed increasing symptoms and had a reduced development. Moreover, in the same experiment, they were able to see leaf lesions only in seedlings inoculated with fumonisin producing strains of *F. verticillioides*. This means that fumonisin are an important contributor to the amount of disease levels and they are required to induce the leaf lesion typical of *Fusarium* seedling disease (Williams et al., 2007).

In filamentous fungi genes involved in the synthesis of mycotoxins, and other secondary metabolites, are often organized in clusters (Proctor et al., 2003). Fumonisin pathway derives from a gene cluster of 42 Kb consisting of 15 co-regulated genes (Desjardins and Proctor, 2007).

Fumonisins are synthesized by enzymes encoded by the fumonisin biosynthetic (*FUM*) gene cluster located on Chromosome 1. These genes are designated *FUM1*-FUM3 (previously *FUM5*, *FUM12*, and *FUM9*), *FUM6-FUM8*, *FUM10*, *FUM11*, and *FUM13-FUM19* (Butchko et al., 2006). *FUM1*,

FUM6 and *FUM8* are essential genes in fumoinisin biosynthesis since their deletion blocked mycotoxins accumulation. Several gene-deletion studies have been performed to investigate the role of each gene-product on the fumonisin biosynthetic pathway. The synthesis starts from acetate which is converted to mycotoxins during several reaction steps. Most of field isolates of *F. verticillioides* produce four fumonisin analogs: B1, B2, B3 and B4. As shown by **Figure 2**, fumonisin B1, which is the most important analog from a toxicological perspective, is derived by fumonisin B3 while the B2 analog is derived by the B4. *FUM3* is the gene responsible of the end of the synthesis of fumonisin, and its deletion produces the B3 and B4 analogues that lack the OH group on the C_5 (Butchko et al., 2006).

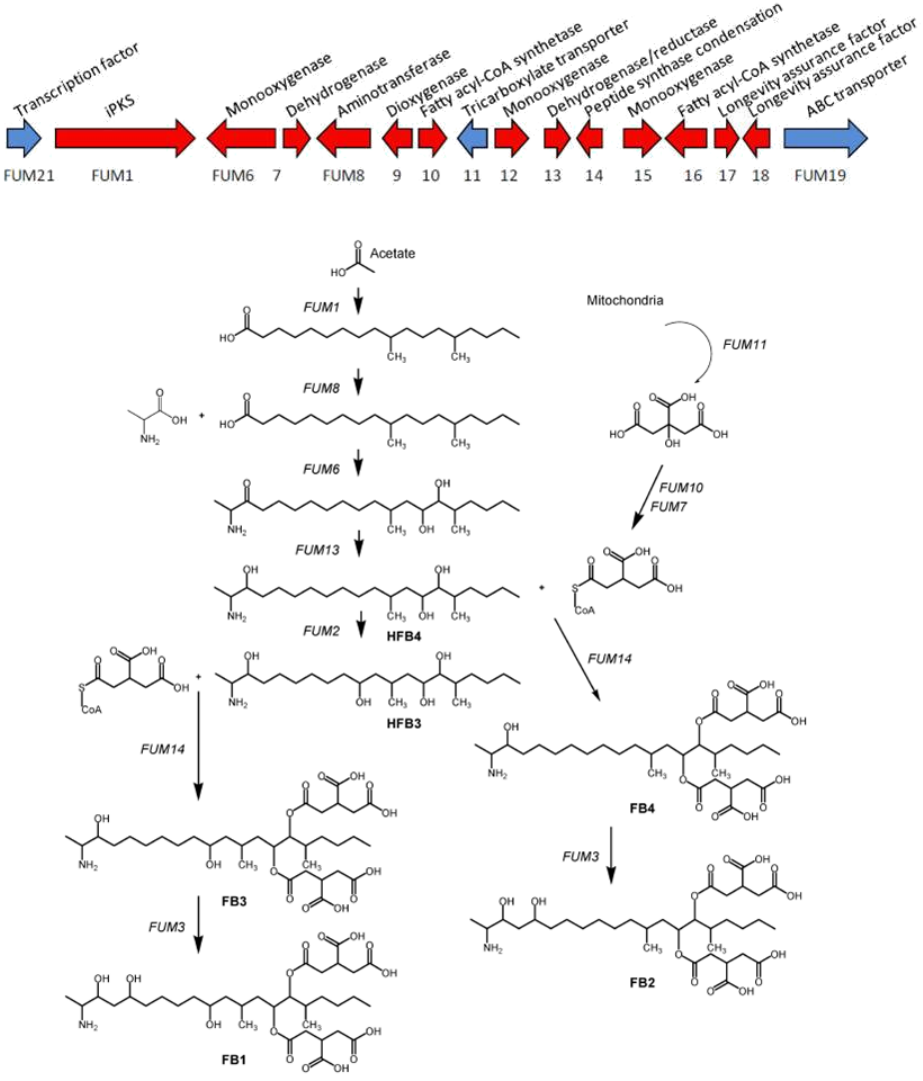

Figure 2. Proposed fumonisin biosynthetic pathway (Butchko et al., 2006).

3.2. Zearalenones

Zearalenones (ZEN) have not been associated with fatal mycotoxicoses and have no acute toxicity. Chemically they are non-steroidal estrogenic compounds derived by the cyclization of resorcyclic acid lactone and are structurally similar to antibiotic metabolites synthetized by several fungi (Desjardins and Proctor, 2007).

They are produced by several *Fusarium* species: *F verticillioides*, *F. graminearum*, *F. equiseti*, *F. culmorum*, *F. cerealis* and *F. incarnatum.*

The pathogenesis starts in the field in cool and moist conditions, but can continue also in post-harvest if storage conditions are inadequate. ZEN are heat-stable toxins that exert their toxicity associated with the reproductive cycle due to a greater affinity to estrogen receptor (Marin et al., 2013).

3.3. Trichotecenes

Trichothecenes comprise a large family of compounds derived by cyclization of sesquiterpene with several alternative possible esterification or oxygenation at various positions (Desjardins and Proctor, 2007); at least 170 trichothecenes have been identified and divided in four classes (A-D). These molecules are generally stable and not degraded by storage, milling, cooking or high temperature exposure (Marin et al., 2013).

These toxins are strongly associated with chronic and fatal toxicoses in humans and animals all over the world. Trichothecenes toxicity is explicated at the ribosomal level with protein synthesis inhibition by binding to the 60S ribosomal subunity and inhibiting the enzyme peptidyltransferase (Desjardins and Proctor, 2007; Marin et al., 2013).

One of the most known trichotecens is deoxynivalenol (DON); the principal producers of DON are *F. graminearum*, *F. culmorum* and *F. cerealis* typically found in temperate climate.

3.4. Other *Fusarium* toxins

These toxins include other secondary metabolites like fusaproliferin, enniatins, beauvericin, moniliformin (Marin et al., 2013), butenolide, equisetin and fusarins (Desjardins and Proctor, 2007); few information are available on these new toxins because of their recent identification and recognition as mycotoxins.

Fusaproliferin is a sesterpene toxic for humans, insect cells and chicken embryos. It is produced by *F. proliferatum* and *F. subglutinans*.

Enniatines and beauvericin are non-ribosomal, cyclic hexadepsipeptides with cation chelating, ionophore and antibiotic activity (Desjardins and Proctor, 2007; Marin et al., 2013); they are apolar cytotoxic molecules that enter the cellular membranes disturbing the ionic balance (Marin et al., 2013).

Moniliformin is a strong acid produced by *F. verticillioides, F. subglutinans, F. avenaceum, F. tricinctum* and *F. proliferatum*, it inhibits thiamine pyrophosphate dependent enzymes interrupting the gluconeogenesis; it is also able to inhibit glutathione peroxidase and reductase (Marin et al., 2013).

Butenolide is an acid lactone produced by *F. graminearum* and others trichotecens-producing *Fusaria*; it is associated with noninfectious problems in cattle (Desjardins and Proctor, 2007).

Equisetin is derivative of *N*-methyl-1,4-pyrrolidone; it is of interest because of its role against the human immunodeficiency virus. It is produced by *Fusarium equiseti* and *F. semitectum*.

Fusarins are 2-pyrrolidones with different substitutions. They are produced by *F. verticillioides* and *F. graminearum*; fusarin C resulted mutagenic in the Ames test while fusarin A and D are not, however, the role of this toxins in human or animal disorder remains unclear (Desjardins and Proctor, 2007).

4. Breeding for *Fusarium* ear rot resistance

Few rescue treatments are available for corn diseases and fungicides are not fully efficient to control the mycotoxin risk and because their harmful effects, on human health and environment, their use should be seriously restricted in the near future (Sweets and Wright, 2008; Atanasova-Penichon et al., 2016). To durably solve the problem plant breeding for disease resistance is an important and environmentally safe method to control plant diseases and reduce mycotoxins in the grain chain (Santiago et al., 2015). A deeper understanding of the corn-*Fusarium* pathosystem will provide relevant information for plant breeding.

Fusarium ear rot resistance and fumonisin accumulation in maize have a highly complex genetic base and low heritability measured on single plant but resistance calculated on family mean is moderately-high heritable. Moreover genotypic correlation between disease resistance and fumonisin contamination is reported. This means that selecting visually for disease resistance results in low-fumonisin containing material (Zila et al., 2013).

Resistance to *Fusarium* depends on plant ability in contrasting initial infection and containing the development of the fungus; resistance to mycotoxin contamination is linked to the capacity of host tissues in counteracting accumulation of fungal toxins. This capacity is the result of two mechanisms: the metabolic transformation of the toxin in any less toxic molecule and the inhibition of toxin biosynthesis due to the action of host metabolites (Atanasova-Penichon et al., 2016).

Five main types of resistance have been classified; mechanisms associated to any one of the five steps may be host specific:

I. Resistance against the infection of the flower: silk resistance in maize.
II. Resistance to limit the spread of the infection within the host plant.
III. Resistance to grain infection.
IV. Tolerance and ability to maintain yield.
V. All mechanisms useful for resistance to mycotoxin accumulation.

The last resistance step can be divided in two components. The first (V-1) represents resistance to toxin accumulation operated by metabolic transformation performed by enzymes like UDP-glycosyltransferase, gluthathione-S-transferase or cytochrome P450 mono-oxygenase. The second (V-2) corresponds to resistance due to the inhibition of mycotoxin biosynthesis operated by plant endogenous compounds both constitutively produced or induced by pathogen infection (Atanasova-Penichon et al., 2016).

The success of maize breeding for *Fusarium* resistance relies on the variability observed about *Fusarium* infection and fumonisin accumulation in diverse maize materials.

Research and breeding to improve resistance to ear rots and mycotoxin contamination should focus on:

- Accurate techniques for phenotyping ear rots and mycotoxin contamination.
- Identification of novel host germplasm sources and resistance traits.
- Development of genomic technologies for marker assisted selection (MAS) programs.
- Application of biotechnological approaches to breeding for reducing susceptibility to ear rot and mycotoxin contamination (Lanubile et al., 2014a).

The identification of the characteristics linked to *Fusarium* resistance and low fumonisin accumulation is fundamental to perform indirect selection of developing materials to increase plant resistance. Several phenotypic traits easy to select are linked with *Fusarium* resistance (see 4.2. paragraph).

4.1. Artificial infection and phenotyping techniques for *Fusarium* ear rot

There are no defined criteria on how to perform selection for ear rot resistance (Lanubile et al., 2014).

The selection for sources of *Fusarium* resistance can be performed under natural infection condition when the presence, dispersion and quantity of fungal inoculum are ensured and then making infection rates of the ears at harvest maturity.

Since under natural infection many factors can contribute to inoculum dispersion, in the case of *F. verticillioides* wind, rain splash and insect larvae, it is necessary to screen materials under artificial inoculation field experiment where is possible to control the amount and time of inoculation. The most used way for *F. verticillioides* inoculation is through the silks rather than through the kernels. There are several issues regarding the more appropriate inoculation technique, time and inoculum amount and inoculation techniques are not equally efficient (Lanubile et al., 2014).

The best inoculation protocol results in adequate ear infection to discriminate among resistant and susceptible genotypes without resulting in fungal overgrowth that makes differences not appreciable.

Artificial inoculation techniques can be divided in two groups, if there is or not mechanical assistance.

- Toothpick method and derivatives: a toothpick colonized by fungal mycelium is inserted in in the silks channel or in the ear through the husk perpendicularly to the axis and midway from the ear base to the top. Toothpicks or other devices are left in the ear until harvesting.

- Pinbar-inoculation: devices similar to forks or with sewing pins are immersed in the spore suspension and then inserted in the kernels through the husk (King and Scott, 1981) and a different number of kernels is inoculated according to the device used.
- Spray: a spore suspension is sprayed on the silks after flowering, allowing the fungus to grow in the silks channel and colonize the ear.
- Ear-tip inoculation: the inoculum is dispensed to flood the kernels at the tip of the ear (Chungu et al., 1996).
- Injection: the spore suspension is injected in the silks channel or through the husk into the kernels (Lanubile et al., 2014); a drill can be used to create a wound in the cob tip to insert the inoculum (Chungu et al., 1996).
- Exposed kernels: on one side of the ear the husk is peeled back to expose kernels that are subsequently sprayed with the inoculum, the bracts are then repositioned, secured and the ear is covered with bags to prevent bird damages (King and Scott, 1982). This method was proposed for *A. flavus* evaluation.
- Pipe-cleaner inoculation: a nail is used to pass through the husk and kernels to reach the cob and create a tunnel; subsequently a pipe-cleaner saturated with spore suspension is inserted in the tunnel (Chungu et al., 1996).

Chungu et al. (1996) proposed also an inoculation method that combines wounding the ear and then spraying the inoculum on the wounded area.

Methods based on mechanical devices simulate insect attack; these methods are able to screen materials for their ability to counteract the pathogen during spreading from the inoculation point in other tissues. Inoculation methods that not require mechanical damages of the plant simulate the natural inoculation by wind or rainfall splash and can discriminate the germplasm based on his ability to counteract initial penetration.

Regarding the time of inoculation it is performed early (4-6 days) or late (19-23 days) after silking: sooner inoculation results in increased disease severity, while late infection results in lower disease (Lanubile et al., 2014a).

Another method, called Rolled towel assay (RTA) is available to screen germplasm although it has not been applied to screen maize kernels for disease resistance. RTA is a bioassay, a technique that uses living organisms, from cells to whole organisms, to determine the effect and biological activity of substances. This assay is useful to determinate the toxicity of substances. RTA has been applied to test soybean genotypes for resistance to *F. graminearum* and aggressiveness of *F. oxysporum* isolates (Ellis et al., 2011; Ellis et al., 2012; Ellis et al., 2014; Lanubile et al., 2015); to test the effect of strain of Ni-resistant and plant promoting bacteria on seedling growth of Indian mustard (*Brassica juncea*) (Rajkumar and Freitas, 2008) and to test the promoting role of *R. solani*

antagonist in pepper (Rajkumar et al., 2008). This assay implies the placing of a certain number of seeds on germinating paper, inoculating the seed with the substance or fungal strain of interest and after a proper time of incubation to score the effect on seed germination and seedling growth between inoculated seeds and non-inoculated control seeds.

4.2. Plant characteristics useful for *Fusarium* resistance

Genetic variation on resistance level to FER is reported for inbred and commercial maize hybrids, but there is no evidence of complete immune genotypes. Several lines are reported to show good levels of resistance against FER, in some cases these lines cannot be used for direct breeding because they are unadapt to the environment (tropical lines have difficulties in flowering in temperate maize growing area) or they are agronomically unacceptable (Lanubile et al., 2014a).

4.2.1. Earliness

Short cycle lines and hybrids reduce disease susceptibility as they mature rapidly and kernel moisture drops below minimum levels required for pathogen growth. On the opposite side, in late maturing hybrids kernel moisture decreases slowly below the threshold required for fungal growth. Irrespectively to class maturity, hybrids with a slow kernel humidity decreasing rate are the most prone to FER and consequently fumonisins accumulation (Santiago et al., 2015).

4.2.2. Husk coverage

Husk coverage has been strongly related to *F. verticillioides* susceptibility and generally to ear rot infection severity. The husk has the task to cover and protect the ear from the surrounding environment and reduce possible threats to developing kernels. If the tip of the ear is not well protected by bracts, it represents an easy entrance way for insects and pathogenic microorganisms (Santiago et al., 2015). Hybrids with tight and adherent husks are more resistant to ear rot (Lanubile et al., 2014a). The behavior of the ear during maturation may play a role: it is reported that ear maturing upright are more susceptible to ear rots than ears maturing downward (Sweets and Wright, 2008).

4.2.3. Silk characteristics

Maize silks are one of the possible infection ways used by *Fusarium* species to colonize the ear. Which are the exact conditions that allow silk-infection are not known, but silks moisture is a predisposing factor (Munkvold and Desjardins, 1997) and other physical or chemical characteristics of silks may play a key role in pathogenesis.

Retarded silks senescence is thought to be a resistance related trait for some genotypes, as silks can act as a barrier towards fungal penetration: inbreds with silks that are green and actively growing at the time of inoculation have less symptomatic and asymptomatic kernel infection (Lanubile et al., 2014; Santiago et al., 2015). On the other hand, Reid et al. (2002) report that accelerated senescence is favorable to resistance. Brown silks, having a reduced humidity, are favorable because the fungus is blocked by unfavorable conditions. Other authors reported that there is correlation between fumonisin contamination and duration of silking period and silks humidity. The amount of phenolic and flavones compound in silks is not an indicator of maize resistance to *Fusarium* though it is reported that after *F. verticillioides* infection there is the induction of production of 3-deoxyantocianidins in the resistant lines (Santiago et al., 2015).

4.2.4. Kernel characteristics

Kernel color can be associated with *Fusarium* resistance. Pericarp and cob color are controlled by the *P1* locus conferring pigmentation due to the accumulation of phlobaphene which is a flavonoid pigment.

Generally, yellow kernel hybrids accumulate less mycotoxins than white ones; however the opposite situation can be found in some years. Moreover, white kernel lines with comparable disease behavior to the most resistant yellow lines can be found (Santiago et al., 2015).

The accumulation of flavonoid pigments, particularly phlobaphenes, in kernels is able to reduce the accumulationof fumonisin B1, the development of pigmented sweet, pop and polenta corn varieties can help farmers to keep mycotoxin level under the established threshold for human consumption (Pilu et al., 2011).

Soon after pathogen infection there is the production of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) involved in signaling pathways. These molecules create perturbation of the oxidative status of the tissue that can interfere with fungal metabolism. Therefore, the role of some antioxidants in disease resistance was tested. Among the evaluated antioxidants, α-tocopherol, lutein, zeaxanthin (which is also a phytoalexin), lutein, ferulic acid and β-carotene, α-tocopherol is

more powerful than ferulic acid in inhibiting fumonisin production; although ferulic acid is more abundant than α-tocopherol in tissues (Santiago et al., 2015).

Moreover, the biosynthetic pathway of FBs and other mycotoxins is characterized by several oxygenation steps; therefore, all changes with the oxidative status of the tissue may interfere with toxin synthesis (Atanasova-Penichon et al., 2016) and explain the role of cereal secondary metabolite with antioxidant capacity in disease resistance mechanisms.

Phenolic acids are toxic to *Fusarium* species and many other fungi; the toxicity of these compounds may vary for different strains of the same species but it seems that the lipophilic propertiy of the phenolic compound is a key factor in antifungal activity (Atanasova-Penichon et al., 2016).

Flavones and flavanones are less toxic to *Fusarium* species than phenolic acids. Their toxicity is due to their ability to irreversibly bind nucleophilic amino-acid in fungal proteins; unsubstituted flavones and flavanones are more efficient antifungal substances than hydroxylated counterparts (Atanasova-Penichon et al., 2016).

Benzoxazinones, as DIMBOA and DIM2BOA, have an allelochemical function and provide protection against pathogenic fungi, bacteria and insects. *F. verticillioides* can detoxify benzoxazinones to 2-aminophenol (AP). The bacterium *Bacillus mojavensis*, an endophytic bacterium, is a valid help against *F. verticillioides* since it produces a pigment, 2-amino-3H-phenoxazin-3-one (APO) which blocks fungal AP production increasing host resistance (Lanubile et al., 2014a). *Fusarium* species responsible of FER and GER show a wide range of responses to 6-methoxybenzoxazolin-2(3H)-one (MBOA) and benzoxazolin-2(3H)-one (BOA): the most resistant are *F. verticillioides, F. subglutinans* and *F. graminearum* (Atanasova-Penichon et al., 2016).

Secondary metabolites have also an effect in mycotoxin production. Cinammic acid and derivatives are able to inhibit trichotecenes biosynthesis, flavonoids (rutin) are aflatoxin B1 inhibitor, but their role on fumonisin production is poorly understood. It is reported that sub-lethal doses of α-tocopherol are able to significantly reduce fumonisin synthesis (Atanasova-Penichon et al., 2016). It is necessary to consider that during kernel development the antioxidant profile is modified several times before harvest maturity. *Fusarium* infection usually happens sooner during ear development and the antioxidant onset that the fungus has to face is extremely different to that found in mature grains (Atanasova-Penichon et al., 2016).

Regarding the role of lipids in *Fusarium* resistance it is reported that polyunsaturated fatty acids released by the action of lipases on the cell membranes as a response to pathogen attack, have a role in plant-pathogen interaction as free fatty acids or as precursors of oxylipins. Fatty acids and oxylipins can prime the production of fumonisins: studies with the 9-oxylipin-deficient maize mutant have demonstrated that in the mutant the level of fumonisin is reduced by 200-fold with respect to the control (Santiago et al., 2015). Hybrids which accumulate linoleic acid in the kernel

are more susceptible to fumonisin accumulation than hybrids with a higher oleic-linoleic acid ratio (Dall'Asta et al., 2012).

In infected maize embryos several antioxidant enzymes were found as well as detoxification enzymes, protein-folding related proteins, lipid transfer proteins, ribosomal proteins, aldolases, dehydrogenases, glucanases and chitinases; glyoxalase, trypsin inhibitor, late embryogenesis abundant proteins and heat shock proteins can contribute to *F. verticillioides* resistance (Santiago et al., 2015).

Kernels of MI82 and Tex6 contain proteins that inhibit fungal growth and aflatoxin accumulation. A 14kDa protein in MI82 kernels may reduce fungal growth by limiting the production of α-amylase. In Tex6 kernels is reported the activity of chitinase and β-1,3-endoglucanase and a small peptide extracted from maize kernels is able to inhibits *in-vitro* germination and hyphal elongation of *F. graminearum* and *F. verticillioides* (Moore et al., 2004).

The pericarp is the protective tissue of the kernel thus it plays an important role in seed protection. It is reported that the stylar canal is the only way to enter the pericarp from outside. It was suggested the possibility to screen maize lines according the stylar canal and correlate this trait with kernel infection, although this kind of assay is difficult to perform on a large scale (Duncan and Howard, 2010)

The role of pericarp is important since it protects the starchy reserve of the grain and it is the last barrier before the fungus can reach the endosperm. A thin pericarp can be an unfavorable character, since it is easy to overpass by the fungus, however, contrasting evidences about thin or thicker pericarp are reported. It is reported that resistant and intermediate groups of lines have a thicker pericarp than susceptible inbred (Hoenish and Davies, 1994), while Ivic et al. (2008) report that there is no correlation between pericarp thickness and disease susceptibility in Croatian materials. Moreover, a thicker pericarp can reduce drying speed creating a comfortable environment for the fungus (Santiago et al., 2015).

Waxes present on pericarp surface are a good barrier to fungal penetration and lines with higher wax amount on the pericarp are more resistant to fumonisin accumulation.

Phenolic compounds are important in biotic and abiotic stress responses. Diferulates, formed linking two ferulate monomers, create a crosslink of cell-wall polysaccharides conferring hardness and forming a physical barrier against penetration. Fungal enzymes can break down diferulates producing ferulic acid that has a direct role in fumonisin production inhibition.

The endosperm is the starchy reserve of the seed and is an optimum substrate for fungal development and toxins production. In Europe the cultivated corn belongs to two different types: dent (*Zea mays* var. *indentata*) and flint (*Zea mays* var. *indurata*). Dent and flint corn differ on kernel morphology: flint kernels have a hard endosperm layer all around the soft core-endosperm,

while in dent corn there is no thick endosperm at kernel top. It is reported that flint corn is more resistant than dent corn to fungal infection, but different or opposite situation were also found (Santiago et al., 2015).

Several starch and protein mutants of commercial value exist in corn and they show different susceptibility. The waxy mutant (which accumulates only amylopectin in the kernel) is more susceptible than amylose extender, which accumulates more than 50% amylose. The *opaque-2* mutants that accumulate high levels of lysine and tryptophan in the endosperm are more prone to *F. verticillioides* infection, however, it is reported that *opaque-2* lines have less fumonisins than their corresponding wild type (Santiago et al., 2015).

Shrunken 2 (sh2) mutants accumulate sucrose in the kernel instead of starch thus increasing the susceptibility to *F. verticillioides* (Santiago et al., 2015).

4.3. Genes providing resistance to fungal infection

Some genes that provide resistance to *Fusarium* infection are known. The defective lipoxygenase *lox3* maize mutant accumulates low levels of fumonisin B1, reduces the conidiation of *F. verticillioides* and provides resistance to some other fungal species. The lipoxygenase pathway is responsible of the production of oxylipins that are involved in defense mechanisms. In lines resistant to *Gibberella* ear rot there is a higher level of expression of a guanylyl cyclase.

The maize gene *An2* encodes a protein similar to *An1* that is involved in gibberellin production. Even though *An2* is induced by *Fusarium* infection, the role of the copalyl diphosphate synthase encoded by this gene is not clear in pathogen defense response.

Several genes have expression changes after *F. verticillioides* infection. It is demonstrated that, in resistant inbreds, defense related genes are transcribed also before infection meaning that there is a basal defense. In susceptible lines the expression of disease related genes starts only after the infection event (Lanubile et al., 2012; Lanubile et al., 2014a; Maschietto et al., 2016).

Several changes were identified in protein production after *Fusarium* infection in kernels and seedlings. Antioxidant enzymes like Cu/Zn-superoxide dismutase, glutathione-S-transferase, catalase and proteins responsible of synthesis and protein-folding and stabilization processes showed expression changes after *Fusarium* infection (Lanubile et al., 2014b).

A transcriptomic study performed using the resistant CO441 and susceptible CO354 inbred reveals around 7 thousand of differentially expressed genes. In the resistant inbred there is a high level of expression, particularly of genes involved in secondary metabolism. After infection the response is similar in the two genotypes but the magnitude of increase is higher in the resistant genotype compared to the susceptible. As a response to the fungus several genes are induced, like those

encoding transcription factors, or involved in signaling, defense and jasmonate/ethylene mediated defense responses. Secondary metabolism genes are highly expressed in the resistant rather than in the susceptible line, meaning that these genes play a relevant role in defense to *Fusarium* (Lanubile et al., 2014b).

4.4. QTLs for FER and fumonisin resistance

High levels of genetic resistance to mycotoxigenic fungi in maize are not available, even though significant progress has been made for *Fusarium*, *Gibberella* and *Aspergillus* ear rots (Warburton and Williams 2014; Lanubile et al., 2015).

Globally, the genetic architecture of resistance to *F. verticillioides* and fumonisin contamination appear complex with many quantitative trait loci (QTLs) of small effects that control the phenomenon. Studies revealed that genes and QTLs conferring resistance to *F. verticillioides* and or fumonisins accumulation have also effects on *A. flavus* and *F. graminearum* infection (Xiang et al., 2010; Mideros et al., 2014). There are differences among maize genotypes and resistance traits have moderate to high heritability, so phenotypic selection is useful to increase resistance. Unlikely phenotypic selection for ear rot has some technical problems: diseases can be evaluated only in some particular growing stages. For ear rot it is necessary to wait harvest maturity in addition inoculum calibration is necessary to expose genotypes to the same amount of pathogen, assessing mycotoxin accumulation is expensive and time consuming and many environmental factors can influence genotype evaluation (Lanubile et al., 2014a).

PCR-based DNA markers related to resistance traits and QTL mapping are powerful methods to improve resistance.

FER and fumonisin accumulation resistance are polygenic and QTLs are not conserved in populations (Maschietto et al., 2017).

Robertson-Hoyt et al. (2006) reported 12 QTLs for FER resistance in two different segregating population obtained by GE440 x FR1064 and NC300 x B104. Ding et al. (2008) reported two QTLs on chromosome 3 with the major QTL explaining 13-22% of the phenotypic variation.

Advanced back-crossed (BC) lines between the inbred GE440, resistant but with low agronomic value, and the susceptible elite line FR1064 are more resistant to ear rot, accumulate less fumonisin and provide good grain yield comparable to the elite parent (Eller et al., 2010).

From the cross of CO441 (resistant) x CO354 (susceptible) 188 F_3 lines were derived and phenotypically evaluated for FER and fumonisin accumulation resistance in two years and two sowing dates per year. Inoculation was performed following the toothpick and pin-bar method and fumonisin were detected using Near-Infrared Spectroscopy. Fifteen QTLs for FER and seventeen

QTLs for fumonisin B1 accumulation were detected; nine QTLs were in common between FER and fumonisin accumulation. Moreover a low positive correlation between silking date and fumonisin accumulation was reported (Maschietto et al., 2017).

On chromosome 1 are reported two QTLs responsible of resistance to *Fusarium* and *Gibberella* ear rot, three on Chromosome 2, four on Chromosome 3, one in Chromosome 4 and 6, two on Chromosome 5 and 7; for *Fusarium* and *Aspergillus* ear rot 9 QTLs are reported on chromosomes 3, 4, 5, 6; regarding aflatoxins and fumonisins accumulations on Chromosomes 1, 3, 4, 5 and 8 ten QTLs are reported (Santiago et al., 2015).

Some SNPs were reported in two genome wide association studies for *F. verticillioides* resistance using two different populations, one of about 300 inbred and one of about 1,700 inbred. In the first study three SNPs were associated with 3-12% of trait variation (Zila et al., 2013). In the second study seven SNPs were identified in six genes, each SNP identified was associated with a trait variation between 1 and 3% (Zila et al., 2014).

5. Defense mechanisms in plants

Plants, and other higher organisms, show a wide range of associations with microbes. At one extreme the mutualistic relation, as that between nitrogen-fixing bacteria and legumes. At the other end the parasitism of biotrophs and necrotrophs that are able to cause disease, while endophytic fungi are able to grow systemically in host tissues, without causing evident disease symptoms and sometimes providing beneficial effect to their host, producing compounds useful against herbivore insects or providing stress tolerance (Balint-Kurti and Holland, 2015).

Plants are continuously exposed to abiotic and biotic stresses like pests and fungi. An adaptive immune system comparable to that of vertebrate has not been detected in plants; however vegetables have evolved a multi-layer defense system to counteract biotic stresses (Pandey et al., 2015; Ellis and Jones, 2003). Plant response is based on two different strategies: constitutive and induced plant defense. Constitutive defense relies upon waxes, thick protective coatings of bark and cuticle to prevent pathogen penetration, phytoanticipins to control pathogen growth, and secondary metabolites (Pandey et al., 2016).

The inducible plant defense is mediated by pathogen recognition that relies on a complex interaction system between the invading pathogen and the host cells.

Plant pathogens can be classified in three different groups: biotrophs, necrotrophs and hemibiotrophs. Biotrophic pathogens obtain their nutrients through alive host cells, these pathogens produce effectors to suppress the plant defense mechanism. Necrotrophic pathogens derive their nutrients through dead host tissue and they produce a wide range of molecules to promote the infection.

Hemibiotorophic pathogens are able to behave as both biotrophs and necrotrophs at different disease stages (Pandey et al., 2016). At the beginning of infection cycle the pathogen suppresses the immune and cell death system of the plant to invade tissues with iphae. After invasion, during the necrotrophic phase, the pathogen secretes toxins to kill the host tissues (Koeck et al., 2011).

Plants have evolved a sophisticated mechanism to recognize and respond to pathogens at different levels during infection.

The ability of a pathogen to cause disease symptoms in host plant is referred as *virulence* while the inability to cause symptoms is called *avirulence*.

Studies in flax rust suggested five general indications to explain plant-pathogen interactions: i) resistance to a pathogen can be determined by a single gene, called resistance gene, different between susceptible and resistant host genotypes; ii) resistance is commonly dominant over susceptibility; iii) multiple resistance genes, each one conferring resistance to one or more pathogens, can be present in a species; iv) different resistance genes to a particular pathogen can be

present more times in host genome; v) plants are polymorphic for resistance (Ellis and Jones, 2003). Regarding the pathogens, virulence/avirulence can be caused by a single gene polymorphism in different isolates with avirulence most commonly dominant over virulence; multiple avirulence genes are found in pathogens.

In many host-pathogen interactions it has been observed a gene-for-gene interaction where the resistance gene of the plant encodes a receptor that detects the product of the corresponding avirulence gene in the pathogen. Avirulence genes are genes of the pathogen that confer the ability to be recognized by the host plant (Ellis and Jones, 2003).

There are two types of plant resistance, the first one is vertical, qualitative or host resistance, while the second is horizontal, quantitative or non-host resistance. Vertical resistance is temporal since it's overcame by pathogen race evolution, while horizontal is a permanent type of broad spectrum resistance and more complicated than vertical resistance; however overlaps between vertical and horizontal resistance exist (Ali and Yan, 2012; Gill et al., 2015).

Resistance types are differentiated based on the pathogen adaptation to a species (host) or lack of adaptation to another (non-host). Both host and non-host resistances are the result of the effect of plant immune system (Gill et al., 2015).

Among the plant immune system basal defense is the first line of defense and is initiated during the early phase of pathogen detection. It is reported that exist overlaps between basal and non-host resistance because it is possible that host and non-host plant species recognize analog factors to start the defense response (Gill et al., 2015).

5.1. Host and non-host resistance

Qualitative, vertical or host resistance follows the gene-for-gene model, confers a high level of, generally race-specific, high resistance and is governed by single dominant or recessive genes that are termed R-genes. Over the past years several R genes, conferring resistance to various pathogens in several plant species, have been cloned. This type of pathogen resistance is largely deployed in crops and describes the various adaptations that a plant evolves to improve its survival strategy against pathogens (Ellis and Jones, 2003; Wisser et al., 2005; Ali and Yan, 2012). When the interaction between pathogen and host plant is possible, disease symptoms develop and plant susceptibility is more common than plant resistance. The capacity of a pathogen to cause disease is evaluated on the basis of the host reaction, while the susceptibility or resistance of the host species is evaluated regarding the effect on microorganism development (Ali and Yan, 2012).

When the pathogen is able to overcome the plant defense barrier is defined as homologous pathogen, and plant-microbe interaction is called homologous or basic compatibility.

Quantitative, horizontal or non-host resistance is partial and race-nonspecific, it is due by the effect of few or several genes each of those is responsible of a small part of the entire resistance. Quantitative resistance is very useful in the agronomic perspective since its durability and broad spectrum effects. Identifying genes responsible of quantitative resistance is challenging because of the small phenotypic effects of these genes (Wisser et al., 2005; Ali and Yan, 2012). Non host resistance is the most durable kind of resistance. It is the case when all isolated of a certain pathogen are tested on a determined plant species which doesn't show any symptom of disease after infection. The species is called non-host plant and the pathogen is referred as heterologous pathogen (Ali and Yan, 2012). The interaction between constitutive and inducible responses is responsible of non-host resistance.

Non-host resistance is a multi-level barrier against pathogen penetration; possible obstacles are presence/absence of signals from the plants, barriers such as cell wall, cuticle, phytoanticipins, lignin accumulation, production of antimicrobial compounds, hypersensitive response (HR) and production of pathogenesis-related (PR) proteins (Gill et al., 2015).

The subsequent barrier to pathogen invasion is the inducible plant defense mechanism. In this phase molecules of the phytoalexins family play a key role. The third obstruction that the pathogen has to face out is the plant signaling mechanism mediated by plant hormones as ethylene and salicylic acid. The fourth component involved in resistance is broad spectrum disease resistance genes (Ali and Yan, 2012).

Non-host resistance against bacteria, oomycetes and fungi has been classified in two different types. Non-host resistance type I does not produce any kind of visible symptoms and involves the presence of passive or pre-constituted barriers and/or active defense induced as a response to general elicitors such as Pathogen Associated Molecular Patterns (PAMPs) (Gill et al., 2015), resembling therefore PAMP-triggered immunity (PTI).

Type II is always associated with a rapid and localized necrosis and cell death related to HR. Type II is an effector triggered reaction and resemble effector-triggered immunity (ETI) (Gill et al., 2015). It is possible that a pathogen can trigger both the two types of non-host resistance on different plant species. Non-host resistance is important to find genes responsible of this phenomenon and transfer these helpful characteristic in economically relevant host species (Ali and Yan, 2012).

5.2. PAMP and PTI

The external surface of the host cells is responsible of the first recognition, during the inducible defense. Plants possess pathogen recognition receptors (PRRs) that are able to recognize PAMPs that leads to PTI.

PAMPs recognized by the plant immune system are a wide range of chemical compounds like proteins, lipids or carbohydrates essential to the pathogen life cycle as the bacterial abundant protein, elongation factor Tu, flagellin and chitin which is the fungal cell wall polysaccharide. PRRs activate a response cascade that leads to oxidative burst, ethylene production and cell wall modifications. Plants are also able to recognize and react to endogenous molecules released by the pathogens during host tissues invasion. Molecules derived from the degradation of the cell wall or cuticle fragments are known as danger associated molecular patterns (DAMPs).

PAMP induced defense is not sufficient to arrest the infection, therefore it is referred as basal resistance. Initiation of plant defense response and the counter attack of pathogen are explained by the zig-zag model, where numerous PRRs of the plant are able to recognize PAMPs and initiate the defense mechanisms.

Some well adapted pathogens have evolved systems to produce others virulence factors, called effectors, to suppress PTI and facilitate pathogenesis (Koeck et al., 2011; Ali and Yan, 2012; Gill et al., 2015; Pandey et al., 2016).

5.3. Effectors and ETI

The second kind of recognition starts when the plant recognizes specific pathogen effector protein produced to suppress PTI and allows effector triggered susceptibility (Gill et al., 2015).

Plants have developed a new system to detect pathogen effectors, also called Avr proteins, using NB-LRR receptor protein, generally known as R proteins, that directly or indirectly interact with the effectors and start a stronger and typical defense response called ETI (Koeck et al., 2011; Gill et al., 2015).

ETI is stronger than PTI and blocks the pathogen through cell death. This strategy is very useful against biotrophic pathogens that require alive host cells for their survival (Koeck et al., 2011).

5.4. *R* genes

R genes confer resistance to a wide spectrum of pathogens such as fungi, bacteria, viruses and nematodes and are responsible of qualitative resistance. Despite the wide range of pathogen taxa, *R* genes encode a limited number of R proteins formed by a different number of conserved domains. *R* genes can specifically recognize pathogen strain or race-specific factors; they permit the setting-up of cultivars with race-specific resistance. Inside host tissue the pathogen is blocked by *R* genes products that recognize specific avirulence dependent signals, this leads to a signal transduction chain that end with the activation of defense mechanisms (Ali and Yan, 2012).

Avirulence genes (*Avr*) are genes of the pathogen that produce a protein which is directly or indirectly recognized by plants that contain the complementary resistance gene. If the pathogen *Avr* gene correspond to the plant *R* gene the plant resist the disease, if one is missing the pathogen is able to develop.

Avr genes products are considered to be virulence factors necessary for host colonization, those molecules function as elicitors to activate the plant defense (Ali and Yan, 2012).

Most of *R* genes encode protein with three domains: the C-terminal leucine-rich repeat (LRR) domain implicated in the binding of pathogen effector and in the regulation of signal transduction, the central nucleotide binding site (NBS) with conserved motifs which is responsible of the binding and hydrolysis of ATP and GTP in R proteins and the Toll/ interleukin-1-receptor or the coiled-coil N-terminal domain.

The NBS domain was firstly defined by the presence of kinase 1a (P-loop), kinase 2 and kinase 3a, after *R* gene cloning five additional domains were found and the NBS domain renamed to NB-ARC domain (Ellis and Jones, 2003).

LRR domains are common to NBS-LRR resistance proteins and other NBS non-resistance proteins.

The LRR domain can be either intracellular, as part of NBS-LRR protein or extracellular in transmembrane receptor-kinase protein or in receptor-like proteins. It is composed by variable repeats and LRR domain assignment is sometimes difficult (Ellis and Jones, 2003; Ali and Yan, 2012). LRR domain is responsible of the direct or indirect interaction with molecules produced by the pathogen. Transmembrane domain (TM) and protein kinase (PK) can be present in this kind of protein. *R* genes are so grouped in four subclasses: NBS-LRR, receptor-like kinases (RLK), LRR-TM and LRR-CC.

Studies that examined the evolution of *R* genes in the same species have found that in LRR regions some codons of the leucine repeated motif are subjected to amino acid variation. Variations can introduce new or more efficacious recognition specificities supporting the fact that this region is involved in the recognition of pathogen derived molecules (Ellis and Jones, 2003).

The largest group of *R* genes is represented by NBS-LRR which encodes a N terminal of about 200 amino acids (aa) connected to a NBS domain of 300 aa and a variable tandem array of 10-40 short LRR. Moreover NBS-LRR genes are classified basing on the different motifs that can be present at the N-terminal (Ali and Yan, 2012).

According to the N terminal domain NBS-LRR proteins are divided in two subgroups: TNL or TIR-NBS-LRR if they encodes for the Toll/ interleukin-1-receptor and CNL or CC-NBS-LRR if is present a coiled-coil domain (Ellis and Jones, 2003, Cheng et al., 2012).

TIR domain is an intracellular protein-protein interaction domain consisting of 125-200 conserved residues. TIR domain is involved in determining the specificity of signaling and resistance. In plant and animals this domain is found in protein with function related to innate immune pathways; in bacteria the TIR domain has a different function while it is absent in fungi, archaea and viruses (Cheng et al., 2012).

The CC domain is involved in protein-protein interaction and signaling and can be divided in two or three subclasses (Ellis and Jones, 2003).

Leucine zipper (LZ) and non-motif domain can also be present (Ali and Yang 2012; Cheng et al., 2012; Ve et al., 2015).

Since NBS disease resistance genes have a key role in defending plant from pathogens they were studied in many important plant species.

Cheng et al. (2012) classified maize NBS-encoding genes in four subgroups basing on which domain was encoded: CC-NBS-LRR, NBS-LRR, CC-NBS and NBS. The TIR domain was not found in maize; this is similar to what is reported for others monocots such as sorghum and rice. Globally, 109 NBS encoding gens were reported in maize. Among these only two were considered non-regular since the NBS ORF was not complete. Phylogenetic comparison of these genes to that of other species revealed that maize NBS genes possess similar function to known NBS genes in other species; moreover NBS gene duplication in maize was lower than in other species with an increase in the functional diversity of maize NBS genes.

Song et al. (2015) in their work identified and characterized 151 NBS-LRR and 226 LRR-RLK (leucine rich repeat-receptor like kinases). The 151 NBS-LRR were subdivided according to their C-terminal: 147 genes were classified in the CC-NBS-LRR group, while the remaining 4 were classified as TIR-NBS-LRR.

The distribution of these gene is not random and not uneven among chromosomes, is possible to find single or gene cluster and there is the tendency to find these genes at the distal end of the chromosome (Song et al., 2015).

5.5. Hypersensitive Response

Pathogens are able to suppress and overcome the basal resistance of plants. In this case the plant may respond in another way which is the HR.

HR was described around 100 years ago and can be defined as an area of cell death that forms at the point of attempted pathogen ingress and which correlates with the exhibition of resistance (Mur et al., 2008).

HR is a type of plant response against pathogens, fungi, bacteria, virus and nematodes, where the plant responds quickly to the pathogen at low quantity of inoculum. The response results in the death of the cells around the site of infection to counteract the pathogen and limiting the access to water and nutrients. Despite HR is drastic compared to basal response, the death of few cells can be crucial for plant survival.

HR is more pathogen-specific than basal resistance and is often triggered when plant cell receptors recognize the presence of specific disease-causing effector molecules introduced into the host by the pathogen. At the same time the plant activates other morphological or biochemical strategies such as suberification and lignification around the dead tissues. Different metabolisms such as phenols, chitinase and glucanase synthesis can be activated in the surrounding tissues to create unfavorable condition for pathogen diffusion (Freeman and Baeattie, 2008).

5.6. Systemic Acquired Resistance

At the beginning of the 20^{th} century scientists understood that plants previously infected by a pathogen are able to better counteract subsequent pathogen attacks.

After pathogen invasion, plant develops an enhanced resistance to subsequent pathogen penetration also in non-inoculated organs. This new resistance is called Systemic Acquired Resistance (SAR).

After SAR activation the plant is in a readiness state helpful to quickly and effectively activate defense response mechanisms in a second time it comes in touch with a pathogen.

SAR has some positive characteristics such as that it is activated in all the plant and also in organs distal from the first inoculation point, and it is active to a wide range of possible plant pathogens (fungi, oomycetes, viruses and bacteria). SAR effect is prolonged during time from few weeks to months and even to the entire growing season (Conrhat, 2006).

It is possible to induce SAR artificially spraying plant with chemicals called plant activators (Freeman and Baeattie, 2008).

Several molecules are considered important in SAR signaling transmission in the plant: salicylic acid (SA), lipid-derived molecules, hydrogen peroxide and gaseous methyl salicylate. The latter,

produced by tobacco leaves inoculated with tobacco mosaic virus, functions as an airborne signal which can activate non infected tissue of the inoculated and neighboring plants (Conrhat, 2006).

5.7. The role of plant hormones and other molecules in plant defense response

Plants produce a wide range of molecules with hormonal function such as auxins, gibberellins, abscisic acid, cytokinins, SA, ethylene (ET), jasmonates (JA), brassinosteroids, peptide hormones and stringolactones.

These hormones have fundamental roles in several growth and developmental processes and in biotic and abiotic stress responses (Bari and Jones, 2009). After the perception of a pathogen by plant receptors the host activates a signaling cascade mediated by plant hormones (Pandey et al., 2016).

Plant infection with different pathogen species results in the change of various phytohormones level. During the evolution, pathogenic microorganisms have developed the ability to handle the defense-related regulatory mechanism producing phytohormones or their functional mimics. This manipulation results in hormonal imbalance and activation of inappropriate defense responses (Bari and Jones, 2009).

5.7.1. Phytoalexins

Phytoalexins are secondary metabolites of low molecular mass which possess antimicrobial activity and synthetized during stress condition playing a relevant role in plant defense. Phytoalexins are a wide group of molecules belonging to several chemical families such as phenolics, terpenoids, furanoacetylenes, steroid glycoalkaloids, sulfur-containing compounds and indoles (Jeandet, 2015). They explicate their activity towards several pathogenic microorganisms, since that are thought to be disease associated markers (Ahuja et al., 2012).

The hypothesis of the presence of phytoalexins was made at the beginning of 1940s when potatoes tuber tissues infected with an incompatible race of *Phytophthora infestans* were resistant to a subsequent infection with compatible race of the same fungus. The supposition was that tuber tissues, as a response to incompatible infection, produced some substances that protected the same tissues against subsequent infection by compatible strains of the same pathogen (Ahuja et al., 2012).

Phytoalexins have also health promoting effects like antioxidant, anticancer, cardiovascular protective activity, antidiabetics, vasodilator, anti-aging and anti-inflammatory effects as reported for phytoalexins found in *Brassica* vegetables, peanuts, soybean, sorghum and grapevine (Ahuja et al., 2012).

As a response to pathogen attacks crop species of the *Poaceae* family accumulates avenanthramides (oat), diterpenoids and the flavonoid sakuranetin (rice) and 3-deoxyanthocyanidins (sorghum) (Ahuja et al., 2012). Phytoalexins found in corn belong to the family of terpenoids like zealexins and kauralexins and benzoxazinoids like DIMBOA and HDMBOA (Poloni and Schirawski, 2014).

Zealexins are a group of sesquiterpenoids related to β-macrocarpene of at least five members termed zealexin A1, A2, A3, B1 and C3 that accumulate after *F. graminearum* infection; other compounds of unknown identity were also found (Ahuja et al., 2012; Poloni and Schirawski, 2014). Phytoalexins are also accumulated after *A. flavus* and *Rizophus microsporus* infection in maize. *A. flavus* and *F. graminearum* are inhibited by zealexin A1 and A3; zealexin A1 is also able to block *R. microsporus*. Zealexin synthesis required the activation of terpene synthase 6 and 11. These two enzymes were found up-regulated in maize infected by *U. maydis*, *S. reilianum* f. sp. *zeae* and *F. graminearum*. Silencing of these two enzymes resulted in an increased susceptibility to *U. maydis* indicating that zealexins are a key component of disease response in maize (Poloni and Schirawski, 2014).

Kauralexins are a group of six *ent*-kaurene related diterpenoids named kauralexin A1, A2, A3, B1, B2 and B3. The gene *AN2*, which encodes *ent*-copalyl diphosphate synthase anther ear 2, is involved in the synthesis of kauralexins; *AN2* is also expressed after attacks of *O. nubilalis* that increases the concentrations of JA and ET. Exogenous application of ET and JA are sufficient to stimulate the kauralexisn pathway.

Attacks from *R. microsporus* and *Colletotrichum graminicola* in maize stalks cause the accumulation of six *ent*-kaurene-related diterpenoids to inhibit the growth of the pathogen. Kauralexin production is preceded by the accumulation of fungal-induced kaurene synthase 2; in kauralexin regulation is demonstrated a synergistic role of JA and ET (Ahuja et al., 2012; Poloni and Schirawski, 2014). During pathogen attacks zealexins and kauralexins are co-induced and co-produced.

Other compound produced by maize leaves are part of the benzoxazinoid hydroxamic acid family: HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-glucoside), DIMBOA-Glc (4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one-glucoside), HMBOA-Glc (2-hydroxy-7-methoxy-1,4-benzoxazin-3-one-glucoside), DIM2BOA-Glc (2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside), HBOA (2-hydroxy-2H-1,4-benzoxazin-3(4H)-one), DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) and TRIBOA (2,4,7-trihydroxy-2H-1,4-benzoxazin-3(4H)-one). DIMBOA-Glc is predominant in maize seedling and during plant growth its concentration decreases, while DIMBOA can be produced as a response to pathogen infection. Herbivore insects triggers the conversion of DIMBOA-Glc to HDMBOA-Glc with an increasing of plant resistance, however, in some samples this is associated with susceptibility (Poloni and Schirawski, 2014).

5.7.2. Ethylene

ET is a gaseous hydrocarbons with widespread effects on plant development such as germination, senescence, formation of vascular system, fruit ripening, organ abscission and responses to biotic and abiotic stresses (Pandey et al., 2016; Bouchez et al., 2007).

ET is associated with the defense against necrotrophic pathogens (Pandey et al., 2016; Bari and Jones, 2009); it modulates plant cell death on local or large scale. The best known plant cell death is the HR that limits the growth of microorganisms and is regulated also by ET. Exogenous treatment with ET can increase both plant resistance or susceptibility depending on the pathogen type and on plant-pathogen interaction (Bouchez et al., 2007). In response to pathogens ET has a negative interaction with the pathway related to defense mechanisms against biotrophs and hemi-biotrophs while has a positive effect against necrotrophs (Bari and Jones, 2009).

5.7.3. Salicylic acid

It is important for the activation of defense mechanisms against biotrophic and hemi-biotrophic pathogens (Bari and Jones, 2009), as well as it is necessary to establish both local responses and SAR in tissues far from the first infection point. It has been shown that transgenic tobacco plant expressing a salicylic acid hydrolase are not able to accumulate SA and establish SAR (Conrhat, 2006).

It is reported that mutants that show problems in SA accumulation or are unresponsive to SA or have enhanced susceptibility to biotrophic and hemibiotrophic pathogens.

In pathogen-threatened tissues there is an increasing of SA level that induces the expression of PR-proteins. Exogenous application of SA results in the accumulation of PR proteins and in an increased resistance to a broad range of microorganisms (Loake and Grant, 2007; Bari and Jones, 2009).

SA is generally antagonist of JA-signaling pathway but there are evidences of a more complex mechanism with both positive and negative interactions (Loake and Grant, 2007).

5.7.4. Jasmonate

JAs are a class of lipid-derived molecules also known as oxylipins and involved in several physiological processes. The biosynthesis of JA starts form linolenic acid which is oxidized and cyclized via the octadecanoic pathway (Ballarè, 2011; Pandey et al., 2016). It is involved in defense mechanisms against necrotrophic pathogens and herbivores insects as caterpillars, beetles, thrips, leafhoppers, spider mites, fungal gnats and mired bugs (Bari and Jones, 2009).

JA is also involved in many developmental processes like seed germination, root growth, tuber formation, tendril coiling, fruit ripening, leaf senescence and stomatal opening (Bari and Jones, 2009).

The bioactive compound related to JA is jasmonoyl-isoleucine (JA-Ile); its perception is achieved by the ubiquitin ligase SCFCOI1 complex. The F-box protein CORONATINE-INSENSITIVE1 (COI1) recognizes JA-Ile, and triggers the ubiquitination and subsequent proteosomal degradation of JASMONATE ZIM DOMAIN (JAZ) proteins. This relieves JAZ-mediated repression of gene expression, resulting into the activation of JA responses (Ballarè, 2011). Plant response is affected by the ecological context of the plant itself. The defense activated depends by the nature of the pathogen, the vitality of the attacked organ, the proximity of competing plants, the history of previous interactions with pathogenic microorganisms and herbivores insect, as well as by the association with beneficial organisms (Ballarè, 2011).

It is reported that plant mutants affected in the JA biosynthesis or perception have compromised resistance against herbivore insects (Paschold et al., 2007; Zarate et al., 2007). As for SA, the application of exogenous JA results in the activation of defense mechanisms (Bari and Jones, 2009).

SA and JA induced defense mechanisms are antagonistic. Plants infected by biotrophs prioritize the SA pathway suppressing the JA defense; vice-versa plants attacked by necrotrophs enhance JA pathways suppressing SA defense related mechanisms (Bari and Jones, 2009; Ballarè, 2011); however, some synergistic interactions have been reported (Bari and Jones, 2009).

ET is another relevant regulator of JA induced defense response. In this case ET and JA have a positive interaction in inducing plant defense mechanisms. This is due to the fact that there is the activation of common transcription factors like APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) in *Arabidopsis*. ET has also the capacity to remove the downregulation of JA responses caused by SA. This means that the defense mechanisms against necrotrophs and herbivorous insects, that induce at the same time JA and ET pathways, is not suppressed by a subsequent attack carried out by biotrophs (Ballarè, 2011).

Since the defense signaling pathway activated by the plant depends on the pathogen type and pathogen lifestyle is not easy classifiable as simply biotrophic or necrotrophic, the positive or

negative cross-talk between SA and JA/ET is regulated by the type of pathogen (Bari and Jones, 2009). In addition, in the natural environment, plant have to face up with multiple attacks at the same time and so there are complex regulatory mechanisms to prime effective defense response against various pathogens and pests (Bari and Jones, 2009).

5.7.5. Auxin

Auxin has a fundamental role in plant development. Auxin allows the degradation of a family of transcriptional repressor termed Auxin/Indole-3-acetic acid (Aux/IAA). Aux/IAA binds to the auxin response factors (ARFs) and inhibits the transcription of specific auxin response genes.

During plant growth and development auxin can induce the expression of three groups of genes Aux/IAA, GH3 and auxin-up RNA (SAUR) family.

GH3 genes have a role in defending plant (*Arabidopsis* and rice) by pathogens. GH3.5 has a double function modulating SA response and auxin signal during pathogenesis; GH3-8 is involved in defending rice from *Xanthomonas oryzae* pv. *oryzae*. The infection resulted in auxin production that promotes the synthesis of expansins resulting in the loosening of cell wall; this can potentiate the growth of the pathogen. The inhibition of auxin signal can suppress the production of expansin resulting in an increased resistance.

Exogenous application of SA results in the repression of auxin responsive genes; after the induction of *SAR* genes induced by auxin were found to be repressed (Bari and Jones, 2009).

Exogenous application of auxins result in increased disease caused by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi*; in *Arabidopsis* the co-inoculation with *Pseudomonas syringae* pv. *maculicola* 4326 and auxin resulted in increased disease and pathogen growth while the *Arabidopsis* auxin resistant *axr2-1* mutant is more resistant to the same pathogen than wild type plants (Bari and Jones, 2009). Several studies report that auxin promotes disease susceptibility and down regulation of auxin signaling could result in enhanced resistance (Bari and Jones, 2009).

In *Arabidopsis*, regarding the necrotrophic pathogens *Botrytis cynerea* and *Plectosphaerella cucumerina*, the repression of auxin metabolism, through mutation in signaling or transport component, results in a compromised resistance. For necrotrophic fungi auxin is an important component in plant response (Bari and Jones, 2009).

Viruses are able to interfere with the auxin metabolism too. In *Arabidopsis* Tobacco Mosaic Virus (TMV) disrupts Aux/IAA function to reprogram cellular metabolisms in useful way for its replication and spread.

5.7.6. Abscisic acid

Abscisic acid (ABA) is one of the most known plant hormone, it is a 15-C weak acid firstly isolated as a growth inhibitor from abscising cotton fruit and from leaves of sycamore (*Ficus sycomorus*) induced to dormancy. ABA is involved in many other aspects of plant metabolism like seed dormancy, germination, embryo maturation, floral induction, leaf senescence, stomatal aperture, cell division and elongation, adaptation and response to environmental stresses such as drought, salinity, cold, pathogen attack and UV radiation (Bari and Jones, 2009; Finkelstein, 2013).

ABA is not directly involved in abscission, but its presence in abscising organs is due to its role in promoting senescence and/or stress responses, processes which precede abscission. ABA plays a relevant role also in young tissues; mutants deficient in ABA metabolism appear to be rachitic (Finkelstein, 2013).

It is reported that ABA has a complex role also in plant defense according to different type of plant-pathogen interaction (Bari and Jones, 2009). In abiotic stress response ABA has generally a positive role while in pathogen response ABA may promote both resistance and susceptibility according to the type of pathogen and mode of infection (Finkelstein, 2013). Generally it is involved in the negative regulation of plant defense against different biotrophic and necrotrophic pathogens and application of exogenous ABA enhance susceptibility to diseases (Bari and Jones, 2009).

In *Arabidopsis* is reported an antagonistic effect between ABA and SAR resulting in SAR suppression; so far ABA has a negative effect on disease response.

However positive roles of ABA during pathogen attacks are reported: tobacco plants infected by TMV show increased ABA levels and ABA application has resulted in increased resistance and plant ABA deficient mutants are more sensitive to the infection by *Pythium irregulare*, *Alternaria brassicicola* and *Leptosphaeria maculans* (Bari and Jones, 2009).

As an activator of stomatal closure ABA plays a positive role in plant defense blocking major point of entrance for pathogens (Bari and Jones, 2009; Finkelstein, 2013). Treatment with ABA protects plants against the necrotrophic pathogens *A. brassicicola* and *P. cucumerina* (Bari and Jones, 2009); ABA can also enhance resistance to fungal infection promoting callose deposition in the apoplast so interfering with pathogen penetration (Finkelstein, 2013). ABA has also functions in the production of ROS and in transcriptional reprogramming of plant cell metabolisms to regulate defense gene expression (Bari and Jones, 2009).

The understanding of ABA effects is complicated by the cross-talk with other hormones that regulate defense responses (SA, JA, ET) and by the fact that increases in endogenous ABA are usually related with exposure to abiotic stresses, which can compromise plant viability. Generally,

ABA promotes early defense responses but inhibits late defense responses by suppressing salicylic acid-dependent responses and modulating JA and ET-dependent defenses (Finkelstein, 2013).

5.7.7. Brassinosteroids

Brassinosteroids (BRs) are plant hormones structurally similar to animal steroids hormones. They are implicated in various physiological processes: seed germination, cell division and elongation, flowering, development of reproductive organs, senescence and abiotic stresses response. Their role in biotic stresses response is not clear.

BR-mediated resistance does not require SA biosynthesis or PR genes expression.

In tobacco BRs enhances resistance to TMV, *P. syringae* pv. *tomato,* and *Oidium* spp.; in rice against *Magnaporthe grisea* and *Xanthomonas oryzae*. Exogenous application of a BR protected tomato plants against *Verticillium dahliae* and potatoes against *Phytophthora infestans* in association with accumulation of ABA and ET levels.

BRI1-associated kinase 1 (BAK1) is a key component in brassinosteroid signaling for the activation of basal defense and programmed cell death. This gene is up-regulated in response to some PAMPs, *Arabidopsis bak1* mutants are compromised in PAMP responses and susceptible to netrotrophic pathogens as *A. brassicicola* and *Botrytis cynerea*, while they are resistant to biotrophic pathogens as *Hyaloperonospora parasitica*; in this case exogenous application of BR failed to restore resistance to pathogens.

BR has also the capacity to interact with the expression of genes involved in disease resistance and in the biosynthesis of other hormones (ET and JA) (Bari and Jones, 2009).

5.7.8. Gibberellins

Gibberellin (GA) was isolated from the fungus *Gibberella fujikuroi* that causes the bakanae disease in rice.

GAs are plant hormones with a tetracyclic diterpenoid structure and they work at all plant development stages regulating germination, root, leaf, stem and fruit growth, greening of leaves, flower and seed development (De Bruyne et al., 2014)

GA promotes plant growth through the degradation of negative growth regulators called DELLA proteins. Since GAs are produced not only by plants but also by fungi and bacteria it is supposed that they are secondary metabolites used as signaling factors and useful to establish interactions in host plants. It is reported that DELLA proteins in *Arabidopsis* functions as controllers of plant immune response controlling SA and JA mediated defense response (Bari and Jones, 2009).

Studies on GA role in plant defense report that there is an ambivalent role played by these hormones: in some cases they contribute to plant susceptibility while in some others they enhance resistance.

De Bruyne et al. (2014) report that in monocot species this dual face is conserved, experiments with wheat and barley resulted in increased susceptibility to biotrophs and resistance to necrotrophs.

In *Arabidopsis* the situation is reversed: exogenous GA application increased resistance to *P. syringae* pv. *tomato* and susceptibility to *A. brassicicola* indicating that GAs act as virulence factors for necrotrophs (Bari and Jones, 2009).

DELLA proteins modulate the production of ROS during stress periods inducing genes responsible of ROS detoxification and restraining cell death. During necrotrophs infection the reduction of cell death resulted in increased resistance (De Bruyne et al., 2014).

5.7.9. Cytokinins

Cytokinins (CK) are plant hormones important for diverse processes such as stem-cell control, differentiation, chloroplast biogenesis, seed development, root, shoot and inflorescence growth and branching, leaf senescence, nutrient balance and stress tolerance. Regarding the role of CK in disease response there are some evidences about the involvement of CK in plant response against pathogens (Bari and Jones, 2009). Genes involved in cytokinin homeostasis resulted down-regulated in *Arabidopsis* plants infected by *Plasmodiophora brassicae* the causal agent of clubroot disease. The overexpression of cytokinin oxidase/dehydrogenase in transgenic *Arabidopsis* plants resulted in the resistance against *P. brassicicola*. *A. tumefaciens* interferes with CK metabolisms to promote tumor growth in the plant (Bari and Jones, 2009). In *Arabidopsis* the constitutive activation of an *R* gene resulted in morphological abnormalities caused by the accumulation of CK; this indicates a possible role of CK in some *R* gene mediated responses (Bari and Jones, 2009).

5.7.10. Peptide hormones

This is a new class of plant hormones involved in the regulation of various aspects of growth and metabolism comprising plant defense response against pathogens. Defense related peptide hormones are systemin, hydroxyproline-rich glycopeptide systemins and AtPep1. These hormones are 18-23 amino acids longer processed from larger precursor polypeptides induced by JA and wound and have a crucial role in the activation of both local and systemic reactions against pests and wounding.

Systemin and hydroxyproline-rich glycopeptide can activate the expression of anti-herbivore proteinase inhibitor and polyphenols oxidase as a response to wounding and methyl-jasmonate; the role of this hormones in plant defense remains unclear, current evidences suggest a role in the amplification of signals caused by wounding and elicitors (Bari and Jones, 2009).

6. Association mapping

The fundamental aim of genetics is to connect a genotype with the corresponding phenotype (Oraguzie and Wilcox, 2007).

This objective has been one of the major focus on human, animal and plant studies; for plants our ability to map QTLs important for disease resistance, grain yield and stress tolerance in breeding materials, landraces and inbred collection have a great potential for germplasm security and future trait improvement and can give help in understanding adaptive processes (Zhu et al., 2008; Brachi et al., 2011; Huang and Han, 2014).

Association mapping has been defined in several ways: "association genetics", "association studies" and "linkage disequilibium (LD) mapping" (Oraguzie and Wilcox, 2007).

Gene mapping uses genomic information to dissect complex traits in mendelian loci or quantitative trait loci (QTL) and identify genetic markers important for crop improvement. The possibility to discover genetic determinants in a mapping study depends on the availability of proper populations with high genetic diversity and recombination density (Dell'Acqua et al., 2015).

The first step in traditional method for mapping QTLs in plant requires to generate a segregating population (F2, backcross or recombinant inbred) from a biparental cross of two founders of different phenotypes; subsequently, it is necessary to collect the different phenotypes of interest showed by the individuals and genotype the population with genetic markers throughout the genome (Flint-Garcia et al., 2005). The genome of the progeny is reconstructed by the haplotypes of the founder lines (Dell'Acqua et al., 2015). Phenotypic and genotypic data are then analyzed via linkage mapping (Flint-Garcia et al., 2005).

Linkage mapping has been useful in dissecting complex trait, but it has some limits due to the resolution of the mapping population. These populations have generally a small size caused by the cost of propagating and evaluating large population and a limited number of recombination events resulting in a resolution of 10-30 cM; even if the resolution is reduced to few cM it still corresponds, in maize, to millions of bases and hundreds of candidate genes. Another limit of biparental crosses is that at a single locus only two alleles are available (Flint-Garcia et al., 2005; Zhu et al., 2008, Brachi et al., 2011, Soto-Cerda and Cloutier, 2012).

An alternative method to linkage mapping is association mapping (AM). AM has the potential to identify a single polymorphism inside a gene which is responsible of a phenotypic difference and involves searching for genotype-phenotype correlation among unrelated individuals (Soto-Cerda and Cloutier, 2012). A comparison between requirements and goals of QTL and association mapping is reported in **Table 1**.

Table 1. Comparison of requirements and goals of Association genetics and QTL mapping (Oraguzie and Wilcox, 2007).

Attribute	QTL mapping	Association genetics
Detection goal	Quantitative trait *locus* i.e., wide region within specific pedigrees within which a QTN is located	Quantitative trait nucleotide i.e. physically as close as possible to causative sequence (s)
Resolution of causative trait polymorphism	Low-moderate density linkage maps only required	High-disequilibrium within small physical regions requiring many markers
Experimental population for detection	Defined pedigrees, e.g, backcross, F_2, RI and two-three generation pedigrees/families, half sib families etc	Linkage disequilibrium (LD) experiments: unrelated individuals ("unstructured" populations), large numbers of small unrelated families (e.g., transmission disequilibrium tests, TDT)
Marker discovery cost	Moderate	Moderate for few traits, high for many traits
Extend of inference	Pedigree specific, except where species has high extant LD	Species or subspecies wide
Number of markers required for genome coverage	10^2,-low 10^3	10^5 for small genomes- 10^9 for large genomes

AM is based on LD and uses ancestral recombination events in natural population to associate markers and genotypes and evaluate if a certain marker is found with a certain phenotype more frequently than expected (Flint-Garcia et al., 2005).

AM has some advantages respect to linkage mapping: it is possible to use existing natural populations instead to generate a new population, this is relevant especially for species that require more years to be in the reproductive state; at a single locus more allele can be tested at the same time while for linkage mapping only two alleles are examined, mapping resolution is higher, functional variations are searched in a larger germplasm background, less time is required to conduct the analysis and establish marker-trait association and it is applicable in breeding programs. AM guarantees a higher resolution exploiting the historical recombination accumulated in natural population or collection of inbred, landraces or breeding materials; mapping resolution can be

increased to few thousands base pair (bp) in maize inbred lines (Flint-Garcia et al., 2005; Zhu et al., 2008; Soto-Cerda and Cloutier, 2012).

AM analysis relies on common variants which are able to explain 5-10% of the variation in human diseases; the role of rare alleles is becoming more important. New AM methods are being developed to account for rare alleles since currently method allocate their statistical power on higher frequency alleles.

Since most of the characters of agricultural interest are highly quantitative, the interest in using AM methods is increasing (Soto-Cerda and Cloutier, 2012).

Conversely to human or animal studies association analysis is more promising in plant science, because geneticists can easily produce large number of different progenies or population and conduce replicate studies with immortal individuals (inbred or recombinant inbred lines -RILs) (Zhu et al., 2008).

AM is divided in two categories: candidate-gene association mapping, where genes that are presumed to control a certain trait are sequenced. This approach is useful in species with low LD. The second category is Genome Wide Association Study (GWAS) genome-scan or genome wide association mapping places single nucleotide polymorphisms (SNPs) markers on the entire genome and looks for signals of association for various complex traits; this approach is useful in population with moderate or extensive LD (Flint-Garcia et al., 2005; Zhu et al., 2008).

Genome wide association analysis of important human diseases have confirmed the result found in candidate gene studies and has found new important loci previously unknown. The same approach is now widely used in plants species since genotyping costs are very reduced than in the past (Zhu et al., 2008).

Association mapping uses the genetic diversity of natural populations to resolve complex traits variation to the gene or nucleotide level. Using linkage analysis with population derived by bi-parental crosses produces important results that, however, tends to be specific to the population or to genetically related materials. Contrarily, results from association mapping are applicable to a broader germplasm.

One of the fundamental step in association studies is the choice of the germplasm: genetic diversity, extend of genome-wide LD and relatedness within the population determine the marker density, mapping resolution, mapping power and statistical method to apply.

The populations used in association mapping can be classified in five groups:

- ideal sample with small population structure and familial relatedness;
- multi-family sample;
- sample with population structure;
- sample with population structure and familial relationship;

- populations with severe familial relationship and population structure.

Plant material can also be classified according to the source of materials, germplasm bank collection, as synthetic population or as elite materials.

For many plant species, due to local adaptation, selection and breeding history, populations used for association studies have both population structure and familial relationship (Zhu et al., 2008).

A set of unlinked and selective neutral background marker is used to characterize the genetic composition of the population. Different marker technologies were available during the time: random polymorphic amplified DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite (SSR) and SNPs. Due to their higher genome density and low detection cost in populations SNPs are the markers chosen for association studies; since they are generally biallelic they are less informative than SSRs, therefore more SNPs are needed to estimate population structure and relatedness (Zhu et al., 2008).

The advent of high-density SNPs typing has allowed whole-genome scans to detect also small haplotype blocks that are significantly associated with quantitative trait variation. In plant studies this has led to identify loci relevant for crop improvement (Brachi et al., 2011).

AM refers to the significant association between a marker and a phenotype trait, and it is an application of LD which is the non-random association between alleles. Markers in LD are not necessarily correlated with a particular phenotype. QTL mapping and AM are both based on LD between molecular markers and functional loci, but in the case of QTL mapping LD is generated by the mating design used to generate the population, while for AM, LD reflects the history of the germplasm collection (Soto-Cerda and Cloutier, 2012).

The main advantage of AM with respect to QTL mapping is the high potential resolution in localizing a QTL, and resolving it to gene and polymorphism level for a trait of interest. AM has the potential to identify superior alleles and provide detailed marker data ready to be used in breeding programs. The germplasm examined is usually composed of diverse and important materials where it is possible to find segregation for interesting phenotypes. Furthemore, it is not necessary to have available a population as big as required for QTL mapping (Soto-Cerda and Cloutier, 2012). Although AM have numerous advantage some limitations exist. AM is limited when the trait of interest is strongly related to the population structure: it is the case of traits that are under local adaptation. Therefore, it is necessary to correct for population structure before searching for marker-trait association, as a consequence several polymorphisms responsible of the phenotype remain undetected and AM power is decreases.

AM requires a huge amount of markers, while few markers are necessary to run QTL mapping. The number of markers depends on genome size and LD decay. Such high number of markers can be

achieved by Genotyping by sequencing (GBS) techniques (Elshire et al., 2011; Soto-Cerda and Cloutier, 2012).

AM is also influenced by allele frequencies: it is reported that high percentages of alleles are rare and they can't be evaluated properly because are present only in few individuals and their resolution power is low. New analysis methods to account for rare alleles are proposed (Soto-Cerda and Cloutier, 2012).